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Melatonin Attenuates Free Radical Load and Reverses Histologic Architect and Hormone Profile Alteration in Female Rat: An *In vivo* Study of Pathogenesis of Letrozole Induced Poly Cystic Ovary

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Abstract

Objectives: The present study was designed and conducted for obtaining information about the role of melatonin (Mel) in pathophysiology of polycystic cystic ovarian syndrome (PCOS) which generally leads to infertility in human females.

Methodology: Letrozole, a non-steroidal aromatase inhibitor supplemented (1 mg/kg/body weight for 28 days) for induction of (PCOS). Treatment of exogenous melatonin (200 µg/kg/body weight) was given to PCO and normal rats. After completion of experiment gravimetric analysis, Lipid peroxidation (LPO) in terms of thiobarbituric acid reactive substance (TBARS), histological slide preparation following hemotoxylene-eosin (HE) double staining method for ovarian tissue was done and results were documented. Hormonal assay estrogen (E), progesterone (P), and Melatonin (Mel), luteinizing hormone (LH) and follicle stimulating hormone (FSH) was performed using ELISA kit.

Major findings: Letrozole induced PCOS exhibited increase in ovarian weight, lipid peroxidation level (LPO). Histopathology of PCO rats showed sub-capsicular cysts and capsicular thickening. Circulatory hormone profiles showed a significant decrease in plasma level of E, P, Mel. Plasma testosterone (T) level was noted significantly high whiles an unsteady ratio of LH) and FSH in PCOS rats. Melatonin treatment to the PCO rats showed recovery in ovarian weight, significant decrease in lipid per oxidation (LPO), withdrawal of presence of cyst from ovarian histology, reversal of plasma circulatory hormone profile to the control group of rats.

Conclusion: The finding update about similarity of ovarian cysts in rats to that observed in human PCOS and their regression following exogenously melatonin administration. The present findings may indicate and novel therapeutic approach based on the modulation of pathogenicity of PCOS in female rats through melatonin to improve the functional ovarian physiology as a possible future molecule among human females to treat the infertility. Such clinical trials may really prove to be highly beneficial for women with PCOS.

Keywords: Melatonin; Letrozole; PCOS; Oxidative stress; Estrogen; Progesterone; Testosterone

Introduction

Melatonin (N-acetyl-5-methoxytryptamine) an indoleamine is the principal hormone produced by the pineal gland [1,2]. Its release is mainly due to pineal secretion but 25% of melatonin production is of extra-pineal origin [3]. The circadian rhythm of melatonin levels is high at night and important for the synchronization of the reproductive response to appropriate environmental conditions in photoperiodic animals [4]. The participation of the pineal gland in the regulation of seasonal, photoperiodic dependent reproduction has been firmly established but is poorly understood. Reproductive physiology among vertebrates is compactly synchronized via various intrinsic like hormones and cytokines and extrinsic factor corresponding environmental stressors [5,6].

Polycystic ovarian syndrome (PCOS) is a common endocrine disorder that causes infertility due to anovulation in women of reproductive age [7]. Besides infertility, women with PCOS manifest clinical features such as hyperandrogenism, hyperinsulinemia, insulin resistance, hirsutism, obesity, chronic anovulation, and polycystic ovaries (PCO). Not only anovulation but also decreased oocyte and embryo quality may be a cause of infertility in women with PCOS [8]. The ROS induced oxidative stress may be responsible for poor oocyte quality. Free radicals have a dual role in the reproductive tract and are key signaling molecules for various ovarian functions [9]. Specifically, free radicals function in the microenvironments of oocytes, sperm, and in the follicular fluid (FF) [10]. Free radicals mediate their actions through a variety of pro-inflammatory cytokines with these processes having been proposed as a common underlying factor for endometriosis, ovarian cancer, polycystic ovary disease (PCOD), and various other pathologies affecting the female reproductive tract [11]. These features can lead to multiple symptoms with systemic as well as organ-specific aberrations. As PCOS is associated with several other diseases/morbidity-related factors such as obesity and other cardiovascular disease (CVD) risk factors, which are becoming more prevalent among females today, further research on the patho-

physiology and the long-term effects of PCOS is of the utmost importance in order to prevent future health problems in the large group of PCOS women.

Melatonin is a documented powerful free radical scavenger and a broad-spectrum antioxidant [12,13]. The use of melatonin as a drug to prevent free radical damage has been widely investigated and its utility as an antioxidant provides opportunity for the management of several diseases including cancer, immunological disorders, Alzheimer's disease, diabetes, and viral infections [14-17]. This neurohormone has a myriad of actions correlated to the neuroendocrine mechanisms involved to govern the waxing and waning of reproductive competence in seasonally breeding mammals [18,19] and directly on the peripheral reproductive organs of non-seasonal breeders as well [20,21]. Melatonin is considered nowadays among pinealogists as extensive 'tool kit' because of a consequence of its wealth of actions. Therefore, this ubiquitously-acting indoleamine has both receptor-mediated actions as well as functions that seem not to rely on typical receptors and signal transduction processes, i.e., its free radical scavenging effects. Nevertheless, it is further very well documented that maturation of the follicle and oocyte is associated with elevated prostaglandin and cytokine production which further results in the activation of proteolytic enzymes finally leading to the generation of toxic oxygen derivatives [22,23]. On the other hand rupture of the ovarian follicle and shedding of the ova is initiated by the luteinizing hormone surge from the anterior pituitary gland. Mechanistically, ovulation has been described as being reminiscent of an inflammatory process [24]. Although the physiological roles of melatonin in regulation of pathogenesis of ovarian dysfunction during poly cystic ovary condition have not been very well understood yet. It is therefore hypothesized that melatonin possibly plays an antioxidant in ovarian internal milieu. The aim of present study was to determine the alteration in level of free radical load, histological cellularity and hormone profiles luteinizing hormone (LH), follicle stimulating hormone (FSH), Testosterone (T), Estrogen (E) progesterone (P) and melatonin(Mel) as well as to check the efficacy the effect of exogenous melatonin administration to letrozole induced poly cystic rat ovaries [25-28].

Materials and Methods

Chemicals

Melatonin (Mel), Testosterone (T), Progesterone (P) Estrogen (E), Luteinizing hormone (LH), follicle stimulating hormone (FSH) and Letrozole (L) were obtained from Sigma Aldrich, USA. Carboxy Methyl Cellulose (CMC), Thiobarbituric acid (TBA),Tris-hydrochloric acid (Tris-HCl), Phosphoric acid and Butylated Hydroxy Toluene (BHT) were purchased from Himedia and are of analytical grade.

Animal maintenance

The animal experiment and all procedure were carried out in accordance with guidelines for care and use of laboratory animals of Institutional Animal Ethics Committee (IAEC), Guru Ghasidas Vishwavidyalaya, Bilaspur (C.G) India (Registration number: 994/a/Re/06/CPCSEA). Female adult albino rats (Wistar strain) weighing 150 ± 10 g of approximately same age were procured from Defence Research and Development Establishment (DRDE) Gwalior. They were housed in polypropylene cages with proper bedding, feeding and water ad libitum. After an adaptation period of two weeks rats were randomly divided into following experimental groups.

Groups	Treatment	Group	No. of Rats
Control	(1% CMC)	I	6
Letrozole	(1 mg/kg body weight, P. O)	II	6
Letrozole + Melatonin	(1 mg/kg body weight, P. O) (200 µg/100 g body weight/day, I.P)	III	6
Melatonin	(200 µg/100 g body weight/day, I.P)	IV	6

Table 1: Different experimental groups.

Drugs and treatment

Melatonin solution was made by dissolving it in few drops of ethanol and then diluted with normal saline (0.9% NaCl) up to the desired concentration. Letrozole was dissolved in 1% CMC (Vehicle solvent) and then the desired volume was added to obtain the concentration (1.00 mg/kg body weight) was prepared. Group I comprising control rats were given orally 1% CMC/100 g body weight/day with the help of oral gauge. Group II female rats were given oral supplementation of letrozole (1 mg/kg body weight) and Group III female rats were those with PCOS condition induced by letrozole and then received melatonin (200 µg/100g body weight/day) for 28 days. Group IV rats were given melatonin (200 µg/100 g body weight/day) alternatively. At the end of the (28^{th} days) animals of each groups were sacrificed following complete anaesthesia (anaesthetic ether) and were subjected to ovarian wet analysis, Histological block preparation and sectioning, MDA assay to note lipid peroxidation (LPO) [25]. Blood plasma was separated by centrifugation and was stored as -20°C for ELISA of melatonin (Mel), Testosterone (T), Estrogen (E) and progesterone (P), luteinizing hormone (LH) and Follicle stimulating hormone (FSH).

Treatment of 1 mg/kg/body weight successfully induces PCOS condition in female rat

In a pilot experiment six female rats were subjected to two doses of letrozole -non-steroidal aromatase inhibitor, to induce (0.5 mg/kg/bodyweight and 1 mg/100 g/bodyweight) to induce polycystic ovarian condition [25-28]. Letrozole concentration 1 mg/kg/bodyweight/day was noted appropriate and sufficient to induce polycystic condition in female rats as observed with the histological preparation and therefore the same was selected for further experiment. Vaginal smear of PCO induced female rats showed presence of cornified cells denoting failure of at least two consecutive estrus cycles and leading to persistent (PE) estrus cycles.

Selection of melatonin dose

Following four doses of melatonin were given for 28 days to check the most effective one. 50 µg/100 g body weight/day; 100 µg/100 g body weight/day; 150 µg/100 g body weight/day; 200 µg/100 g body weight/day. Melatonin treatment with 200 µg/100 g body weight/day was noted to be the most effective dose.

Gravimetric analysis

Ovaries were removed and the adherent fat was removed. The weight of ovaries was taken to observe the changes in ovarian weight

on induction and after the treatment of melatonin in order to compare it with control.

Histological Preparation

Ovaries of all the four groups were harvested, cleaned from adherent fat and connective tissue, and fixed in Bouin's fixative for at least 24 hours. Then the Ovaries were processed and embedded. Ovarian histological sections of 6 µm thick were cut using rotary microtome (Leica RM 2125-RT 5) and then stained with hemotoxylene and eosin. Representative photomicrographs of respective groups were captured in Trinocular research microscope (Leica DM 2000) under 40X, 100X and 400x magnifications.

Lipid peroxidation assay (LPO)

Thiobarbituric acid reactive substances (TBARS) are produced during oxidative damage to cell membrane. Malondialdehyde (MDA), one of the major lipid breakdown product and commonly used parameter to assess lipid peroxidation. Ovaries were excised and weighed for the preparation of 10% tissue homogenates in 20 mM Tris Hydrochloride (HCl) buffer (pH-7.4). The homogenates were centrifuged at 3000 g for 15 min at 4°C and supernatant was subjected to Thiobarbituric acid (TBA) assay by mixing it with 8.1% SDS, 20% acetic acid, 0.8% TBA and boiling for 1 h at 95°C. The reaction mixture was immediately cooled in running water and vigorously shaken with n-butanol and pyridine reagent (15:1) and centrifuged for 10 min at 1500 g. The absorbance of the upper phase was measured at 534 nm. LPO was expressed as TBARS in nmol/g tissue weight by taking 1,1,3,3 tetraethoxypropane (TEP) as standard. The standard curve was calibrated using 10 nM concentration of TEP [29].

Hormone enzyme linked immunosorbent assay

Blood samples were collected directly from heart in a heparinized tube and centrifuged at 1000 × g for 15 minutes to collect the plasma. The blood plasma of each group of female rats were stored at -20°C for subsequent hormonal assay of melatonin (Mel), luteinizing hormone (LH), Follicle stimulating hormone (FSH), Testosterone (T), Estrogen (E) and progesterone (P) following ELISA as per the manual kit of Melatonin (IBL-Germany RE54041), LH and FSH (Monobind Inc., Costa Mesa, U.S.A) Testosterone, Estrogen and Progesterone (DRG International, GmbH, U.S.A). The intra and inter assay coefficient of variance (CV) and recovery were 5.5%, 8.5% and 92% respectively. The sensitivity of the melatonin, LH, FSH, testosterone, Progesterone, estrogen ELISA were 0.3 pg/ml, 0.054 mIU/ml, 0.134 mIU/ml, 0.083 ng/ml, 0.045 ng/ml, 9.71 pg/ml respectively.

Statistics

All the data were presented as means with n indicating the number of animal. Statistical significance of difference between the groups was assayed by two-tailed student t-test and one way analysis of variance. Calculations were performed using commercial software SPSS (IBM).The difference were considered significant $P<0.05$ and $P<0.01$.

Results

Melatonin treatment to PCO rats rears the ovarian weight

A significant increase resulted following letrozole induced PCO in female rats. Melatonin treatment alone and in combination with letrozole restored the normal ovarian weight comparable to the control group of female rat. Development of many ovarian cysts and poorly developed and anovulated ovarian follicles might be the cause of increased ovarian weight (Figure 1).

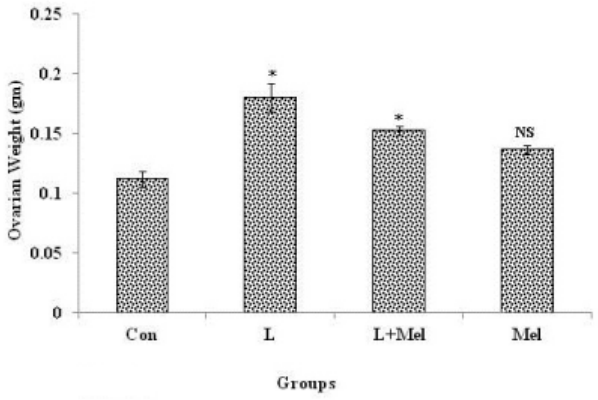

Figure 1: Effect of exogenous melatonin on ovarian weight of letrozole induced (PCOS) showing significant increase in ovarian tissue weight, melatonin treatment showing a reversal to control group. Histogram represents Mean ± SE; n=6, Con=Control, L=Letrozole, L+Mel= Letrozole+Melatonin, Mel= Melatonin. Con vs. L=$p<0.05$, Con vs. L+Mel=$p<0.05$, Con vs. Mel=NS, L vs. L +Mel=NS. NS: Non Significant.

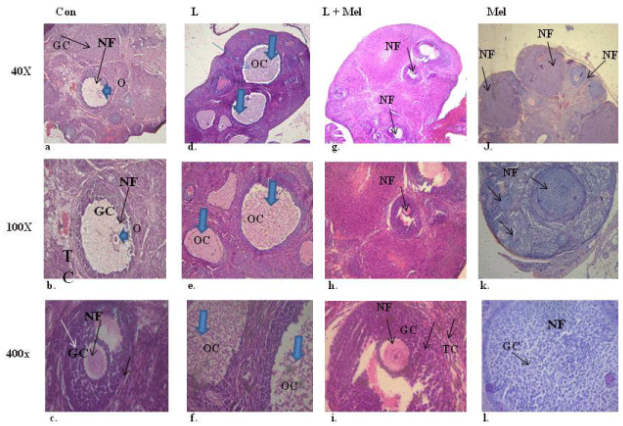

Figure 2: Histomicrograph showing changes of the rat ovarian tissues in Con: Control, L: Letrozole, L+Mel: Letrrozole+Melatonin and Mel: Melatonin groups (40X upper panel; 100 X middle panel and 400X lower panel) **a, b, c)** showing normal follicle growth in Control Group (Con), GC: Granulosa Cell, O: Oocyte, OC: Ovarian Cyst, NF: Normal Follicle, TC: Theca Layer; **d, e, f)** Showing induction of poly cystic ovarian syndrome following letrozole treatment; **g, h, I)** melatonin given to letrozole induced PCO rats showing reversal on healthy ovarian follicles, **j, k, l)** Melatonin treatment maintaining the normal ovarian follicle architecture.

Melatonin treatment to the PCO female rats (200 µg/100 g bw/day) reduces number of cysts and restore follicular architecture.

Histomicrograph of the letrozole treated ovaries clearly showed that letrozole non-steroidal aromatase inhibitor regularly for 28 days resulted in sub-capsicular cysts and capsicular thickening with incomplete luteinisation and reduction in the number of corpora leutea of the subjected female ovaries. The combined treatment of melatonin to letrozole induced poly cystic ovaries (PCO) showed recovered cellularity as that of the healthy follicles of control female rats. Further, melatonin alone treated rat ovaries maintains their healthy histological follicular architecture. This histological data strongly relates decrease number of cyst to the free radical scavenging capacity of melatonin which has provided a healthy internal milieu for further development and maturation to ovarian follicles following melatonin treatment (Figure 2).

Melatonin attenuates increased free radical in letrozole induced PCO rats

Letrozole induced PCO rats showed significant increase in lipid peroxidation (LPO) expressed in terms of thiobarbituric acid (TBARS Level). Melatonin treatment to letrozole induced PCO rats resulted in significantly decreased free radical production (TBARS level). However melatonin treatment alone had reduced TBARS level of ovarian tissue even lower than the ovaries of control female rat ovaries. The increased lipid peroxidation (LPO) in letrozole induced polycystic rat ovaries suggests the involvement of free radicals generation and role of redox imbalance which is main responsible cause for PCO condition as a result of generation of reactive oxygen species (ROS). Melatonin treatment lowered the level of free radical generation because of being strong anti-oxidant and free radical scavenger (Figure 3).

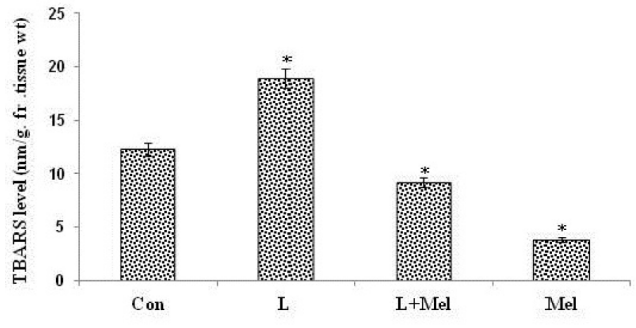

Figure 3: Effect of exogenous melatonin on lipid per oxidation (LPO) in letrozole induced PCO rats showing significant increase in lipid peroxidation (LPO) analyzed in terms of TBARS level; Melatonin treatment effectively and significantly lowered lipid per oxidation (LPO) of PCO rats. Histogram represents Mean+SE; n=6; Con: Control, L: Letrozole, L+Mel: Letrozole+Melatonin, Mel: Melatonin, Con vs. L=p<0.05, Con vs. L+Mel=p<0.05, Con vs. Mel=p<0.05, L vs. L+Mel=p<0.05.

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Melatonin treatment to PCO rats reverses the normal circulatory plasma level of hormones estrogen (E), progesterone (P) and testosterone (T)

Following induction of polycystic ovary in adult female rat by non-steroidal aromatase inhibitor letrozole, a significant decrease in plasma estrogen, progesterone levels were noted. However, contrary to E and P a significant increase in circulatory testosterone level was noted in PCO rats. Treatment of melatonin with a concentration of 200 µg/100 g body weight/day brought a significant increase in plasma estrogen and progesterone level and it was comparable to the control female rats. The testosterone level was also noted significantly decrease following melatonin treatment to the induced polycystic condition in female rat and melatonin alone treated female rats as well. Letrozole being a non-steroidal aromatase inhibitor might have affected the physiological functional pathway of steroidogenesis leading to such hormonal imbalance and irregularities of E, P and T in circulation (Figures 4-6).

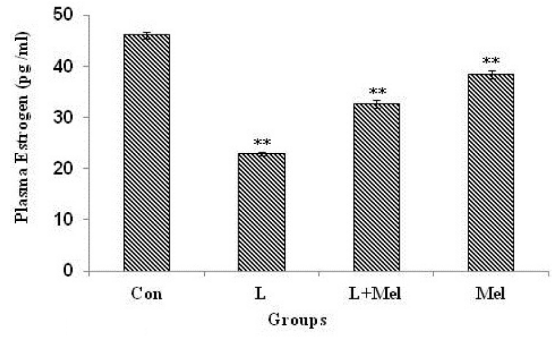

Figure 4: Effect of exogenous melatonin on circulatory level of plasma estrogen level in PCO rats showing recovery of estrogen with significant increase as compared to control rat group. Histogram represents Mean+SE; n=6; Con: Control, L: Letrozole, L+Mel=Letrozole+Melatonin, Mel: Melatonin. Con vs. L=p<0.01, Con vs. L+Mel=p<0.01, Con vs. Mel=p<0.01, L vs. L+Mel=p<0.01.

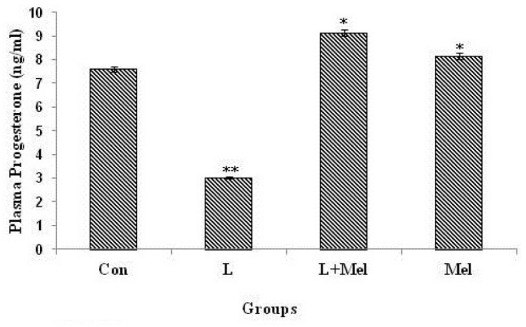

Figure 5: Effect of exogenous melatonin on circulatory level of plasma progesterone in PCO rats showing recovery with a significant increase of progesterone as compared to control groups of rats; Histogram represents Mean ± SE; n=6; Con: Control, L: Letrozole, L+Mel=Letrozole+Melatonin, Mel: Melatonin. Con vs. L=p<0.01, Con vs. L+Mel=p<0.05, Con vs. Mel=p<0.05, L vs. L +Mel=p<0.05.

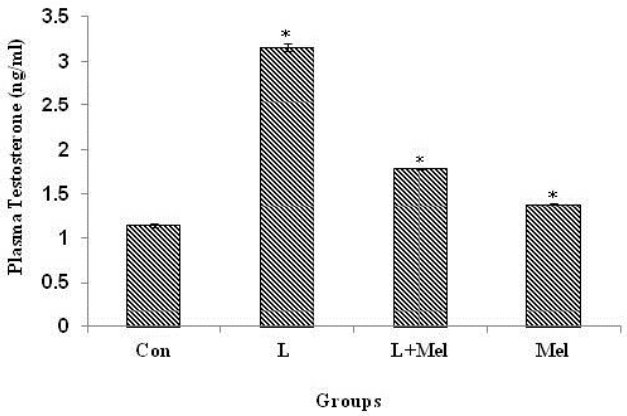

Figure 6: Effect of exogenous melatonin on circulatory level of plasma testosterone in PCO rats showing significant decreased in plasma testosterone as compared to control groups of rats; Histogram represents Mean ± SE; n=6; Con=Control, L=Letrozole, L+Mel=Letrozole+Melatonin, Mel: Melatonin. Con vs. L=p<0.05; Con vs. L+Mel=p<0.05; Con vs. Mel=p<0.05; L vs. L+Mel= p<0.05.

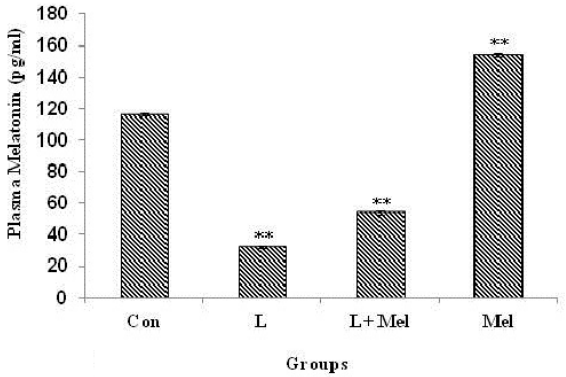

Figure 7: Effect of melatonin on circulatory level of plasma melatonin in PCO rats showing significant decrease in plasma melatonin level. Melatonin treatment maintained its own endogenous plasma level comparable to control groups of rats; Mean ± SE; n=6, Con: Control, L: Letrozole, L+Mel=Letrozole +Melatonin, Mel: Melatonin. Con vs. L=p<0.01; Con vs. L +Mel=p<0.01; Con vs. Mel=p<0.01; L vs. L+Mel=p<0.01.

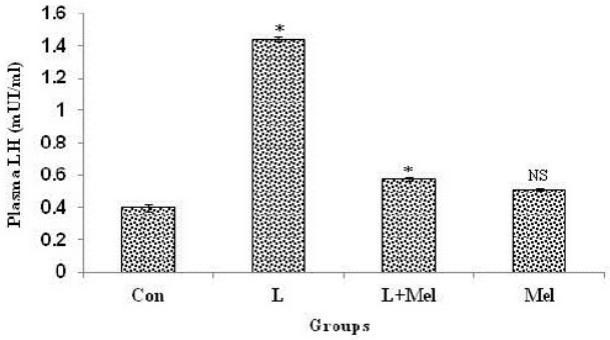

Figure 8: Effect of exogenous melatonin on circulatory level of luteinizing hormone (LH) in PCO rats; showing recovery with a significant decrease in LH level as compared to control groups of rats; Histogram represents Mean ± SE; n=6, Con=Control, L=Letrozole, L+Mel=Letrozole+Melatonin, Mel=Melatonin. Con vs. L=p<0.05; Con vs. L+Mel=p<0.05; Con vs. Mel=NS; L vs. L +Mel=p<0.05. NS: Not Significant.

PCOS condition decreases the circulatory plasma level of melatonin

Letrozole induced polycystic female rats showed a significant decreased level of circulatory plasma melatonin which shows a recovery pattern following melatonin treatment to the letrozole group as well as melatonin alone (Figure 7). Presence of normal circulatory level of melatonin is expected for healthy ovarian follicular growth. The increase in circulatory level of testosterone is expected to inhibit melatonin production because of the reciprocal relationship between testosterone and melatonin. The ovarian tissues following melatonin injections were sensitive and therefore responded to further normal functional status of recovery.

Melatonin maintains the normal level of circulatory plasma Gonadotropins to the tolerable ratio (LH and FSH)

An abrupt and significant increase of LH and FSH was noted. The circulatory level of gonadotropins was noted with the significant increased surge in the female rats with PCOS and an irregular ratio of LH/FSH. However, melatonin treatment in combination with letrozole as well as alone brought the level of circulatory gonadotropins very close to the control group with a recovery to their normal ratio. The hypothalamus-pituitary gonadal axis (HPGA) can be said to be very sensitive with exogenous melatonin treatment and therefore the pulsatile secretion of LH and FSH was restored which ultimately might have influenced on E and P circulatory level (Figures 8 and 9).

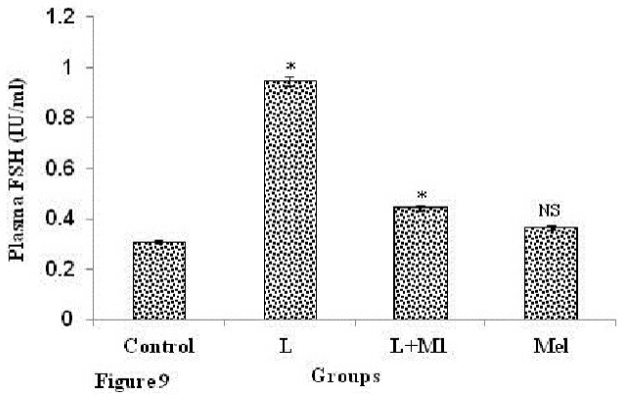

Figure 9: Effect of exogenous melatonin on circulatory level of follicle stimulating hormone (FSH) in PCO rats; showing recovery with a significant decrease in FSH level compared to control groups of rats; Histogram represents Mean+ SE; n=6; Con: Control, L: Letrozole, L+Mel=Letrozole+Melatonin, Mel: Melatonin. Con vs. L=p<0.05, Con vs. L+Mel=p<0.05, Con vs. Mel=NS, L vs. L +Mel=p<0.05. NS: Not Significant.

Discussion

The pathophysiology of polycystic cystic ovarian syndrome (PCOS) is subject of many hypothesis and speculations. Although many studies were undertaken in this regard but pathophysiology of PCOS remains unclear and unexplained. Present study explains about similarity of ovarian cysts in rats to that observed in human polycystic ovary syndrome (PCOS) and their regression following exogenously administered melatonin. PCOS is a common disorder characterized by chronic anovulation and bilateral polycystic ovaries, without truly unique clinical parameters. Female rat shows short length of the estrus cycle (4-5 days) which makes them ideal for investigating numerous physiologic changes happening during reproductive cycle. In the present study persistent estrus cycles was noted during the induction procedure. The induction of ovarian cyst was done by injecting letrozole (a non-steroidal aromatase inhibitor) 1 mg/kg body weight for continuous 28 days and melatonin and neurohormone was supplemented to the PCO rat model hypothesizing the fact that this indoleamine has an anti-gonadotropic action on the anterior hypothalamus [30].

A significant increase in ovarian weight of PCOS induced rats were noted as compared to control groups however melatonin treatment of 200 µg/100 bw/day decreased the ovarian weight. It is expected that treatment of melatonin might have checked at cellular proliferation, differentiation and apoptosis as well as tissue re-modeling to maintain and bringing back the ovarian weight comparable to the normal group of female rats. Present data coincided with the previous report suggesting that daily melatonin administration to rats suppresses body weight, intra abdominal adiposity, and plasma leptin levels [31].

In present study it was noted that induction of PCO condition significantly increased the level of lipid peroxidation (LPO) measured in terms of TBARS. The increase in (LPO) coincides with the previous report that free radical increase is one of the major etiologies for PCOS. Melatonin treatment to the PCOS female rat significantly decreased the free radical noted in terms of LPO and TBARS. It is expected that melatonin being a strong antioxidant and free radical scavenger might have inhibited the free radical generation and therefore lowered the same. The present data is in correlation and of the previous literature considering melatonin as an antioxidant [32,33]. Oxidative stress in the oocyte induces lipid peroxidation of membrane and DNA damage which are expected to cause harmful effects in cell division, metabolite transport and mitochondrial function [33,34]. Although the role of melatonin in reproduction is focused on its direct actions in the ovary, however as melatonin is a lipophilic molecule therefore it can cross the cell membrane and enter inside all tissues irrespective of the tissue specificity and its concentration [33,35,36]. A number of studies demonstrated that melatonin is a powerful direct scavenger of free radicals the high lipophilicity and hydrophilicity of melatonin permits its rapid transfer into other organs and fluid and melatonin can easily pass through cell membrane [33]. Interestingly, the female rats received continuous melatonin treatment resulted in lower free radical generation when compared to that of controlled group this further supports the previous data where melatonin is reported as a strong buffering molecule the ovarian internal milieu required for follicle maturation [37].

Histopathological observation of ovarian tissue showed the formation of cysts in letrozole induced groups. The ovarian cortex exhibited the presence of atritic follicle and formation of more than two cysts in the ovary. The cyst shows attenuated layer of granulosa cells and hyperplasia as reported earlier [38-40]. Histopathological study the PCO females rats receiving melatonin injection daily in evening showed marked recovery and restoration of the ovarian tissue with the presence of normal structure of follicle with normal granulosa and theca layer comparable to control female rats. The ovarian cortex showed the presence of many follicles in various stage of development.

The successful reproduction in all manners depends on the function of hypothalamus-pituitary-gonadal (HPG) axis which regulated the LH and FSH release from the anterior pituitary via the respective gonadotrops these gonadotropins LH and FSH stimulates the specific cells in the ovary leading to ovulation. Considering these neuro-endocrinological regulations of ovarian physiology the circulatory level of testosterone, progesterone, estrogen along with the circulatory gonadotropins (LH, FSH) was measured in all experimental group of female rats. The circulatory level of testosterone was noted to increase significantly when compared to the normal rats, melatonin treatment to the PCOS rats brought circulatory testosterone to normal. The increase in testosterone level of PCO rats can be correlated with the fact that Letrozole being an aromatase inhibitor might have blocked the conversion of testosterone to estradiol lead to hyperandrogenism and anovulatory condition, the clinical feature of PCOS [41,42]. Further, too much of androgen can cause early leutinization of ovarian granular cells, stopping follicular development and growth leading to follicle atresia leading to anovulation and or poor ovulation [37]. In the present finding circulatory level of plasma estrogen and progesterone was noted to be significantly low in PCOS rats as compared to the control group. Estrogen being the regulatory hormone for the development and maturation of the follicle in the perifollicular phase whereas progesterone in the later phase. These data may be explained on the basis of enzyme inhibition of P450 aromatase. This enzyme converts testosterone and androstenedione into estradiol and estrone respectively. The inhibition of the biosynthetic pathway at the conversion point of testosterone to estradiol and progesterone resulted in the disproportionate regulatory gonadal hormones. Exogenous treatment of melatonin to PCO rats showed a tendency towards the recovery of the circulatory level of estrogen and progesterone. The

exact mechanism how melatonin is maintaining the circulatory level may be explained that exogenous melatonin might have reverted back the steroidogenic pathway and enzymatic level *via* its receptor (mt1/ mt2) and enable the normal conversion of testosterone and estrogen. It is reported that melatonin receptors are present in the granulosa and theca cells both of which occur in mature follicles and in lutein cells which promotes the production of sex steroid hormones [43]. Melatonin in association with estrogen is expected to have is contribution in providing a healthy maintenance to the ovarian follicles for development, differentiation and maturation.

In the present finding asymmetrical ratio of LH and FSH (gonadotropins) was noted in induced PCO rats and were noted in regaining the normal ratio with melatonin supplementation. Recovery of LH and FSH following melatonin treatment to the PCO rat can be explained on the basis of specific pulsatile secretory pattern of melatonin to the hypothalamus pituitary gonadal axis via the feed-back loop which has resulted to normalization the LH and FSH ratio. The normalization in the ratio of LH and FSH has further maintained the level of Estrogen and progesterone and restored normal ovarian function of rats. The exact mechanism of melatonin action on the neuroendocrine system effecting reproduction is not known. However, one plausible possibility is that melatonin acts directly by affecting the hypothalamic functions involved in the inhibitory regulation of gonadotrophin- releasing hormone (GnRH) [44]. The release of pituitary gonadotrophic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), often occurs on a rhythmic basis with the period of release ranging from an ultradian (i.e., about 1- 4 h) to a circadian (i.e., about 24 h) and a seasonal (i.e., about 1 year) pattern.

Conclusion

PCOS results in anovulation and therefore impairs the fertility. The histopathological, hormonal and free radical assessment here suggest that melatonin supplementation to PCO rats lead to recovery to the normal histological architecture and circulatory hormone profile (T, E, P) including the ratio of gonadotropins (LH/ FSH) with a marked reduction in lipid per oxidation . Melatonin showed good antiandrogen effect by reducing elevated level of testosterone and thus prevented ovarian dysfunction in PCO rats. Melatonin therefore may be considered as a multifunctional molecule responsible for its efficacy in the management of PCOS. However, the present finding can be used as the baseline data for further investigations. The vast amount of research conducted on the mammalian pineal and melatonin has led to great progress in understanding the molecular processes of its synthetic and secretory abilities at the peripheral level. The evidence that melatonin functions through multiple receptors, both membrane and nuclear, as well as a direct free radical scavenger, a process that requires no receptors, is unequivocal. Furthermore, melatonin increases fertilization rates and reduces oxidative damage in the FF. However, the usage of melatonin for ovarian diseases, such as PCOS, endometriosis, and POF, is limited. Nonetheless, much more studies needed to explore the involvement of melatonin to understand its therapeutic potential during pathophysiology of PCO. The present findings may indicate a novel therapeutic approach based on the modulation of pathogenicity of PCOS in female rats through melatonin to improve the functional ovarian physiology as a possible future molecule among human females to treat the infertility. Such clinical trials may really prove to be highly beneficial for women with PCOS.

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