Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Archives of Applied Science Research, 2011, 3 (2):180-191

(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-508X
CODEN (USA) AASRC9

Neonatal melatonin treatment has favorable quantitative and qualitative influence on adult ovarian functions in rat

Thakkar B.P. and Ramachandran A.V*

Division of Reproductive Endocrinology, Department of Zoology, Faculty of Science, The M. S. University of Baroda, Vadodara, Gujarat, India

---

ABSTRACT

The long-term programming effects of neonatal hypermelatonemia on adult ovarian weight and functions and serum hormone profiles have been studies in the Charles Foster strain of rats. The neonates were administered melatonin (MT) i.p. (40 µg/animal/day) in the evening from day 0 to day 21 post partum. The serum titres of T3, T4, estradiol and progesterone and, ovarian weight and histoarchitecture were assessed on post partum days 22, 45 and 90. Though there was no difference in adult ovary weight between control and MT programmed rats, there was increased density of follicles with significantly higher number of antral follicles and corpora lutea in MT rats. The number of atretic follicles showed a significant decrement. In conclusion, the present observations reveal increased progesterone but decreased estrogen level and increased protection against follicular apoptosis in the adult due to neonatal melatonin programming thereby increasing follicular number and corpora lutea.

INTRODUCTION

Melatonin, the hormone of the pineal gland secreted during night in all vertebrates [1, 2] modulates seasonal reproduction in mammals [3, 4, 5] and exerts control over circadian and annual cyclicities [6, 7, 8]. However, its role in reproduction of non-seasonal breeders is not that marked [9]. The reproductive system of adult rat is reportedly insensitive to exogenous melatonin [10, 11]. Though there are a few reports of varying effects due to either pinealectomy or melatonin infusion in adult rats, a general concept of no major role for pineal has been the understanding [10, 11, 12]. Immature rats nevertheless seem to be responsive to melatonin. There are reports of retardation of development of both male and female reproductive system due to melatonin administration [13, 14, 15, 16, 17]. Based on a number of experiments involving administration of melatonin at different periods in the immature stage, 20-40 days seem to be relatively more sensitive to the inhibitory effect of the hormone [18, 19].

Sexual development and maturation are prolonged processes that commence right from the intrauterine phase and come permanently under the mediatory influence of the hypothalamo-

hypophysial-gonadal (HPG) axis with its ontogeny [20]. Jarringe et al. [21, 22] have studied the role of the maternal pineal gland during pregnancy on the sexual function of offspring. Melatonin treatment during gestation delays sexual maturation of the female offspring of rat [23]. A subsequent study by the same group has indicated that maternal melatonin is necessary for normal somatic growth and postnatal development of reproductive organs of the offspring [24]. Since the influence of melatonin on the development of the reproductive system has been known to commence during the prenatal period and extend into the postnatal life [25], melatonin infusion either in the evening or in the morning in the infantile to pre-pubertal period (10-25 days) has been tested in our laboratory. This study showed decreased body weight and testes weight in the period immediately after melatonin treatment, more pronouncedly in the evening schedule [26].

Recent study from this laboratory on neonatal programming by hypothyroidism demonstrated long- term favorable influence on body weight gain with increased germ cell number in the adult testis and a permanent hyposetting of the central set point of the neuroendocrine reproductive axis [27]. Similarly, neonatal programming by melatonin in male rats led to permanent hyposetting of the neuroendocrine reproductive axis and increased germ cell mass due to decreased apoptosis [28]. Since there is no report on the effect of neonatal programming by hormones in females, the present study evaluates the programming effect of melatonin neonatally in the preweanling period (1-21days) on adult body and ovarian weights, ovarian histoarchitecture and serum hormone profiles.

MATERIALS AND METHODS

Animals and maintenance:

Healthy female laboratory rat neonates (Charles foster strain) maintained at Sarabhai Research Center under a constant temperature range of 21±2°C and alighting regimen of LD 8:16 throughout the experimental period of the study served as the experimental animals. The rats received standard diet (Amrut Rat Feed) and water ad libitum. The treatment was initiated on day 0 (day of birth) and terminated on day 21 post partum.

Preparation of melatonin:

Melatonin (Sigma Co, USA) for administration was prepared by dissolving weighed amount in a few drops of ethanol and diluted with 0.9% saline.

Experimental Protocol:

The experimental setup consisted of two groups of study.

Group I - Control (C):

Female rats maintained until day 90 served as controls. This consisted of two subgroups of 10 animals each as follows :

(i) Control rats (maintained as such)
(ii) Injected i.p with vehicle (0.9% saline) in the evening at 16:30hrs.

Group II-Melatonin (MT):

Ten female rat neonates consisting were injected i.p with 40 µg melatonin/animal/day (MT) from day 0 to day 21 postpartum at 16:30 hrs.

Parameters and Methods of Evaluation:
After treatment for 21 days, the animals were sacrificed on days 22, 45 and 90 and, morphometric, gravimetric, and histocytometric studies carried out. The animals were sacrificed under mild anaesthesia and, blood collected by brachial venipuncture in eppendorff tubes. After centrifugation at 4000 rpm, separated serum was stored at -4°C and various hormones assayed. The viscera was cut open and the ovaries were excised, blotted free of tissue fluids and weighed accurately in a Mettler balance. Relative weight was calculated and expressed as percentage of body weight. The fixed ovaries (Bouin's fluid) were processed for paraffin sectioning.

Histology and histometry:
Paraffin sections of 3µm thickness were cut on a microtome and stained with Haematoxylin-Eosin (HE). For morphometry and enumeration of ovarian follicles, homologous cross sections of entire ovary showing better area of vision were chosen. The section area was calculated by integrating the area inside the traced perimeter and volume calculated by multiplying by the section thickness. The section volume was multiplied by 10 (to count for the number of sections skipped) to give the '10-section' volume, all of 10 section volumes were summed to obtain an estimate of the total ovarian volume in mm^3 [29, 30]. A count of different types of follicles was also made.

Hormone Assays:
Serum T_3 and T_4 were assayed by ELISA using kit purchased from Glaxo (product code H –T_3H-0010 and H-T_4H-0010) and expressed as ng/ml of serum. Estradiol and Progesterone were assayed by using ELISA kits purchased from General Biologicals Corp, Taiwan and expressed as ng/ml of serum.

Statistical Analysis:
All data are expressed as mean±SEM. The data were analyzed by student's t test and analysis of variance (ANOVA) wherever applicable, at 95% confidence limit.

RESULTS

Since there was no significant difference between the values of the two subgroups of controls, the data of vehicle control is considered.

Body and Ovary weight:
The body weight of melatonin treated animals was significantly less at all ages of study. However, there was no significant difference in relative ovarian weights (Table 1). The per day growth rate was significantly lower in melatonin treated rats compared to controls, with almost similar ovarian growth rate (Table 1).

Histology and Histometry:
In general, the ovary of melatonin treated animals showed greater population of follicles at all ages of study compared to corresponding controls (Plate 1A and 1B). Though there was no difference in ovarian volume, a differential count of various follicles has revealed significantly higher number of primordial, primary, preantral and antral follicles in MT rats. In the 90-day-old ovary, there was almost double the number of corpora lutea in melatonin treated rats as compared to controls. A count of atretic follicles has also shown significantly lesser number in melatonin treated rats (Table 2).

Serum Hormone Profile:
The circulating titre of estrogen was significantly less and that of progesterone significantly high in melatonin treated rats of all ages. Both circulating T_3 and T_4 levels were also significantly higher in melatonin treated rats (Fig 1 and 2). In order to avoid the contradiction due to differential levels of estrogen and progesterone during the estrous cycle, the serum levels assayed were of late diestrous of the last cycle before sacrifice, representing an average of 3-6 animals (Figs 3 and 4).

DISCUSSION

In the present study, neonatal melatonin (MT) administration creating a hypermelatonemic state has shown favorable influence on the adult ovarian functions marked by significantly increased number of follicles and greater fecundity of such rats. Though there are no studies on these lines involving melatonin excess during neonatal period, effect of melatonin excess during foetal/prenatal stage has been studied in the rat [31]. This study showed an altered neonatal hormonal status more particularly with reference to LH and prolactin. A sexual difference marked by elevated LH levels until the prepubertal period in female offsprings born to melatonin treated mothers and, decreased LH level in male offsprings was the feature. The authors had concluded that both pinealectomy of the mother or melatonin treatment could affect fetal development and influence the postnatal ontogeny of the hormones involved in the neuroendocrine reproductive axis in developing rats.

Another study involving maternal melatonin treatment or pinealectomy during gestation has also indicated the requirement of maternal melatonin for normal somatic growth and postnatal development of the reproductive organs of the offsprings [24]. In contrast to fetal excess of melatonin, neonatal melatonin excess does not seem to influence gonadal growth, as the present study registers no significant difference in ovarian growth. In recent times, fetal or neonatal hormonal disturbances known to influence the adult phenotype and physiology by inducing plasticity changes at critical phases of development, have gained recognition as hormonal programming [32]. In this context, we have shown previously that neonatal programming by both corticosterone and melatonin shows significant effect on adult physiology by way of altered testicular germ cell kinetics and hormonal axes [33, 28]. The study on neonatal programming by melatonin in male pups failed to show any significant difference in adult testes weight [27]. However, a differential programming effect of neonatal melatonin on body weight gain and ultimate adult body weight is inferable as the above study showed heavier adult body weight as against a lower body weight in females in the present study. Though a possible long-term positive resetting of the hypothalamo-hypophysial-growth hormone axis was the inference in the above study as a possible cause in the light of known ability of melatonin to induce elevation of growth hormone level [34, 35], a negative resetting seems probable in the present case. This might suggest a sexual difference on the programming effect of neonatal melatonin on growth hormone axis. Similar sexual difference with reference to postnatal reproductive hormone axis due to prenatal melatonin administration was shown by Diaz et al. [24, 31].

Though there is no difference in the ovarian weight, neonatal MT treatment has nevertheless a favorable influence on ovarian functions. The histoarchitecture of the ovary studied post-treatment at 22, 45 and 90 days of age have revealed increased number of all follicle types in the melatonin treated rats. Since there is significant increase in primordial, primary, secondary and antral follicles, neonatal MT seems to have a favorable influence on the survival of follicles on a long term basis. In recent times, influence of MT on ovarian functions has gained increasing

recognition. In this context, Lee et al. [36] have shown expression of melatonin receptor gene in the granulose cells of developing female mice. The expression seemed higher in all developing follicles except the primordial and atretic follicles and, based on these observations they envisaged a pivotal role for MT in folliculogenesis. Woo et al. [37] have demonstrated a direct role for melatonin in regulating ovarian functions by way of progesterone production, LH receptor expression as well as GnRH and GnRH receptor gene expression through melatonin receptors in the human granulosa and thecal cells. Apart from the presence of significantly higher number of follicles, the ovaries of melatonin programmed rats in the present study have also shown significantly lesser number of atretic follicles and more number of corpora lutea suggesting increased follicular survival by way of decreased apoptosis. Interestingly, melatonin improves the quality of oocytes by preventing degeneration as well as by preventing intra-follicular lipid peroxidation in the human ovary [38].In another study, melatonin exhibited radio protective action on ovarian follicles against γ irradiation in mice and the degree of this protection was concentration related [39]. Based on the observation of higher level of melatonin in preovulatory follicular fluid, more than even in serum, and the presence of receptors on granulosa cells, have concluded significant protective role for melatonin in folliculogenesis, follicular atresia, ovulation, oocyte maturation, and corpus luteum (CL) formation in humans. They further opined that melatonin could become an important medication for improving ovarian function and oocyte quality, and open new opportunities for the management of several ovarian diseases. Pertinently, Abecia et al. [40] have shown increased lambing process by way of improved luteal support and embryonic survival by melatonin treatment in sheep. Further, Bellipanni et al. [41] showed that melatonin can abrogates hormonal, menopause-related neurovegetative disturbances and restore menstrual cyclicity and fertility in perimenopausal or menopausal women.

In all the above studies, the demonstrated protective action of melatonin occurred with simultaneous or continuous presence of melatonin. In contrast, in the present study, the protective action of melatonin happens as a long-term effect much after the cessation of administration of melatonin. This is a novel observation and the mechanisms of this long lasting protection afforded by neonatal melatonin administration remains a matter of conjecture. The possible explanations could be a permanent genetic reprogramming with reference to ovarian survival/apoptotic factors and/or permanent resetting of neuroendocrine ovarian hormonal axis, a classic example of neonatal programming and developmental plasticity changes and altered adult functional consequence as suggested earlier for male rats [33, 28]. With reference to the former, a number of hormones, growth factors and cytokines, which in turn activate several sub programs involving many genes [42] regulate follicular survival or apoptosis. A possible avenue of future investigations could be the understanding of the possible role of neonatal melatonin programming on permanent resetting of local genetic programs resulting in activation of survival factors and/or inhibition of apoptotic factors. Such long-term programming by neonatal melatonin involves development of the serotonergic systems in extrahypothalamic regions including the hippocampus and the striatum. The second possibility of altered neuroendocrine reproductive axis finds validation from the presently observed estrogen: progesterone ratio and the increased thyroid hormone titres. In this connection, Brzeznski et al. [43] have suggested a role for melatonin in the intra-ovarian control of progesterone production in the human ovary. Further evidences come from the works of Faigon et al. [44] showing disruption of LH negative feedback and diminished LH induced steroid release, of Johnston et al. [45] reporting altered sensitivity to GnRH induced FSH and LH release due to neonatal melatonin administration, and of Nakamura et al. [46] demonstrating increased follicular progesterone production in response to melatonin. Reports of increased luteotrophic effect and increased progesterone output and of

the positive influence of melatonin on hypothalamic-pituitary-ovarian axis provide support to the present programming effect of melatonin.

Based on recent reports on thyroid hormones favoring follicular growth [47] it is also inferable that the presently recorded increased thyroid hormone levels in response to neonatal melatonin may also provide a favorable environment for follicular growth and reduce the degree of follicular apoptosis. Interestingly, perimenopausal and menopausal women showed improvement in thyroid function on melatonin treatment [48]. Studies by Hapon et al. [49] also show definite favorable influence of thyroid hormones on ovarian development in the post-natal periods. The present findings in this context clearly suggest positive influence of neonatal melatonin programming on adult thyroid hormone axis and improved ovarian functions as long-term plasticity changes. In conclusion, it is surmisable from the present investigation that, neonatal programming by melatonin induces plasticity changes resulting in better adult ovarian functions marked by higher follicular survival, ovulation of greater number of ova, and more number of corpora lutea and fecundity and, up regulation of thyroid hormone axis.

Table 1: Chronological alterations in body weight (g) and relative ovarian weight (g/100g) in control and melatonin treated rats

GROUPS	BODY WEIGHT			RELATIVE TESTIS WEIGHT		
	Age in days			Age in days		
	22	45	90	22	45	90
C	51.33±1.873	136.67±4.176	226.167±9.575	0.046±0.0006	0.045±0.0015	0.034±0.0025
MT	46.67±0.843	129.33±2.917	194.83±6.66[a]	0.038±0.0023[b]	0.040±0.0039	0.038±0.0023

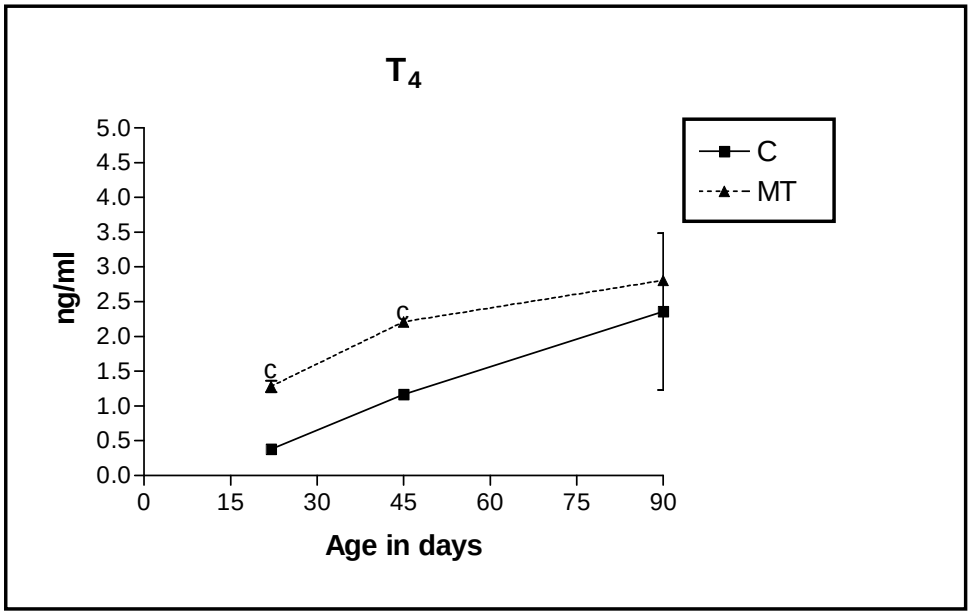
C- Control, MT- Melatonin treated
Values are expressed as Mean±SEM of ten observations.
[a] p<0.05, [b] p<0.005, [c] p<0.0005

Table 2: Differential total follicular count in ovary of control and melatonin treated female rats

Treatment	Age in days	Follicle Type						Ovarian Volume (mm^3)
		Primordial	Primary	Secondary	Antral	CL	Atretic	
C	22	821±48	531±25	350±20	181±04	-	10±0.8	0.58
	45	426±17	162±11	245±10	142±08	-	29±02	0.68
	90	300±13	168±08	96±04	72±05	48±05	36±03	1.31
MT	22	891±42[b]	391±19[b]	452±12[b]	183±05[a]	-	05±0.4[c]	0.45[a]
	45	446±15	229±10[b]	277±28	166±11	-	09±0.1[c]	0.88[b]
	90	373±15[b]	217±12[b]	108±04	96±04[b]	72±06[b]	13±01[c]	1.29

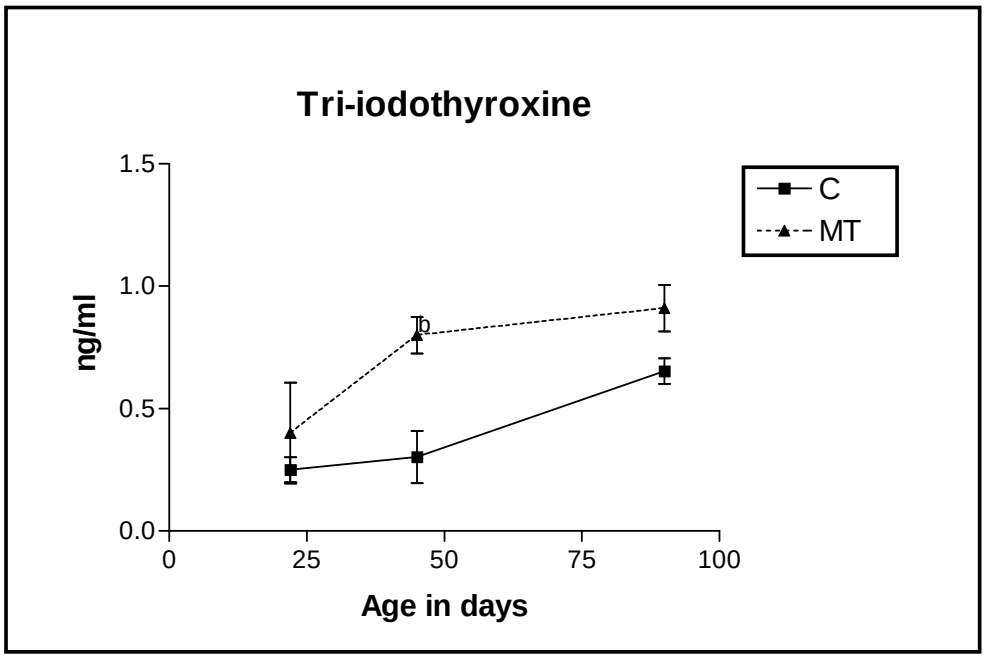
C-Control; MT- Melatonin; CL-Corpus Lutea
Values are expressed as Mean±SEM of ten observations.
[a] p<0.05, [b] p<0.005, [c] p<0.0005

Fig 1: Serum T_4 level (ng/ml) in control and melatonin treated rats.



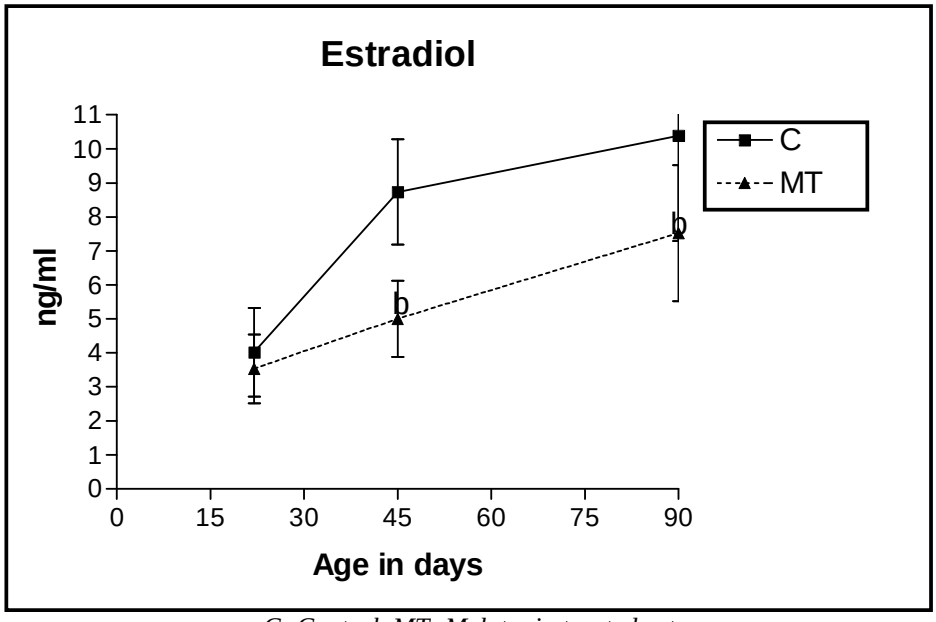
C- Control, MT- Melatonin treated rat
Values are expressed as Mean±SEM of six animals.
[c] p<0.0005

Fig 2: Serum T_3 level (ng/ml) in control and melatonin treated rats.



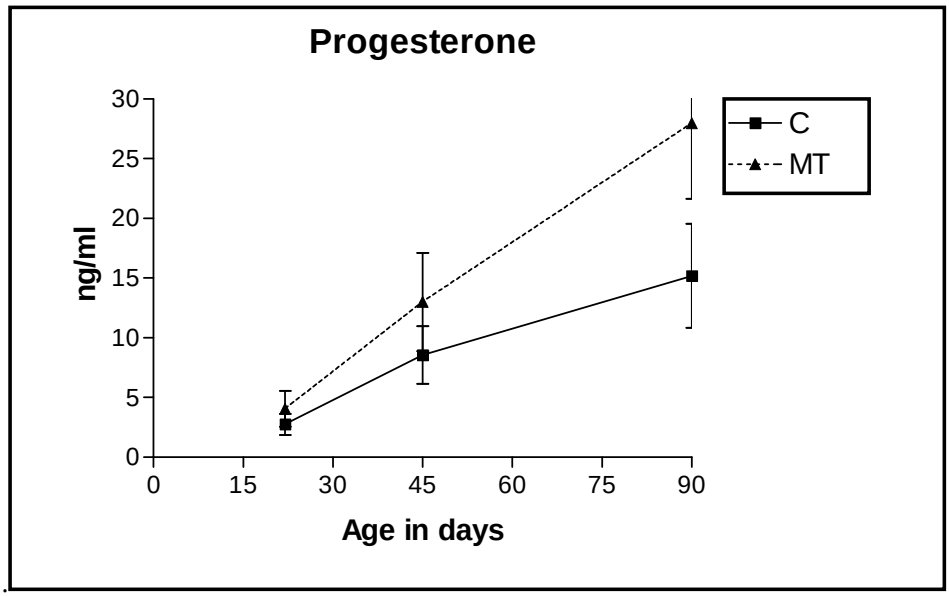
C- Control, MT- Melatonin treated rat
Values are expressed as Mean±SEM of six animals.
[b] p<0.005

Fig 3: Serum Estradiol level (ng/ml) in control and melatonin treated rats.



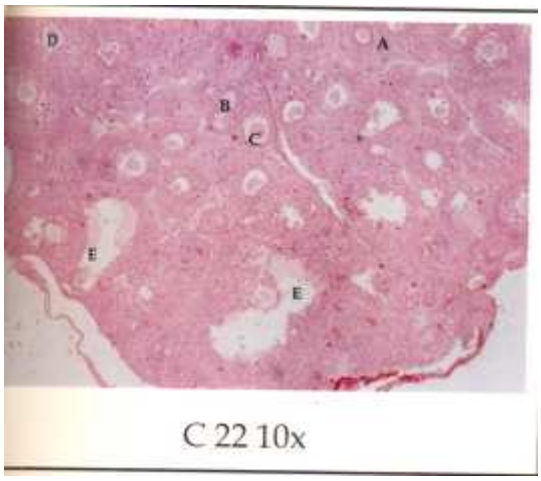
C- Control, MT- Melatonin treated rat
Values are expressed as Mean±SEM of six animals.
[b] p<0.005

Fig 4: Serum Progesterone (ng/ml) in control and melatonin treated rats

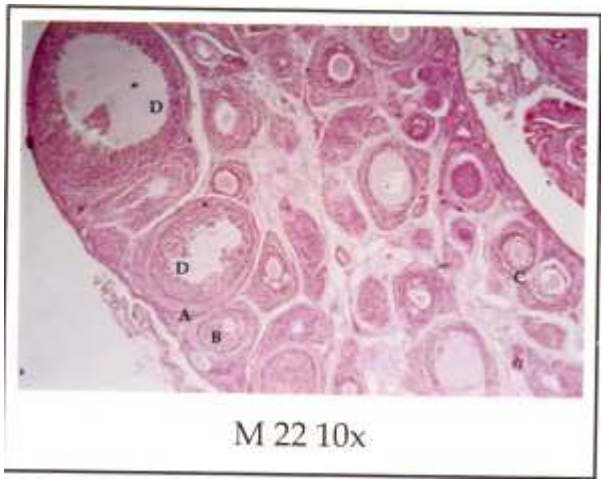


C- Control, MT- Melatonin treated rat
Values are expressed as Mean±SEM of six animals.

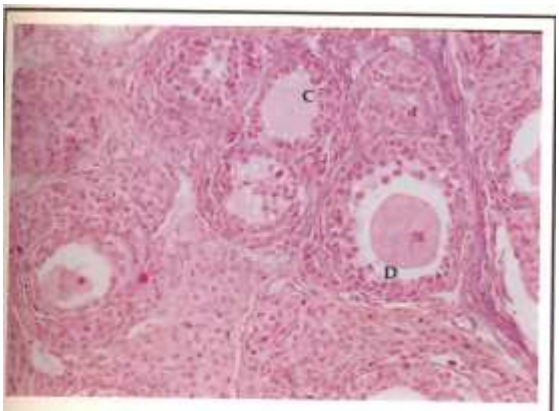
Plate 1A: Photomicrograph of sections of ovary of control and melatonin treated rats.



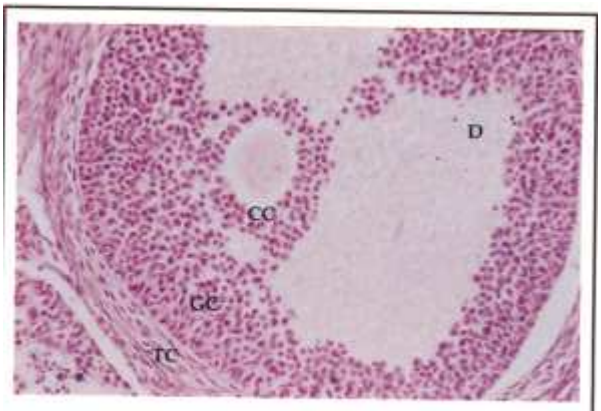
C 22 10x



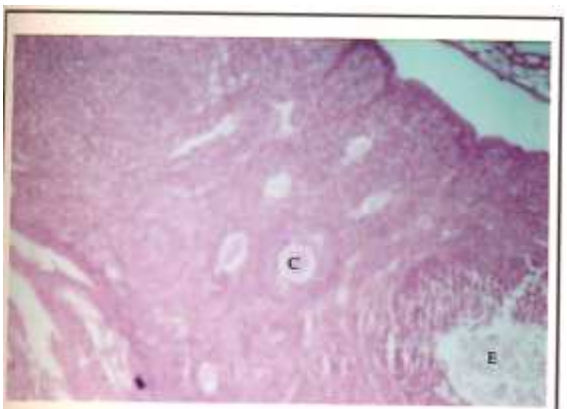
M 22 10x



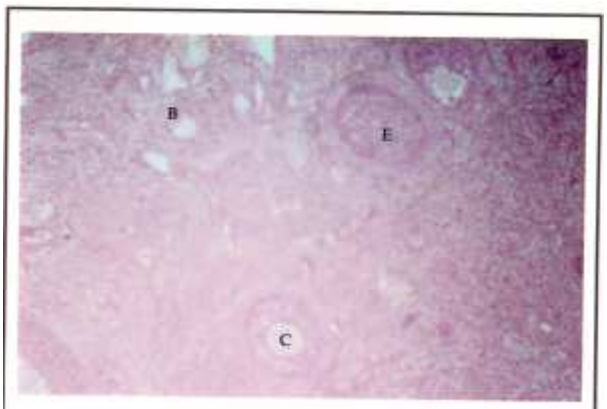
C 22 40x



MT 22 40x



C 45 10x



M 45 10x

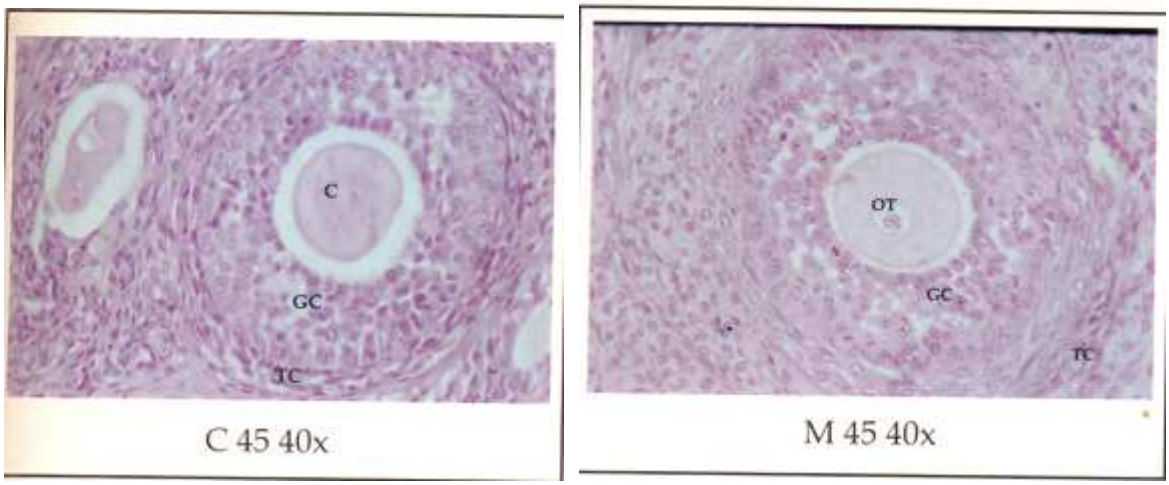
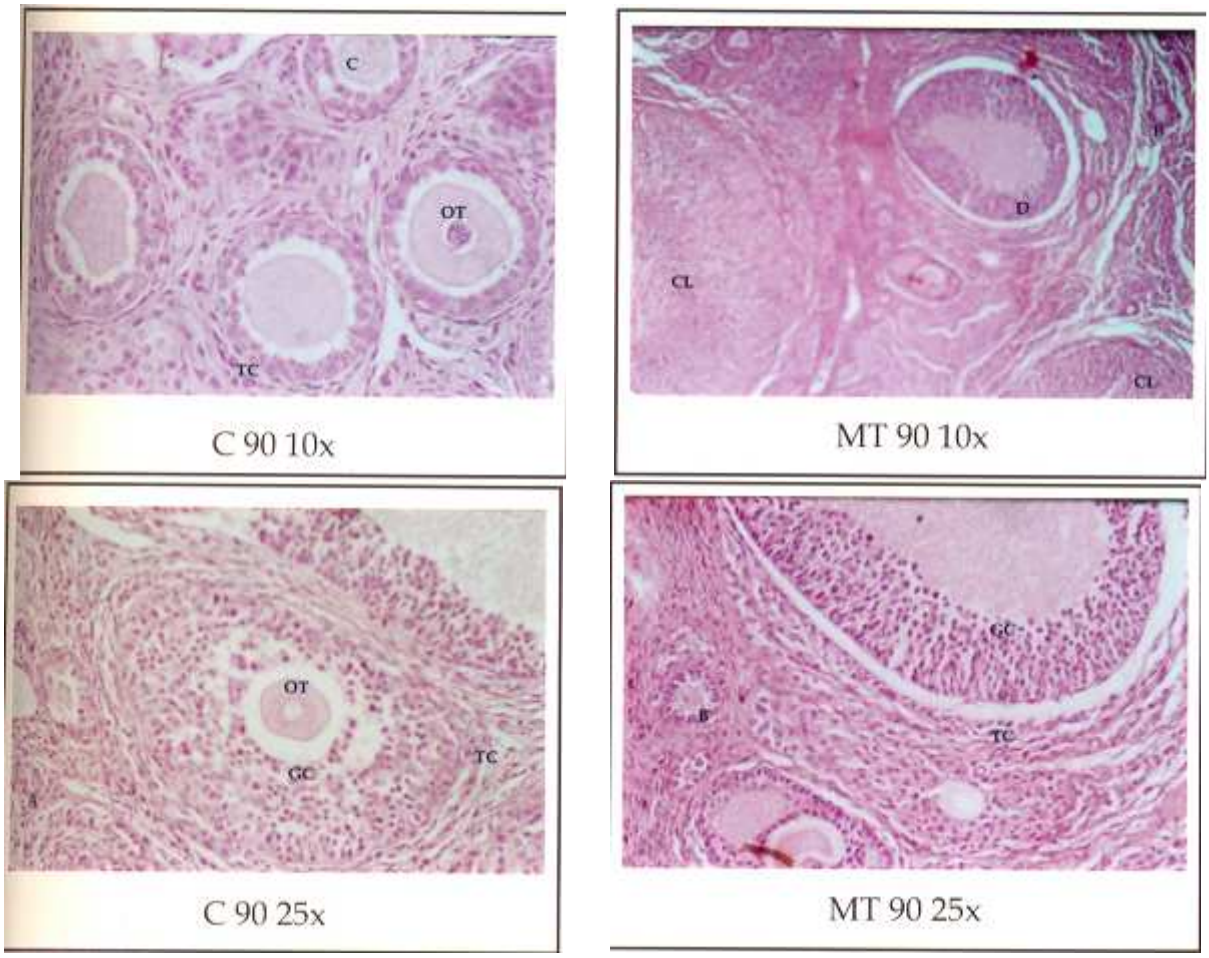


Plate 1A: Photomicrograph of sections of ovary of control and melatonin treated rats.
Sections of ovaries of 22 and 45 day old control rats showing more number of atretic follicles compared to age matched treated groups that shows number of pre antral follicles and fewer atretic follicles.
A- Primordial follicle; B- Primary follicle; C- Secondary follicle; D- Antral follicle; E- Atretic follicle; GC- Granulosa cells; TC- Thecal cells; OT- Oocyte.

Plate 1B: Photomicrograph of sections of ovary of control and melatonin treated rats.



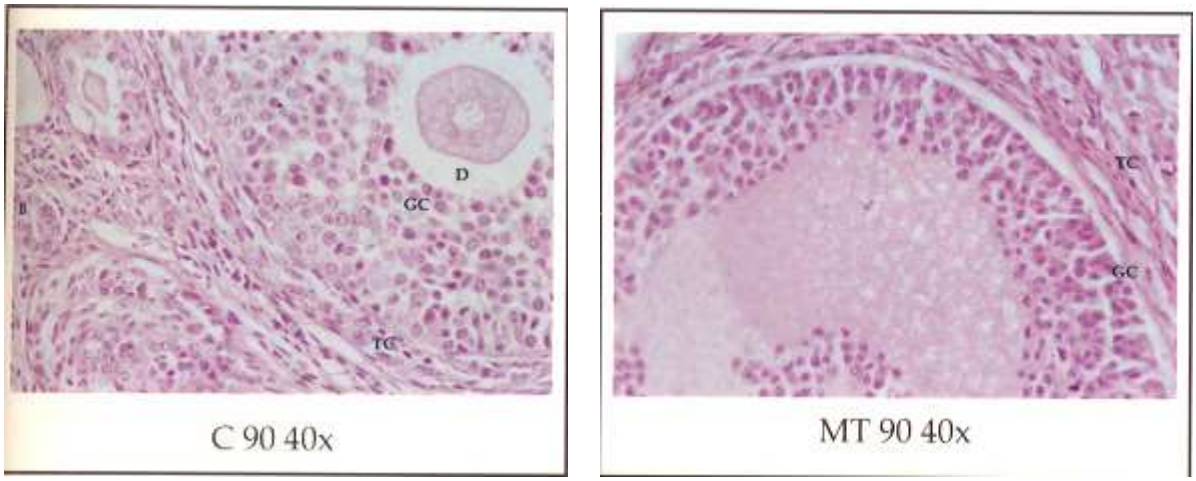


Plate 1B: Photomicrograph of sections of ovary of control and melatonin treated rats.
Sections of ovary of 90 day old control and melatonin treated rats showing more number of secondary and tertiary follicles in melatonin treated rats compared to controls.
A-Primordial follicle; B- Primary follicle; C- Secondary follicle; D- Antral follicle; E- Atretic follicle; GC- Granulosa cells; TC- Thecal cells; OT- Oocyte.

REFERENCES

[1] R. J. Reiter. *Bioessays*. **1992**, 14(3), 169-75.
[2] A. Cagnacci. *J.Pineal Res*. **1996**, 21, 200-213.
[3] E.L.Bittman, A.H.Kaynard, D.R. Olster, J.E. Robinson, S.M. Yelson, F.Karasch. *Neuroendocrinology*, **1985**, 40, 409-418.
[4] M.H.Hastings. *Pharmacol.Ther*. **1991**, 50, 35-71.
[5] T. Misztal, K.Romanowicz, B. Barcikowski. *Acta Neurobiol Exp*. **1996**, 56, 769-778.
[6] J.J.Bartness, J.B.Powers, M.H.Hastings, E.L.Bittman, B.D.Goldman. *J Pineal Res*. **1993**, 15, 161-199.
[7] R.J.Reiter. Experientia. 1993, 49, 654-664.
[8] R. Hardeland, I. Balzer, B. Poeggeler, B. fuhrberg, H. Urja, G.Behrmann, R.Wolf, T.J.Meyer, R.J.Reiter. *J Pineal Res*. **1995**, 18(2), 104-11.
[9] J.Arendt. *Rev. Reprod*. **1998**, 3(1), 13-22.
[10] R.J.Reiter. *Endocr. Rev*. **1980**, 1(2), 109-131.
[11] B.D.Goldman, K.S.Matt, P. Roychoudhury, M.H.Stetson. *Biol Reprod*. **1981**, 24(2), 287-92.
[12] S.A.Binkly. *Endocrinology*. **1983**, 4, 255-269.
[13] R.J.Wurtman, J. Axelford, E.W.Chu, J.E.Fischer. *Science*. **1963**, 141, 277-278.
[14] M. Motta, F. Fraschini, L. Martini. *Proc Soc Expt Med*. **1967**, 126, 431-435.
[15] L.Debeljuk. *Endocrinology*. **1969**, 84, 937.
[16] G.A. Kinson, S. J. Robinson. *Endocrinol* **1970,** 47, 391.
[17] G.A. Kinson, F. Peat. *Life Science* **1971**, 10, 259.
[18] U. Lang, M.L.Aubert, B.S.Conne, J.C. Bradtke, P.C.Sizonenko. *Endocrinol* **1983**, 112, 1578-1584.
[19] U. Lang, R.W. Rivest, L.V. Schlaepfer, J.C. Bradtke, M.L. Aubert, P.C.Sizonenko. *Neuroendocrinol* **1984**, 38, 261-268.
[20] S.A.Chiappa, G.Fink. *J Endocrinol*. **1977**, 72, 211-224.
[21] J.F.Jarrige, O. Tlemcani, D. Boucher. *Acta Endocrinol*. **1987**, 116, 247-252.
[22] J.F.Jarrige, K. Jebbari, D. Boucher. *J Reprod. Fertil*. **1990**, 89, 415-421.
[23] M.D.Colmenero, B.Diaz, J.L.Miguel, M.L.Gonzalez, A.I. Esquifino, B.Marin. *J Pineal Res*. **1991**, 11(1), 23-27.

[24] E. Diaz, C. Fernandez, O.P.Castrillo, A.I.Esquifino, B.Marino, B.Diaz. *Neuroendocrinolgy Letters*. **1999**, 20, 229-236.
[25] D.R.Weaver. Kluwer Academic/ Plenum publishers, New York. **2000**.
[26] M.M.Patel, A.V.Ramachandran. *J.Reprop.Biol.Com.Endocrino*l.**1992**, 4, 63-70.
[27] A.V.Ramachandran, S.K.Lagu, N.G.Bhavsar, B.P.Thakkar. communicated. **2004**.
[28] A.V Ramachandran, N.G Bhavsar, S.K. Lagu. *Annals of Biological Research*, **2010**, 1 (3) : 85-100.
[29] D.R.Plowchalk, B.J.Smith, D.R.Mattison. *Methods in Toxicology*, Academic Press Inc, **1993**, pp 57-66.
[30] J.L.Tilly. *Reproductive Biology and Endocrinology*. **2003**.
[31] D.L.Diaz, M.D.Colmenero, E.D.Rodriguez, A.A. Fraguas, A.I.Esquifino, B.M.Fernandez. *Eur. J. Endocrinol*. **1995**, 132, 765-770.
[32] A.M.Dufty, J. Clobert, A.P. Moller. *Trends in Ecology and Evolution* **2002**, 17, 190-196.
[33] N.G Bhavsar , S.K, Lagu, A.V Ramachandran. *Archives of Applied Science Research*, **2010**, 2 (5):269-284.
[34] B. A. McKeown, T. M. John, J. C. George. *Endocrinol Exp* **1975**, 9, 263.
[35] J. Vriend, M.S.Sheppard, K.T.Borer. *Growth, development and ageing* **1990**, 54, 165.
[36] C.J.Lee, B.R.Do, Y.H.Lee, S.J.Kim, J.K.Kim, S.I.Roh, Y.D.Yoon, H.S. Yoon. *Mol. Reprod.Dev.* **2001**, 59(2), 126-132.
[37] M.M.Woo, C.J.Tai, S.K.Kang, P.S.Nathwani, S.F.Pang, P.C.Leung. *J.Clin. Endocrinol. Metab.* **2001**, 86(10), 4789-4747.
[38] A. Takasaki, Y.Nakamura, H.Tamura, K. Shimamura, H. Morioka. *Repro. Med. Biol*.**2003**, 2, 139-144.
[39] J.K.Kim, C.J.Lee. *Mutat Res*. **2000**, 449, (1-2), 33-39.
[40] J. A. Abecia-, F. Forcada, A. Casao and I. Palacı´n *Animal*, **2008**, 2:3, pp 399–404
[41] G Bellipanni, F Di Marzo, F Blasi, A Di Marzo. *Annals of the New York Academy of Sciences.* **2005**, 1057, 393-402.
[42] E. Markstrom, E.Svensson, R. Shao, B. Svanberg, H. Billig. *Reproduction*. **2002**, 123, 23-30.
[43] A.Brzezinski, T. Fibich, M. Cohen, J.G. Schenker, N.Laufer. *Fertil.Steril*.**1992**, 58(3), 526-529.
[44] M.R.Faigon, J.A.Moguilevsky, B. Szwarcfarb, P. Scacchi. *Prog Clin Biol Res*. **1982**, 112, 149-63.
[45] J.D.Johnston, S.Messager, P. barrett, D.G. Hazlerigg. *J. Neuroendocrinol*. **2003**, 15(4), 405-408.
[46] Y.Nakamura, H. Tamura, H. Takayama, H. Kato. *Fertil Steril*. **2003**, 80(4), 1012-1016.
[47] J.Y.Jiang, M.Umezu, E. Sato. *J.Reprod.Fertil*. **2000**, 119, 193-199.
[48] S.H. Omar, S. Nabi. *Syst Rev Pharm* **2010**, 1,158-71.
[49] M.B. Hapon, G.V.Simoncini, G.A. Jahn. *Reproduction*.**2003**, 126, 371-382.