

## Receptor Cross Talk and Interplay between Melatonin and Ovarian Thyroid Axis in a Letrozole Induced Polycystic (Pco) Rat

Hindole Ghosh, Seema rai\*, Muddasir Basheer and Younis Ahmad Hajam

Department of Zoology, Guru Ghasidas Vishwavidyalaya, Guru Ghasidas University, Chhattisgarh, India

\*Corresponding author: Seema Rai, Department of Zoology, Guru Ghasidas Vishwavidyalaya, Guru Ghasidas University, Chhattisgarh, India, E-mail: drseemakamlesh@gmail.com

Received date: March 26, 2018; Accepted date: May 01, 2018; Published date: May 07, 2018

Copyright: ©2018 Ghosh H, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Abstract

The objective of present study was to establish the interrelationship between thyroid and melatonin during anovulatory/letrozole induced polycystic ovarian condition on female Wister rats. Rats were procured and after acclimatization 20 rats were divided in 4 groups with 5 rats in each. They were divided as Control, Letrozole induced PCO rat (1 mg/kg BW/d), and melatonin alone (200 µg/100 g BW/d). The experiment was conducted for the duration of 28 d. Assessment of gravimetric, hormonal profile and thyroid histology and relative expression of MT1, MT2, and ERα, (thyroid, ovary) Dio2, TRα (Ovary) done followed by standard protocol. Histological observation showed shrinkages in thyroid follicles in PCO rats however exogenous melatonin maintained the cellular architecture and normal thyroid weight. PCO rats showed significantly high circulating testosterone but significant decreased in estrogen and progesterone level. Circulatory gonadotropins (LH, FSH) were noted significantly high in PCO rats. Melatonin injection to the PCO rats however reversed to the control level and restored. Circulatory TSH level in PCO rats were noted suppressed where as T3 and T4 were non-significantly increased suggesting a reciprocal relation between melatonin and thyroxine. Thyroid tissue of PCO rats expressed MT1 and MT2 in way alternate and opposite way being MT1 as up regulated whereas down regulation of MT2. Ovarian tissue of PCO rats showed reverse receptor expression to that of thyroid tissue being MT1 down regulated and MT2 was noted unregulated. Parallel relation was noted between ERα and TRα receptor expression in thyroid and ovarian tissue respectively. PCO rats resulted in up regulation of Dio2, receptor expression in a non-significant manner. Therefore, present finding suggests a fine interplay and cross talk *via* melatonin its two receptor MT1, MT2 with ERα, TRα, and Dio2 thyroid and ovarian tissue as the case between ovarian thyroid axis hence maintaining a physiological trade-offs between these gland with a tonic regulation to maintain melatonin and thyroid homeostasis during polycystic pathogenicity.

**Keywords:** Melatonin; Thyroid gland; Mt1 and Mt2 receptors; Letrozole; Polycystic ovarian (PCO) condition; T3 and T4 hormone

### Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine and principle secreting hormone of pineal gland. This neurohormone is recognized to regulate the seasonal reproduction, the phasing of mammalian circadian rhythm [1,2]. Beside playing the circadian oscillator it has an important role as antioxidant [3,4] being a strong free radical scavenger [5], antiaging [6] and potential anti carcinogenic activities[7], including suppressive effect on secretion and growth processes of the thyroid gland [8,9].

Melatonin regulates its physiological role *via* its membrane bound receptor MT1 and MT2 [10]. Both the receptors MT1 and MT2 are 7 transmembrane Gi/o protein coupled receptor which works *via* cAMP, Protein Kinase C (PKC) and Diacylglycerol Mediated Pathway (DAG) [9,11]. Human MT1 and MT2 receptor showed approximately 60% overall and 75% homology of amino acid with each other within the transmembrane domain.

The thyroid gland is one of the most important glands regulating iodine metabolism. It metabolizes iodine for the synthesis and secretion of its T3 and T4 hormone. The thyroidal anomalies have shown effects on the ovarian physiology. Ovarian atrophy was reported in induced hypothyroid rats [12,13]. The reports of clinical evidences

suggest that women suffering from thyroidal disorders are most frequently associated with disturbances in reproductive, ovarian cycles and impaired fertility. The animal studies in monkey uterus have shown long term steroids influence to the thyroid hormones, Thyroid Stimulating Hormone (TSH) as well as Thyrotropin Releasing Hormone (TRH) receptors [13]. It is also reported that formation of ovarian follicular cysts are also may be caused by the influence of hypothyroidism as it increases ovarian sensitivity to gonadotropin action thus may be followed by consequences like irregular menses, breakthrough bleeding, low endometrial thickness and sometimes non proliferative endometrium [13]. Further ovarian atrophy has been reported following thyroid suppressive drug [14]. Report suggests decreased number of primordial antral and Graafian follicles in hypothyroid rats without any significant changes in reproductive behavior [15]. The induced neonatal hypothyroidism showed disturbed folliculogenesis and absence of corpora lutea [16,17]. The thyroid hormone was reported to be synergized with FSH to exert direct stimulatory effects on granulosa cell function, LH/CG (Chorionic gonadotropin) receptor formation and activation of steroidogenic enzymes [14]. Ovary is known as the prime female reproductive and endocrine organ because of the presence of reproductive steroid (estrogen and progesterone) secreting cells. This syndrome (PCO) may broadly be categorized or classified as clinical, endocrinological, and metabolic. The clinical category evidences irregular reproductive cycle, acne, alopecia, hirsutism, frequent abortions, anovulation which finally leads to infertility. The endocrinological category shows elevated

gonadal steroids, LH [4] and prolactin levels. The metabolic category features lipid abnormality, obesity, insulin resistance and type 2 diabetes [18]. Diseases like hypothyroidism, glucose intolerance, diabetes and cardiovascular may have links with PCOS [19]. Previous studies on women with PCOS showed thyroid volume is associated with age, anthropometry, smoking, iodine status and hyperinsulinemia [20].

Increasing problem of PCOD among human population in the present era becoming alarming day by day and may be one of the causes of infertility. Previous studies proved that hypothyroidism is a very common metabolic and hormonal disorder associated with reproductive abnormalities like menstrual disorders, amenorrhea, frequent abortions and infertility. The autoimmune thyroiditis is a most prevalent cause for hypothyroidism and found in females with PCOS condition [21]. PCOS was previously hypothesized to be responsible for the irregularity of gonadal steroid thus causing autoimmune thyroiditis [21].

The exogenous melatonin proved to be potential to protect the ovarian condition against PCOS [4]. A trade-off relation between the thyroxine and melatonin in Indian palm squirrel has already being proposed by research group of Rai in 2005 [22]. Reproductive abnormalities during PCOS have received much attention but till date least attention has been given to understand the metabolic irregularities and dysfunction during PCOS condition. Therefore, the study was designed to elucidate the physiological cross talk between ovarian and thyroid tissue during pathogenesis of letrozole induced polycystic ovarian (PCO) rat model. The study is designed with an objective to explore the functional correlation between these two-glands following exogenous melatonin treatment by assessing cellular, hormonal and receptor (MT1, MT2, ER $\alpha$ , TR $\alpha$ , Dio2) expression PCO rat.

## Materials and Methods

### Chemicals

Letrozole (Let), Melatonin (Mel), L-thyroxine (T4) were obtained from Sigma Aldrich (USA). Carboxy Methyl Cellulose (CMC) was obtained from, Himedia. All the acquired chemicals were of analytical grade. ELISA kits of LH, FSH, TSH (Monobind Inc., Costa Mesa, U.S.A), Serum T4 kit (Genway), Melatonin (IBL-Germany RE54041) were purchased. PVDF hybond (Amersham, UK) membrane, Mel 1R antibody (goat IgG, sc 13179, santacruz Biotech, USA), horseradish peroxidase conjugated secondary antibody (1: 20,000) (donkey anti-goat, sc 2020, IgG) were acquired for receptor expression by western blot technique.

### Animal model

The animal experiment and all procedures were carried out in accordance with guidelines for care and use of laboratory animals of institutional animal ethical committee (IAEC), Guru Ghasidas Vishwavidyalaya, Bilaspur (C.G) India (Registration number: 994/GO/ERe/S/06/CPCSEA). Female adult albino rats (Wister strain) weighing 150-180 g of approximately same age were procured from Defence Research 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 (Table 1).

Group	Condition/Dose	No. of Rats
Control	1% CMC	6
Letrozole	1 mg/Kg BW	6
Letrozole+Melatonin	(Letrozole) 1 mg/kg BW, (Melatonin)200 $\mu$ g/100 gm BW	6
Melatonin	200 $\mu$ g/100 gm BW	6

**Table 1.** Random division of rats after two weeks adoption period

## Drugs and Treatment

### Induction of polycystic ovary by oral dose of letrozole

Letrozole concentration 1 mg/kg/bodyweight/d 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 [4].

### Treatment of exogenous melatonin on letrozole induced PCO rats

Melatonin solution was made by dissolving it in few drops (approx. 100  $\mu$ l) of ethanol and then diluted with normal saline (0.9% NaCl) up to the desired concentration. Group I comprising control rats were given orally 1% CMC/d with the help of oral gauge. Group II and III female rats were orally supplemented with Letrozole (1 mg/kg body weight/d) to induce PCO condition. Group III females, with PCOS condition, received melatonin (200  $\mu$ g/100 g body weight/d). Group IV rats were given melatonin alone treatment (200  $\mu$ g/100 g body weight/d). The duration of the experiment was for 28 d (Table I). At the end of the experiment animals of each group were sacrificed following complete anaesthesia. Thyroid glands of all experimental groups were dissected and cleaned subjected to thyroid weight analysis. One pair of thyroid gland of each group was fixed in Bouins solution for histological block preparation and sectioning. Blood serum was separated by centrifugation at 3000 rpm for 15 min and was stored in deep freeze (-20°C) for ELISA of melatonin, thyroxine (T4), triiodothyronin (T3), Thyroid Stimulating Hormone (TSH), Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). Further, thyroid and ovary tissues were treated to assay receptor expression of MT1, MT2, TR $\alpha$ , ER $\alpha$  and Dio2.

## Parameters Studied

### Gravimetric analysis of thyroid tissue

Thyroid gland was dissected out in normal saline and adherent fat was removed. The weight of organ was noted and expressed in mg.

### Histological Preparation of thyroid tissue

Thyroids of all four groups were previously fixed in Bouin's fixative for 24 h. Then the tissues were washed in running tap water followed by the process of dehydration by alcohol grades. The dehydrated tissues were cleared with the clearing agent xylene followed by the

paraffin embedding. Thyroid histological sections of 7 μm thick were cut using rotary microtome (Leica RM 212-RT 5), stretched and fixed in gelatin coated glass slides and then stained with hematoxylin and eosin with gradual course of rehydration and dehydration after removing the wax coating from the stretched tissue. Representative photomicrographs of respective groups were captured in Trinocular research microscope (Leica DM 2000) under 100X and 400X magnifications and observation was documented.

## Hormonal Assay

### Enzyme linked immunosorbent assay (ELISA)

Blood samples were collected directly from heart in non-heparinized collecting tube and centrifuged at 3000 rpm for 15 min to collect the serum. The blood serum of each group of female rats were stored for subsequent hormonal assay of Melatonin, Thyroxine (T4), triiodothyronin T3, Thyroid Stimulating Hormone (TSH), Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), Testosterone, estrogen, progesterone following ELISA as per manual kit of LH, FSH, TSH, Testosterone, estrogen and progesterone (Monobind Inc., Costa Mesa, USA), Serum T4, T3 kit (Genway), Melatonin (IBL-Germany RE54041).

### qRT-PCR

qRT-PCR was done for the expression assay of MT1, MT2, ERα, Dio2, TRα by first extracting the total mRNA, followed by cDNA synthesis. The primers were purchased from Imperial life sciences (P) limited (Table 2).

### List of primers

Gene Product	Forward	Reverse
MT 1	5'-CGTTGGTGCTGATGTCG-3'	5'-AGTTTGGGTTTGC GGTC-3'
MT 2	5'-CAATGCTGCTGCGAGGCG-3'	5'-GGCGGTGGTGACGATG-3'
TR α	5'-TGGACCTGGTTCTAGACGATTC A-3'	5'-TCCCGTTCTGCTCAATCA-3'
Er α	5'-TAAGAACCGGAGGAAGAGTTG-3'	5'-TCATGCGGAATCGACTTG-3'
Dio 2	5'-AATTATGCCTCGGAGAAGACCG-3'	5'-GGCAGTTGCCTAGTAAAGG T-3'
β-actin	5'-GGAAATAGGGTTAGCAC-3'	5'-CTCATGTGCGCCTACTTA-3'

Table 2. Gene products along with their primers

### Statistical analysis

Statistical analysis was performed using two tailed student t-test and one way analysis of variance. Calculations were performed using commercial software SPSS.16 (SPSS, Chicago, IL, USA). At a probability value P<0.05, P<0.01 and p<0.001 were considered to be statistically significant.

## Results

### Gravimetric analysis

**Effect of exogenous melatonin on weight of thyroid gland:** The Letrozole induced PCO rats showed significant (P<0.001, 0.01) decrease in the weight of thyroid. Exogenous melatonin to PCO rats showed recovery in the tissue weight significantly (P<0.001, 0.01) comparable to that of control group. However, melatonin alone maintains the healthy weight of thyroid compared to the control (Figure 1).

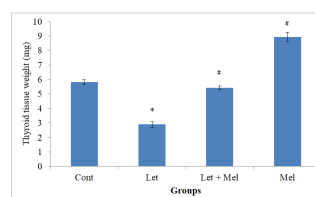


Figure 1: Effect of melatonin on thyroid weight. Histogram represents changes in thyroid tissue weight in PCO condition with treatment of melatonin. Cont vs. Let; (P<0.05=\*) Let vs. Let+Mel, Let vs. Mel; (P<0.05=#). Cont=Normal rat, Let=PCO rats, Let +Mel=Letrozole+Melatonin Mel=Melatonin.

### Histological Observation

**Effect of exogenous melatonin on cellular architecture of thyroid follicle:** PCOS condition resulted in significant decrease in the number of thyroid follicle. PCOS rat receiving melatonin injection showed significant recovery in cellular architecture and the number of thyroid follicle comparable to the normal condition. (Figure 2) melatonin alone resulted in healthy thyroidal architecture.

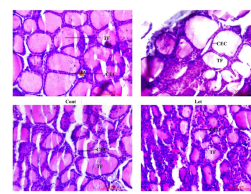
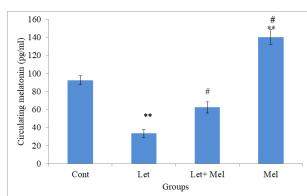


Figure 2: Histomicrograph showing cellular architectural changes of thyroid gland in hematoxyline eosin staining (40X) during PCO condition. Cont=Normal rat, Let=PCO rats, Let+Mel=Letrozole +Melatonin; Mel: Melatonin; CEC: Cuboidal Epithelial Cells; TF: Thyroid Follicles. Letrozole induced PCO rat induced hypothyroid condition that showed decreased number of thyroid follicle per scale and enlarged thyroid follicles with heavy colloid quantity. However, melatonin injection to PCO condition recovers the cellular architecture. Exogenous melatonin however resulted in increase in the number of thyroid follicles with no change in the thyroid cellular architecture.

### Hormonal assay (ELISA)

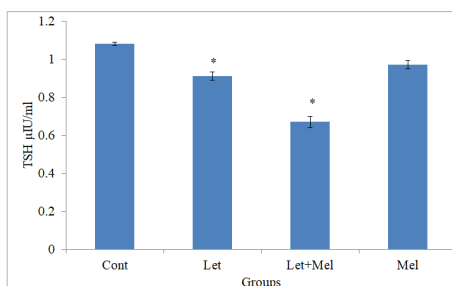
**Effect of exogenous melatonin in circulating hormone levels in induced PCOS rat:** Melatonin: Significant decrease was noted in

circulating melatonin level of PCO rats. Treatment of exogenous melatonin however restored circulating melatonin level comparable to the control group (Figure 3).

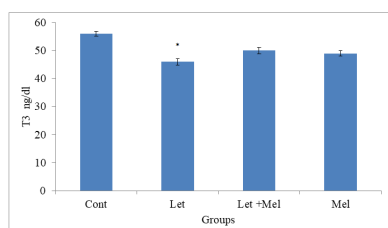


**Figure 3:** Effect of melatonin on plasma melatonin level. Histogram represents Mean+SE; n=6, Cont vs. Let, Cont vs. Mel (P<0.001=\*\*) Let vs. Let+Mel, Let vs. Mel (P<0.05=#). Cont=Normal rat; Let=PCO rats; Let+Mel: Letrozole+Melatonin; Mel: Melatonin.

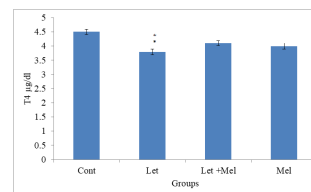
**Thyroid hormones (TSH, T3 T4):** PCO condition resulted in suppression of circulatory TSH level. Melatonin treatment to PCO rats showed further suppression of serum circulatory TSH levels (Figure 4). Non-significant elevation in serum circulatory level of the T3 and T4 levels was noted in letrozole induced PCO rats. Melatonin treatment to PCO could not restore T3 and T4 circulatory levels to of the normal rat (Figures 5 and 6).



**Figure 4:** Effect of melatonin on Serum TSH level. Histogram represents Mean+SE; n=6. Cont vs. Let, Cont vs. Let+Mel. Data were significant at p<0.05. Cont=Normal rat; Let=PCO rats; Let +Mel: Letrozole+Melatonin; Mel: Melatonin.

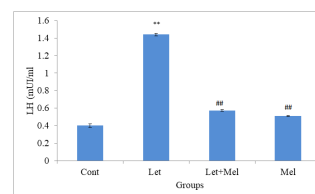


**Figure 5:** Effect of melatonin on Serum T3 level. Histogram represents Mean+SE; n=6, Cont vs. Let, Cont vs. Let+Mel. Data were significant at p<0.05. Cont=Normal rat, Let=PCO rats, Let +Mel: Letrozole+Melatonin; Mel: Melatonin.

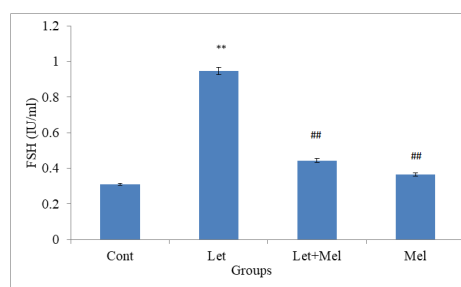


**Figure 6:** Effect of melatonin on Serum T4 level. Histogram represents Mean+SE; n=6, Cont vs. Let, Cont vs. Let+Mel. Data were significant at p<0.05. Cont=Normal rat, Let=PCO rats, Let +Mel: Letrozole+Melatonin; Mel: Melatonin.

**Gonadotropin (LH and FSH):** PCO rats showed significant increase in circulatory serum level of LH and FSH with an irregular ratio. Melatonin treatment to PCO restored the LH and FSH circulatory level comparable to control (Figures 7 and 8).

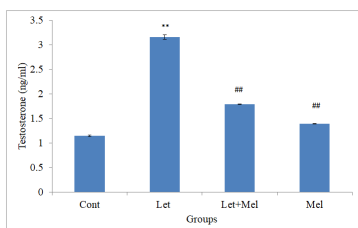


**Figure 7:** Effect of melatonin on plasma LH level. Histogram represents Mean+SE; n=6, Cont vs. Let, Let vs. Let+ Mel (P<0.01=\*\*), Let vs. Let+Mel, Let vs. Mel (P<0.01=##). Cont=normal rat, Let= PCO rats, Let+Mel: Letrozole+Melatonin; Mel= Melatonin.

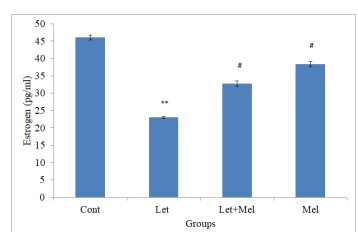


**Figure 8:** Effect of melatonin on plasma estrogen level. Histogram represents Mean+SE; n=6, Cont vs. Let, Let vs. Let+Mel (P<0.01=\*\*), Let vs. Let+Mel, Let vs. Mel (P<0.01=##). Cont=normal rat, Let= PCO rats, Let+Mel: Letrozole+Melatonin; Mel= Melatonin.

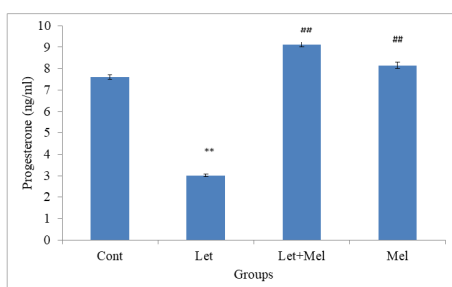
**Gonadal Hormones (Testosterone, estrogen, Progesterone):** PCO rats resulted in significantly high serum circulatory testosterone but estrogen and progesterone levels were noted significantly decreased. Melatonin supplementation to the PCO rats were resulted in normal circulatory level of gonadal steroid (Figures 9-11).



**Figure 9:** Effect of melatonin on plasma testosterone level. Histogram represents Mean+SE; n=6, Cont vs. Let, Let vs. Let+Mel ( $P<0.01=**$ ), Let vs. Let+Mel, Let vs. Mel ( $P<0.01=##$ ). Cont=Normal rat, Let=PCO rats, Let+Mel: Letrozole+Melatonin Mel: Melatonin.

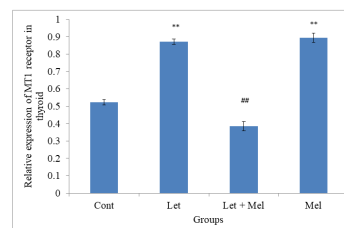


**Figure 10:** Effect of melatonin on plasma FSH level. Histogram represents Mean+SE; n=6, Cont vs. Let, Let vs. Let+Mel ( $P<0.01=**$ ), Let vs. Let+Mel, Let vs. Mel ( $P<0.05=#$ ). Cont=Normal rat, Let= PCO rats, Let+Mel: Letrozole+Melatonin Mel: Melatonin.

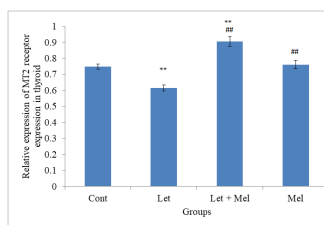


**Figure 11:** Effect of melatonin on plasma progesterone level. Histogram represents Mean+SE; n=6, Cont vs. Let, Let vs. Let+Mel ( $P<0.01=**$ ), Let vs. Let+Mel, Let vs. Mel ( $P<0.01=##$ ). Cont=Normal rat, Let= PCO rats, Let+Mel: Letrozole+Melatonin Mel: Melatonin.

**Effect of exogenous melatonin on MT1, MT2, ER $\alpha$  receptor expression on thyroid:** Letrozole induced PCO condition showed a significant ( $P<0.01$ ) up regulation of MT1 but down regulation of MT2 receptor expression on thyroid tissue, Injection of melatonin however, down regulated the relative expression comparable to the normal level (Figures 12 and 13).

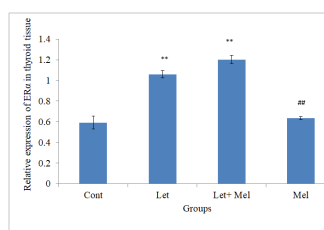


**Figure 12:** Histogram represents relative receptor expression of MT2. The data are expressed as mRNA expression level in thyroid. Data are expressed as mean  $\pm$  SEM, N=6 females per group, Cont vs. Let, Let vs. Let+Mel ( $P<0.01=**$ ), Let vs. Let+Mel, Let vs. Mel ( $P<0.01=##$ ). Cont=Normal rat, Let=PCO rats, Let+Mel: Letrozole +Melatonin Mel: Melatonin.



**Figure 13:** Histogram represents relative receptor expression of MT2. The data are expressed as mRNA expression level in thyroid. Data are expressed as mean  $\pm$  SEM, N=6 females per group, Cont vs. Let, Let vs. Let+ Mel ( $P<0.01=**$ ), Let vs. Let+Mel, Let vs. Mel ( $P<0.01=##$ ). Cont=Normal rat, Let=PCO rats, Let+Mel: Letrozole +Melatonin Mel: Melatonin.

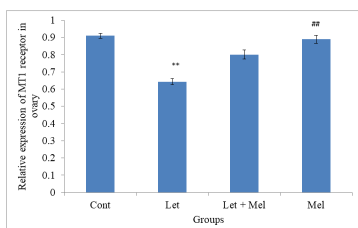
Thyroid tissue of PCO rats showed upregulation of ER $\alpha$  receptor expression. Injection of exogenous melatonin further recovered ER $\alpha$  relative expression by up regulating the same (Figure 14).



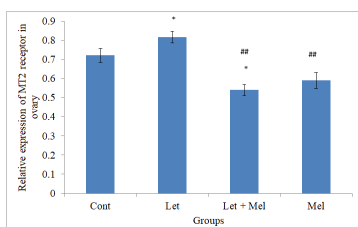
**Figure 14:** Histogram represents relative receptor expression of ER $\alpha$ . The data are expressed as mRNA expression level in thyroid. Data are expressed as mean SEM, N=6 females per group, Cont vs. Let, Let vs. Let+Mel ( $P<0.01=**$ ), Let vs. Let+Mel, Let vs. Mel ( $P<0.01=##$ ). Cont=Normal rat, Let=PCO rats, Let+Mel: Letrozole +Melatonin Mel: Melatonin.

**MT1, MT2, Dio2 and TR $\alpha$  receptor expression in ovary:** Down regulation of MT1 and up regulation in MT2 receptor expression was noted on ovarian tissue of letrozole induced rats. However, exogenous

melatonin restores the relative expression comparable to the normal level (Figures 15 and 16).

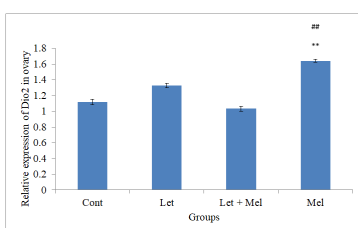


**Figure 15:** Histogram represents relative receptor expression of MT1. The data are expressed as mRNA expression level in ovary. Data are expressed as mean  $\pm$  SEM, N=6 females per group, Cont vs. Let, Let vs. Let+ Mel ( $P<0.01=*$ ), Let vs. Let+Mel, Let vs. Mel ( $P<0.01=##$ ). Cont=Normal rat, Let=PCO rats, Let+Mel: Letrozole +Melatonin Mel: Melatonin.



**Figure 16:** Histogram represents relative receptor expression of MT2. The data are expressed as mRNA expression level in ovary. Data are expressed as mean  $\pm$  SEM, N=6 females per group, Cont vs. Let, Let vs. Let+ Mel ( $P<0.05=*$ ), Let vs. Let+Mel, Let vs. Mel ( $P<0.01=##$ ). Cont=Normal rat, Let= PCO rats, Let+Mel: Letrozole +Melatonin Mel: Melatonin.

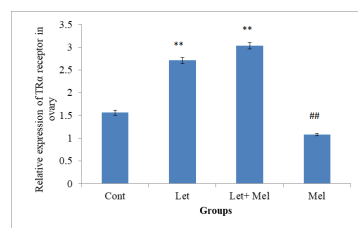
Receptor expression for Dio2 on ovarian tissue of PCO rats were found down regulated. PCO rats treated with exogenous melatonin however resulted in non-significant restoration (Figure 17).



**Figure 17:** Histogram represents relative receptor expression of Dio2. The data are expressed as mRNA expression level in ovary. Data are expressed as mean  $\pm$  SEM, N=6 females per group, Cont vs. Let, Let vs. Let+ Mel ( $P<0.05=*$ ), Let vs. Let+Mel, Let vs. Mel ( $P<0.01=##$ ). Cont=Normal rat, Let=PCO rats, Let+Mel: Letrozole +Melatonin Mel: Melatonin.

Ovarian tissue of PCO rats showed upregulation in TRa receptor expression when compared to control. However supplementation of

melatonin to PCO could not restored significantly the TRa receptor expression (Figure 18).



**Figure 18:** Histogram represents relative receptor expression of TRa. The data are expressed as mRNA expression level in ovary. Data are expressed as mean  $\pm$  SEM, N=6 females per group, Cont vs. Let, Let vs. Let+ Mel ( $P<0.05=*$ ), Let vs. Let+Mel, Let vs. Mel ( $P<0.01=##$ ). Cont=Normal rat, Let=PCO rats, Let+Mel: Letrozole+Melatonin Mel: Melatonin.

## Discussion

The Polycystic Ovarian Syndrome (PCOS) has many hypothesis and speculations about its pathophysiology which may further lead to metabolic diseases such thyroid dysfunctions. In present study letrozole induced PCOS female rats resulted a significant decrease in thyroid gland weight. Further, histological photomicrograph of thyroid gland evidenced in decrease in the cellular density and scanty of thyroid follicles in letrozole induced PCOS female rats. However the treatment of exogenous melatonin showed a normalization of the condition by increasing the density and quantity of the thyroid follicle comparable to the normal condition. The exogenous melatonin treatment to the normal rats reduced the number of thyroid follicles in the thyroid gland. Decrease in thyroid gland weight can be correlated with the decrement of lesser cellular density of thyroid follicle in PCO rats as compared to the control group. PCO condition might have resulted stress induced by hyperandrogenism during PCO condition and hence led to thyroid follicular damage. Melatonin supplementation to PCO rats resulted in restoration of thyroid gland weight as well as the thyroid cellularity. The melatonin being virtue of its strong antioxidant and free radical scavenger might have rescued and protected the thyroid gland during PCO pathogenicity. [9]. The thyroid gland weight might be decreased due the oxidative stress produced by the PCOS condition [4]. Melatonin which is a potent antioxidant is synthesized and received by the thyroid gland that may regulate the thyrocyte function and thyroid volume [9]. Our finding is in agreement of the previous reports suggesting thyroid volume correlation and variation of circulatory gonadotropin levels during in the PCOS endocrinopathy [20]. Our finding also presents the disturbance in the pulsatility and ration of gonadotropins (LH and FSH). The changes in the gonadotropin level might be expected to impact negatively to the thyroid follicles. Results suggests the sensitivity of the thyroid gland for exogenous melatonin which in turn be acting on the hypophyseal-pituitary ovarian axis and hence regularizing the serum circulatory gonadotropin level in PCO rats receiving melatonin injections and therefore thyroid follicle density restored.

Present finding provides information that PCO rats present significant decrease the circulatory serum level of thyroxine. However the exogenous melatonin treatment balances the T4 secretion

comparable to the normal level. The decreased T4 secretion might be resulted by the PCOS condition as it was previously reported that hypo and hyperthyroidism is related with the reproductive abnormality [23,13]. Earlier finding suggest impairment of ovarian function in hypothyroid condition which is reversible by the treatment of exogenous thyroxine injections supporting the present ovarian-thyroid correlation confirming that PCO condition may cause thyroid dysfunction specifically hypothyroidism. The exogenous melatonin reducing the T4 level in PCOS condition might have effect on recovering the reproductive capability of female rats. The tradeoff relation between melatonin and thyroxine might be the key mediator providing a tonic regulation to maintain thyroid hormone homeostasis during PCOS condition following melatonin injection [22]. Nonetheless, it has also been reported that melatonin administration influences the reproductive physiology by controlling iodothyronine-deiodinase followed by circulating thyroxine levels [9].

The mammalian reproductive function depends upon the Hypothalamus-Pituitary-Gonadal Axis (HPG) which regulates the gonadotropins (LH and FSH) from anterior pituitary *via* gonadotrophs. Considering the neuroendocrine and reproductive connection of PCOS, in the present experiment we measured the gonadotropin and melatonin levels in the circulation in all experimental groups. The asymmetrical ratio of gonadotropins was noted in the PCOS condition. The LH ratio was noted higher in the PCOS condition whereas the FSH was noted to be down regulated significantly. The melatonin supplementation in the PCOS rats regained the normal ratio of the gonadotropin circulation. The regulation of gonadotropins in the PCOS condition by melatonin can be hypothesized that melatonin inhibits the secretion of the GnRH from the hypothalamus [24]. The letrozole is an aromatase inhibitor that prevents the conversion from testosterone to estradiol in the ovary, this might intern affect the gonadotropin secretion thus the variation of the circulatory level [4]. As a result we get a significantly high elevation on the testosterone level. In case of hypothyroidism it is reported that the gonadotropins, gonadal steroids and Sex Hormone Binding Globulin (SHBG) gets a higher surge. As other reports also says that the hyperthyroid may also cause gonadotropin dysfunction that may explain the reason behind the gonadotropin dysfunction and a surge in thyroxine level in case of letrozole induced PCOS condition [13]. However the exact mechanism of the regulation by melatonin is not known. The melatonin level was showed a down regulation in the circulation in the PCOS condition. The decrease might be hypothesized and linked with the increased thyroxine level at the same condition. The thyroxine secretion and melatonin shows a trade-off relation [22] that might be responsible for the down regulation of the melatonin level in circulation. The up regulation of melatonin and the down regulation of thyroxine were noted in the treatment of exogenous melatonin in PCOS condition confirming trade-off relation between the two hormones.

In the present work opposite and crisscross expression of MT1 and MT2 was noted on thyroid follicle during PCO pathogenicity. The MT1 expression on thyroid tissue during PCO condition was upregulated whereas MT2 showed a down regulation but not significant. Interestingly ovarian tissue also showed similar trade for MT1 and MT2 receptor expression respectively. The MT2 (Figure 16) receptor expression reciprocates with the expression pattern of MT1 receptor expression in ovary. The affinity binding might have result the reciprocal relationship between MT1 and MT2 receptor gene expression in case of PCOS condition.

A parallel relation was noted towards the expression of ER $\alpha$  on thyroid tissue and TR $\alpha$  on ovarian tissue. Presence of Dio2 receptor was noted on the ovarian tissue which was upregulated during PCO condition. The comparative receptor expression of thyroid ER $\alpha$  and ovarian Dio2 and TR $\alpha$  has a parallel relationship in spite of these functional association are with thyroid and ovary respectively. Presence of MT1 and MT2 receptor and variation in there expression in PCO rat indicates there involvement in regulation of ovarian function *via* melatonin. However, MT1 and MT2 expression are regulating thyroid function in trade off manner similar to circulating melatonin and thyroxine. Previous studies revealed presence of MT1 receptors along with AANAT and HIOMT activity on thyroid follicular cells which further supports our present results [25]. Further, literatures provide evidences that melatonin mediates it impact either *via* MT1/MT2 receptor of thyroid cells or directly influencing thyroid physiology through TTF1 and PAX 8 pathway [9] but these pathway don not involve MT1/MT2 proteins. Possibly TSH, T4 and PAX8 are in an agreement to control the thyroidal function as the increased T4 indicates the role of TSH. The endogenous melatonin either pineal or non-pineal origin along with other physiological agents might be involved in regulation of the thyroid function [26]. The main hormone responsible for PCOS condition is estrogen focusing this fact we observed relative expression of ER $\alpha$  receptor on thyroid tissue. The receptor expression for ER $\alpha$  showed up regulated during PCO pathogenesis. Present result can be correlated with variation in circulatory thyroxine level being decreased in letrozole induced PCO rats. Upregulation of ER $\alpha$  during PCO pathogenicity indicates influence toward HPT axis regarding further synthesis of thyroxine therefore is the signal for ER $\alpha$  mediated cell proliferation as a consequence cascade to elevated expression of the same.

The decrease in the circulatory levels of T3, T4 and TSH insisted to check the relative expressions of TR $\alpha$  and Dio2 enzyme in the ovary as these two polypeptides are the key regulatory molecule to regulate the functions of thyroid hormones in the target tissue. Dio2 shows a greater relative expression in case of letrozole induced PCOS condition. Ovarian tissue needs more triiodothyronine in case of PCOS condition as a result of the decreased secretion of T3. Dio2 converts more T4 to T3 to regulate the homeostasis of the HPG and HPT axis cross talk. The expression of TR $\alpha$  also shows significant increase in the relative expression in case of PCOS condition comparable to the normal rat. The increased expression of thyroid receptor also indicates the correlation between these two tissues. This result initiates a logical evidence and explanation about the crosstalk between thyroid and ovarian axis. In nut shell, as depicted in the results the present study shows a close relation between thyroid and ovarian machinery. In addition we found a relation between gonadotropins and thyroid hormone interplay with underlying regulation by melatonin in thyroid physiology and a hint of redox control mechanism. However, further studies may show the perfect machinery for the melatonin receptor pathway in the thyroid gland in induced PCOS condition. Nevertheless, further investigations are required to note the underlying mechanism of melatonin and PCOS in thyroidal physiology and pathologic conditions.

## Acknowledgment

Authors are thankful for financial assistance Under the DBT BUILDER Program (Sanction number BT/PR7020/INF/22/172/2012) by Department of Biotechnology New Delhi for Junior Research Fellowships to M Basheer and H Ghosh. Equipment and financial

assistance from Department of Zoology, Guru Ghasidas Vishwavidyalaya, Bilaspur India, University Grants Commission-Major Research Project [(E.No.41-94/2012 (SR)] and UGC-BSR Start-up Grant [(No.F.20-1/2012 (BSR)/20-3(3)/ 2012(BSR)] New Delhi to Dr. Seema Rai is highly acknowledged.

## Conflict of Interest

Authors declare no conflict of interest.

## References

- Rai S, Haldar C (2006) Adaptive significance of Annual variation in immune parameters and endogenous hormones (Melatonin and thyroxine) of a tropical rodent *Funambulus pennanti*. *J Endocrinol Reprod* 10: 111-116.
- Revel FG, Pevet MM, Pevet P, Mikkelsen JD, Simonneaux V (2009) Melatonin Controls Seasonal Breeding by a Network of Hypothalamic Targets. *Neuro Endocrinol* 90: 1-14.
- Reiter RJ, Paredes SD, Manchester LC, Tan DX (2009) Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin. *Crit Rev Bio Chem Mol Biol* 44: 175-200.
- Rai S, Basheer M, Ghosh H, Acharya D, Hajam YA (2015) Melatonin attenuates free radical load and reverses histological architect and hormonal profile alteration in female rat: An *in vivo* study of pathogenesis of letrozole induced poly cystic ovary. *J Clin Cell Immunol* 6: 384.
- Goswami S, ChandanaHaldar C (2016) Melatonin Pre-treatment Alletes UVA Radiation Induced Oxidative Stress and Apoptosis in the Skin of a Diurnal Tropical Rodent *Funambulus pennant*. *J Nucl Med RadiatTher* 8:318.
- Reiter RJ, Tan DX, Mayo JC, Sainz RM, Lopez BS (2002) Melatonin, longevity and health in the aged: an assessment. *Free Radic Res* 36: 1323-1329.
- Lissoni P (2007) Biochemotherapy with immunomodulating pineal hormones other than melatonin: 5-methoxytryptamine as a new oncostatic pineal agent. *Pathol Biol (Paris)* 55: 198-200.
- Lewinski A, Karbownik M (2002) Melatonin and the thyroid gland. *Neuro Endocrinol Lett* 23: 73-78.
- Marin GR, Santos JMF, Bernal JM, Martinez FG, Roman VV, et al. (2015) Melatonin in the thyroid gland: Regulation by thyroid stimulating hormone and role in thyroglobulin gene expression. *J Physiol Pharmacol* 66: 643-652.
- Dubocovich ML, Cardinali DP, Delagrang PRM, Krause DN, Strosberg D, et al. (2000) The IUPHAR Compendium of Receptor Characterization and Classification. Melatonin Receptors. In: Girdlestone D (ed.).
- Reppert SM, Weaver DR, Ebisawa T (1994) Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron* 13: 1177-1185.
- Ortega E, Rodriguez E, Ruiz E, Osorio C (1990) Activity of the hypothalamo-pituitary ovarian axis in hypothyroid rats with or without triiodothyronine replacement. *Life Sci* 46: 391-395.
- Gaberscek S, Zaletel K, Schwetz V, Pieber T, Obermayer-Pietsch B, et al. (2015) Mechanisms in Endocrinology: Thyroid and Polycystic Ovary Syndrome. *Eur J Endocrinol* 172: 9-21.
- Asahara S, Sato A, Aljonaid AA, Maruo T (2003) Thyroid Hormone Synergizes with Follicle Stimulating Hormone to Inhibit Apoptosis in Porcine Granulosa Cells Selectively from Small Follicles. *Kobe J Med Sci* 49: 107-116.
- Meng L, Rijntjes E, Swarts HJM, Keijer J, Teerds KJ (2017) Prolonged hypothyroidism severely reduces ovarian follicular reserve in adult rats. *J Ovarian Res* 10: 19.
- Dijkstra G, de Rooij DG, de Jong FH, van den Hurk R (1996) Effect of hypothyroidism on ovarian follicular development, granulosa cell proliferation and peripheral hormone levels in the prepubertal rat. *Eur J Endocrinol* 134: 649-654.
- Shivaprasad KS, Dutta D, Jain R, Kumar M, Maisnam I, et al. (2013) Huge bilateral ovarian cysts in adulthood as the presenting feature of Van WykGrumbach syndrome due to chronic uncontrolled juvenile hypothyroidism. *Indian J Endocrinol Metab* 7: 164-166.
- Tsilchorozidou T, Overton C, Conway GS (2004) The pathophysiology of Polycystic ovary syndrome. *Clin. Endocrinol* 60: 1-17.
- Bhuvaneshwari S, Poornima R, Averal HL (2015) Management of obesity in polycystic ovary syndrome induced albino rats with Pergulariadaemia. *Int J Appl Res* 1: 779-783.
- Cakir E, Sahin M, Topaloglu O, Colak NB, Karbek B, et al. (2012) The relationship between LH and thyroid volume in patients with PCOS. *J Ovarian Res* 5: 43.
- Janssen OE, Mehlmauer N, Hahn S, Offner AH, Gaertner R (2004) High prevalence of autoimmune thyroiditis in patients with polycystic ovary syndrome. *Eur J Endocrinol* 150: 363-369.
- Rai S, Haldar C, Singh SS (2005) Trade-off between L-thyroxine and melatonin in immune regulation of the Indian palm squirrel, *Funambulus pennant* during the reproductively inactive phase. *Neuroendocrinology* 82: 103-110
- Mochizuki M (1997) Thyroid gland menstrual disorders. *Obstetr Gynecol Therap* 36: 41-45
- Roy D, Belsham D (2002) Melatonin Receptor Activation Regulates GnRH Gene Expression and Secretion in GT1-7 GnRH Neurons. *J Biol Chem* 277: 251-258.
- Garcia-Marin R, Miguel MD, Fernandez-Santos JM, Carrillo-Vico A, Utrilla JC, et al. (2012) Melatonin-Synthesizing Enzymes and Melatonin Receptor in Rat Thyroid Cells. *Histol Histopathol* 27: 1429-1438.
- Mocchegiani E, Santarelli L, Costarelli L, Cipriano C, Muti E, et al. (2006) Plasticity of neuroendocrinethymus interactions during ontogeny and ageing: role of zinc and arginine. *Ageing Res Rev* 5: 281-309.