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Immunonohistochemical analyses of melatonin (MT1) and estrogen (ER-α) receptors in rat ovary and uterus during estrus and diestrus phase

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Abstract

The reproductive endocrinology governs all these dynamic changes occurring both in ovary and uterus. We performed this study to note the differential expressional pattern of melatonin and estrogen receptors in ovary and uterus of albino Wistar rat, *Rattus norvegicus* under two different phases of estrous cycle. The immunohistochemical analyses of our present study showed that during the estrus phase when the circulatory level of estrogen is high, the estrogen receptor (ER-α) immunopositive cells were also more in number. The ER-α immunopositive cells were more prominently noted in uterine endometrium as well as in epithelial cells of luminal space lining of uterus. Melatonin receptor (MT1) immunoreactive cells were noted in granulos cells as well as in oocyte cytoplasm of estrus phase ovary while the diestrus phase ovary showed less number of immunoreactive cells in the corpora lutea. Similarly, during the estrus phase the ER-α immunopositive cells are more intense in uterus especially in epithelial cellular lining of space and in endometrium while during the diestrus phase it showed decrease expression. During the diestrus phase the MT1 immunopositive cells were more prominent in the endometrium and luminal epithelial cell whereas it showed weak immnunopositive cells in uterus during the estrus phase. Thus, we can say that there is differential expression of estrogen and melatonin receptors during the estrus and diestrus phase of reproductive cycle. Thus it can be hypothesised that melatonin therapy may become a new treatment for improving oocyte and uterus quality to treat infertile women.

Key Words: Melatonin, estrogen, ovary, uterus, estrus, diestrus

Introduction

Female reproductive cycle involves various steps such as ovarian follicle growth followed by ovulation and luteinisation that leads to events like successful fertilization and implantation. The reproductive endocrinology governs all these dynamic changes occurring both in ovary and uterus. The pubertal onset in female results from an orderly

arranged sequential events following establishment of a luteinizing hormone (LH) pulsatile release that is important for ovarian maturation (Andrews and Ojeda 1981). Ovulation occurs in the young adult laboratory rat every four to five days (Ojeda and Urbanski 1994). Estrogen acts through its receptor ERα to mediate its effect on ovary and uterus. During estrus phase estrogen level is high while during diestrus phase progesterone level is high (Walmer et al, 1992).

Melatonin, a hormone of darkness (Lerner et al. 1958) regulates many physiological functions such as reproduction, immunity in various species (Gupta and Haldar, 2017; Vishwas and Haldar, 2012 ; Mukherjee and Haldar, 2014). Melatonin acts through its well characterized G protein coupled receptors (GPCRs) named MT1 and MT2 (Dubocovich and Markowska, 2005). These receptors for melatonin are documented in ovary and uterus suggesting its potential role in regulation of ovarian and uterine physiology at local level (Brzezinski et al., 1987; Ronnberg et al., 1990). Both receptors were detected in human (Niles et al., 1999) and murine granulos cells (Clemens et al., 2001) also. Melatonin regulates uterine growth and functions in rats and mice (Hertz-Eshel and Rahamimo, 1965; Hipkin, 1970) where it inhibits prostaglandin synthesis (Gimeno et. al., 1980) and stimulates the progesterone synthesis (MacPhee et. al., 1975). Further, melatonin also regulates uterine estrogen and prolactin receptor activities in hamsters and minks (Danforth et. al., 1983). Pinealectomy results in increased number of abortions in rats, where it also inhibited blastocyst implantation (Guerra et. al.,1973; Berria et. al., 1989;

May and Mead 1986) thus revealing the importance of melatonin in regulation of reproductive function. Therefore, we performed this study to note the differential expressional pattern of melatonin and estrogen receptors in ovary and uterus of albino Wistar rat, *Rattus norvegicus* under two different phases of estrous cycle.

2. Materials and Methods

All experiments were conducted in accordance with Institutional practice and within the framework of experimental animals (Scientific Procedure) Act 2007, of the Committee for the Purpose of Supervision and Control on Experiments on Animals (CPSCEA), Government of India on animal welfare.

2.1 Animal procurement and maintenance

The Adult female rats (average weight 150±10 g) were kept in commercial polypropylene cages and provided with commercial rodent food pellet and tap-water ad libitum and exposed to 12/12 h light/dark cycles at 25 ◦C. The animals had 4 or 5-day estrous cycles. Only those animals which had exhibited at least two consecutive 4-day cycles were used. The female rats (N=5 for each group) showing estrus and diestrus phase of estrous cycle were selected randomly for the study. Animals were sacrificed under deep ether anaesthesia. Ovaries and uterus were excised and randomly fixed in 10% neutral formalin for immunohistochemical studies.

2.2 Immunohistochemical studies

The immunohistochemical analyses were performed according to published method [Verma and Haldar, 2016]. Briefly, 7µm thick sections of ovary and uterus were mounted on gelatine coated slides and sequentially rehydrated. Endogenous peroxidase activity was blocked by immersing the slides in methanol containing 0.1% hydrogen peroxide for 30 minutes at room temperature. After washing thrice (10 minutes each) in phosphate buffered saline (PBS, pH 7.4), the slides were incubated in horse serum (1:100 in PBS,Vectastain ABC Kit,PK-6200,Vector Laboratories, Burlingame, CA) for 1 hour at RT. The sections were then incubated in primary antibodies against ERα (1:200, Thermo Scientific, Waltham, MA, USA; Catalogue no PA1-309) and MT1R (Mel 1a, 1:50, Santa Cruz Biotechnology Inc, Dallas, TX, USA, R-18, Catalogue no sc-13186) overnight at 4˚C. Sections were then washed thrice in PBS and incubated with biotinylated universal secondary antibody (1:50,Vectastain ABC Kit, PK-6200, Vector Laboratories, Birlingame, CA) for two hours at RT before washing them in PBS (thrice) and conjugated with pre-formed avidin-biotin complex (Vectastain ABC Kit, PK-6200,Vector Laboratories, Burlingame, CA) for 30 minutes at RT. Immunoreactivity was visualized with 0.03% peroxide substrate, 3,3-diaminobenzidine (DAB, Sigma Aldrich, St Louis, Missouri, USA) in 0.01M Tris-HCl (pH 7.6) and 0.1% H_2O_2.Counter-staining was done using Ehlrich's haematoxylin for MT1 (membrane bound receptor) except for ERα (nuclear antigen). The sections were dehydrated, cleared and mounted with DPX and observed under Nikon E200 research microscope (Japan).

3. Result

Intense immunopositivity for estrogen receptor-alpha (ERα) was noted in granulosa cells and oocytes of estrus phase (figure 1A,1C) whereas decreased intensity of immunopositive cells was noted in the ovarian sections of diestrus phase that mainly consists of large sized corpora lutea (figure 1B). Immunopositive cells for melatonin receptor (MT1R) were noted in the granulosa cells as well as in oocyte cytoplasm of estrus phase ovary (figure 2A). However weak immunostaining was observed diestrus phase ovary showing few immunoreactive cells in the corpora lutea (figure 2B, 2C).

Immunohistochemical analyses of ERα and MT1R in uterus of rats reveals that there is intense immunoreactivity for ER-α during estrus phase utrerine endometrial cells as compared to diestrus phase uterus (figure 3). While an intense immunoreactive cells for MT1R were noted in the uterus of diestrus phase mainly in the endothelium lining of lumen and endometrial cells of uterus as compared to estrus phase uterus (figure 4).

4. Discussion

Estrous cycles are specified by distinct morphological changes in female reproductive tract including ovaries, uterus and vagina (Goldman et al., 2007). The

phases of the estrous cycle in rats can be studied from the histoarchitectural appearance of the ovary and uterus that shows developmental stages of ovarian follicles and endometrial growth in uterus which are well synchronized with cyclic changes in hormonal milieus (Walmer et. al., 1992).

Melatonin receptors have been documented in the reproductive system of female where the two subtypes MT1 and MT2 are present in the cumulus-oocyte complexes, granulos cells and luteal cells of the ovary in humans and rats (El-Raey et. al., 2011; Niles et. al., 1999;Soares et. al., 2003). However, studies concerning estrous cycle dependent immunohistochemical expressional variation of melatonin and estrogen receptors are lacking.

The intra-follicular environment plays a pivotal role in oocyte development and differentiation. Follicular fluid contains anti-oxidants (Oyawoye et. al., 2003) which protect oocytes from damages caused by reactive oxygen species (ROS) which are generated as by products of cellular metabolism. Any imbalance in the production and elimination of these ROS may lead to abnormal development of the oocytes and thus will reduce fertility in females. Melatonin, the potent antioxidant molecule and free radical scavenger has been reported in ovarian follicular fluid (Brzezinski et al., 1992; Ronnberg et al., 1990), suggesting a direct effect of this hormone in ovarian functions such as to promote cumulus cell expansion, in vitro oocyte maturation, and embryo development (Bahadori et. al.,2013; Tamura et. al., 2012).

The immunohistochemical analyses of our present study showed that during the estrus phase when the circulatory level of estrogen is high (Walmer et al, 1992) the ER-α immunopositive intensed cells were noted in ovarian follicular granulos cells as well as in oocytes revealing its importance in the process of folliculogenesis. The ER-α immunopositive cells were more prominently noted in uterine endometrium as well as in epithelial cells of luminal space lining. MT1 immunoreactive cells were noted in granulos cells as well as in oocyte cytoplasm of estrus phase ovary while the diestrus phase ovary showed less intense immunoreactive cells in the corpora lutea. Similarly, during the estrus phase the ER-α immunopositive cells are more intense in uterus especially in epithelial cellular lining of space and in endometrium while during the diestrus phase it showed decrease expression. During the diestrus phase the MT1 immunopositive cells were more prominent in the endometrium and luminal epithelial cell whereas it showed weak immnunopositive cells in uterus during the estrus phase. Thus, we can say that there is differential expression of estrogen and melatonin receptors during the estrus and diestrus phase of reproductive cycle. Hence, utilization of melatonin as a therapeutic agent in women may regulate ovarian and uterine physiology by virtue of its well defined antioxidant and direct free radical scavenging potential under various pathological conditions leading to ovarian dysfunctions. Thus it can be hypothesised that melatonin therapy may become a new treatment for improving oocyte and uterus quality to treat infertile women.

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References:

Andrews WW and Ojeda S R (1981). A detailed analysis of the serum LH secretory profiles of conscious free-moving female rats during the time of puberty. *Endocrinology* **109**, 2032–39.

Bahadori MH, Ghasemian F, Ramezani M and Asgari Z (2013). Melatonin effect during different maturation stages of oocyte and subsequent embryo development in mice. *Iranian J Reprod Med*;11:11–18.

Berria M, Desantis M and Mead RA (1989). Lesions to the anterior hypothalamus prevent the melatonin-induced lengthening of delayed implantation. Endocrinology 125:2897–2904.

Brzezinski A, Seibel MM, Lynch HJ, Deng MH and Wurtman RJ (1987). Melatonin in human preovulatory follicular fluid. J. Clin. Endocrinol. Metab. 64, 865–867.

Brzezinski A, Fibich T, Cohen M, Schenker JG and Laufer N (1992) Effects of melatonin on progesterone production by human granulosa lutein cells in culture. Fertil Steril 58:526–529.

Clemens JW, Jarzynka MJ and Witt-Enderby PA (2001). Down-regulation of MT1 melatonin receptors in rat ovary following estrogen exposure. Life Sci. 69, 27–35.

Danforth DN, Tamarkin L, Do R and Lippman ME (1983). Melatonin- induced increase in cytoplasmic estrogen receptor activity in hamster uteri. Endocrinology; 113:81–85.

Dubocovich ML and Markowska M (2005). Functional MT1 and MT2 melatonin receptors in mammals. Endocrine 27, 101–110.

El-Raey M. Geshi M. Somfai T, Kaneda M, Hirako M, Abdel-Ghaffar AE, Sosa GA, El-Roos ME and Nagai T (2011). Evidence of melatonin synthesis in the cumulus oocyte complexes and its role in enhancing oocyte maturation in vitro in cattle. Mol. Reprod. Dev. 78, 250–262.

Gimeno MF, Landa A, Sterin SN, Cardinali DP and Gimeno AL ()1980. Melatonin blocks in vitro generation of prostaglandin by the uterus and hypothalamus. Eur J Pharmacol 62:309–317.

Goldman JM, Murr AS and Cooper RL (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Res B Dev Reprod Toxicol*, 80:84-97.

Gupta S and Haldar C (2017). Short day length enhances physiological resilience of the immune system against 2-deoxy-d-glucose-induced metabolic stress in a tropical seasonal breeder Funambulus pennant, Horm Behav. 26,89, 157-166.

Guerra MO, Silva NO, Guimaraes CS, Souza JP and Andrade AT (1973). Pinealectomy and blindness during pregnancy in the rat. Am J Obstet Gynecol; 115:582–583.

Hertz-Eshel M and Rahamimo R (1965). Effect of melatonin on uterine contractility. Life Sci; 4:1367–1372.

Hipkin LJ (1970). Effect of 5-methoxytryptophol and melatonin on uterine weight responses to human chorionic gonadotrophin. J Endocrinol; 48:287–288.

Lerner AB, Case JD, Takahashi Y, Lee TH and Mori N (1958). Isolation of melatonin, a pineal factor that lightens melanocytes. J. Am. Chem. Soc. 80, 2587.

MacPhee AA, Cole FE andRice BF (1975). The effect of melatonin on steroidogenesis by the human ovary in vitro. J Clin Endocrinol Metab; 40:688–696.

May R and Mead RA (1986). Evidence for pineal involvement in timing implantation in the western spotted skunk. J Pineal Res; 3:1–8.

Mukherjee A and Haldar C (2014). Photoperiodic rehulation of melatonin membrane receptor (MT1R) expression and steroidogenesis in testis of adult golden hamster, Mesocricetus auratus, J Potochemistry and Photobiology B: Biology, 140, 374-380.

Niles LP, Wang J, Shen L, Lobb DK and Younglai EV (1990). Melatonin receptor mRNA expression in human granulosa cells. Mol. Cell. Endocrinol, 156, 107–110.

Niles LP, Wang J, Shen L, Lobb DK and Younglai EV (1999). Melatonin receptor mRNA expression in human granulosa cells. Mol. Cell. Endocrinol. 156, 107–110.

Ojeda SR and Urbanski HF (1994). Puberty in the rat. In The Physiology of Reproduction, 2nd ed. (E. Knobil and J. D. Neill, eds.), pp. 363–409, Raven Press, New York.

Oyawoye O, Abdel Gadir A, Garner A, Constantinovici N, Perrett C and Hardiman P (2003). Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. *Hum Reprod*;18:2270–2274.

Ronnberg L, Kauppila A, Leppaluoto J, Martikainen H and Vakkuri O (1990). Circadian and seasonal variation in human preovulatory follicular fluid melatonin concentration. J. Clin. Endocrinol. Metab. 71, 492–496.

Soares JMJ, Masana MI, Ersahin C and Dubocovich ML (2003).Functional melatonin receptors in rat ovaries at various stages of the estrous cycle. J. Pharmacol. Exp. Ther. 306, 694–702.

Tamura H, Takasaki A, Taketani T, et al (2012). The role of melatonin as an antioxidant in the follicle. *J Ovarian Res* ;5:5.

Verma R and Haldar C (2016). Photoperiodic modulation of thyroid hormone receptor (TR-α), deiodinase-2 (Dio-2) and glucose transporters (GLUT 1 and GLUT 4) expression in testis of adult golden hamster, Mesocricetus auratus,. J Photochem Photobiol B, 165,351-358.

Vishwas DK and Haldar C (2012). Photoperiodic induced melatonin regulates immunity and

expression pattern of melatonin receptor MT1 in spleen and bone marrow mononuclear cells of male golden hamster, J Photochem Photobiol B, 128, 107-114.

Walmer DK, Wrona MA, Hughes CL and Nelson KG (1992). Lactoferrin expression in the mouse reproductive tract during the natural estrous cycle: correlation with circulating estradiol and progesterone. Endocrinol.; 131:1458–1466.

Figure Legends:

Figure 1: Immunohistochemical localization of estrogen receptor-alpha (ER-α) in ovary of rat. (A) Showing intense immunopositive cells for ER-α in ovarian follicles and corpus luteum of estrus phase ovary. (B) Showing the weak immunopositive cells for ER-α in the corpus luteum (CL) of diestrus phase ovary. (C) Showing intense immunopositive cells for ER-α in ovarian oocyte and its surrounding granulose cells. Magnification: A, B at 10X and C at 40X objective.

Figure 2: Immunohistochemical localization of melatonin receptor (MT-1) in rat ovary. (A) Showing immunopositive cells found to be present in developing follicles and mature follicles during estrus phase. (B) Showing the immunopositive cells for MT-1 in the corpus luteum during diestrus phase ovary. (C) Enlarged view of corpus luteum showing MT-1 receptor expression during diestrus phase ovary. Magnification: A, B at 10X and C at 40X objective.

Figure 3: Immunohistochemical localization of ER-α in uterus of rat. (A) Showing an intense immunopositive cells for ER-α in uterus of estrus phase rat. (B) Showing immunopositive for ER-α during in diestrus uterus of rat. Magnification: A, B at 40X objective.

Figure 4: Immunohistochemical localization of MT-1in uterus of rat. (A) Showing an weak immunopositive cells for MT-1 in uterus of estrus phase rat. (B) Showing intense immunopositive for MT-1 during in diestrus uterus of rat. Magnification: A, B at 40X objective.

Figure 1.

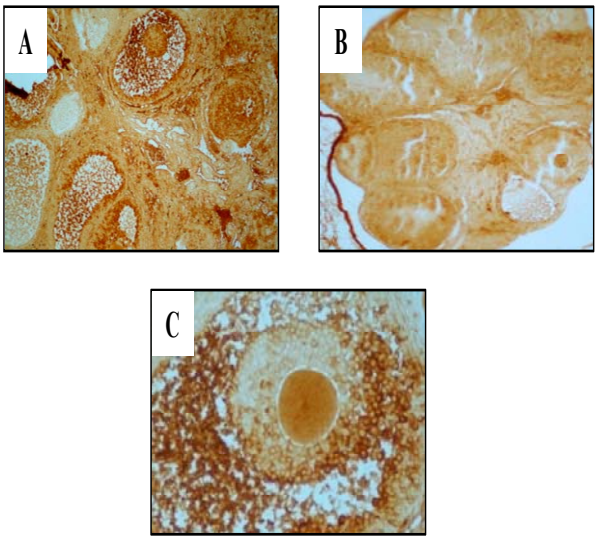

Figure 2.

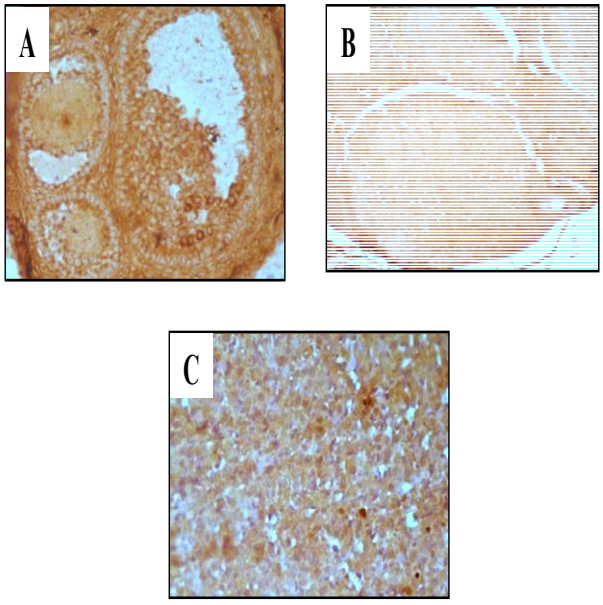

Figure 3.

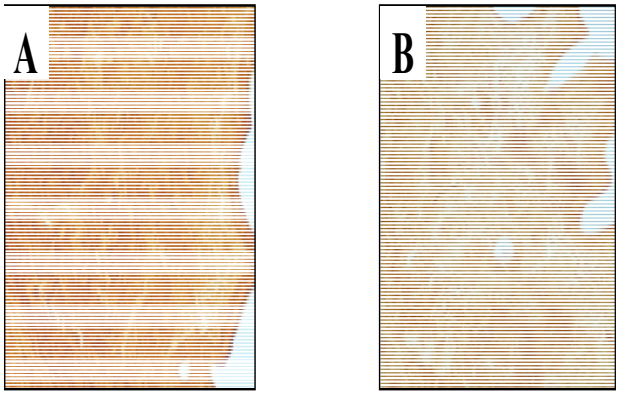

Figure 4.