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**ORIGINAL ARTICLE**

# Effect of stress hormone antagonists on ovarian follicular development in pre-pubertal rat

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Effect of stress on pre-pubertal ovarian follicular development was studied. Fifteen day old female rats were administered under stress (exposed to maternal separation; 6 hours/day) from post-natal day 15 to 21 for 7 days, and appropriate controls were maintained. The time of exposure was randomly changed every day during light phase (7AM to 7 PM) of the day to avoid habituation. There was a significant decrease in serum estrogen levels on post-natal day 21 in stress group rats compared to controls indicating stress response in these rats. However, mean number of healthy follicles in all categories of follicles were significantly lower in stressed rats compared to controls. Concomitant with these changes, mean number of atreitic follicles showed an increase over control values in stressed rats. In contrast administration of Naltrexone (5µg NTX/rat/day), Mifepristone (1 µg MP/rat/day), FSH (10 IU FSH/rat/day) with stressed the significant increases in the relative weight of ovary, uterus, fallopian tube, body weight and the mean number of healthy follicles in the ovary compared to the controls. In the ovary treatment of stressed did not affect primordial follicles. Primordial follicles were reduced in number significantly in the ovary of controls and treated groups when compared with the initial controls whereas there was no significant variation among the controls and the treated groups. The results indicate that stress dose not interfere with the progress of pre-pubertal follicular development. However, it causes increased loss of follicles by atretia.

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*Key words: Naltrexone, Mifepristone, FSH, Stress, Follicular development, Ovary, atretia, pre-pubertal.*

Stress inhibits reproductive functions in different groups of vertebrates (Greenberg and Wingfield, 1987; Pickering *et al.*, 1987; Compbell *et al.*, 1992; Wolfenson *et al.*, 1995; Guillette *et al.*, 1995; Herbert, 1995; Contreras-Sanchez *et al.*, 1998). Despite a number of reports on the inhibitory effects of stress on reproduction, the means by which stress influences reproduction is not clearly understood in vertebrates (Tillbrook *et al.*, 2000). In vertebrates, a major universal response of stress is hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus is excited the pituitary gland to release ACTH under stressful condition. ACTH causes the release of glucocorticoids which affect different physiological processes (Saplosky, 1999).

Glucocorticoids and catecholamines act widely to alleviate the effect of stress (Tillbrook *et al*., 2000). Female reproductive system is highly sensitive to physiological stress (Warren and Perloth, 2001). Ovarian follicle is the functional unit of the ovary and ovarian follicular development is continuous and independent from estrous cycle (Singh *et al*., 1995; McGee and Hsueh, 2000). Stress effects on different aspects of reproduction are reported (Thibier *et al*., 1976; Grey *et al*., 1978; Schillo *et al*., 1978; Tache *et al*., 1978; Barb *et al*., 1982; Paterson *et al*., 1983). In addition, chronic stress inhibits LH secretion (Grey *et al*., 1978; Tache *et al*., 1978; Moberg 1987; Brann and Mahesh, 1999), and follicle development (Christian and le Munyan, 1958; Christian, 1971; Moberg, 1987; Brann and Mahesh, 1999). There are several reports on effect of stress or HPA-axis hormones on follicular development in mammals. Effect of stress on the adult gonad may be reversible but may not be true during initial stages of follicular development (Armstrong, 1986). Influence of stress on ovarian follicular development especially during pre-pubertal period is least investigated. The onset of follicular growth is an important control point in follicular compliment. In case of rodents onset of initial follicular waves occurs immediately after birth and continues throughout the reproductive life span (Pedersone, 1969; Greenwald and Roy, 1994). For instance, stress due to forced swimming exercise during pre-pubertal period delayed onset of puberty in female rats (Pellarian-Massicotte *et al*., 1987), neonatal handling induces an ovulatory estrous cycles in rat (Gomes *et al*, 1999) and affected development of female reproductive function in rats (Rhees *et al*, 2001). Chronic intermittent cold stress decreases total number of follicles (Dorfman *et al*., 2003). Heat stress reduces the duration and intensity of estrous cycle, alters follicular development and increases the rate of apoptosis in the antral and pre-antral follicles (Shimuzu *et al*., 2005). Heat stress during follicular recruitment suppresses subsequent growth to ovulation in goats (Ozawa *et al*., 2005). Heat stresses alters follicular growth (Wilson *et al*., 1998; Roth *et al*., 2000; Hansen, 2009), and disrupt the development and function of the oocytes (Zeron *et al*., 2001; AL-Katanani *et al*., 2002; Sartori *et al*., 2002 and Hansen, 2009). Chronic ACTH inhibits ovulation and follicular development (Christian *et al*., 1964; Hagino *et al*., 1969; Mac Farland and Mann, 1977; Brann and Mahesh, 1999). Heat stress alters the efficiency of follicular selection and dominance, and has adverse effect on the quality of ovarian follicles (Badinga *et al*., 1993) and on follicular steroidogenesis (Howell *et al*, 1994; Roman-Ponce *et al*, 1977; Rosemberg *et al*, 1982; Wolfenson *et al*, 1995; Faust *et al*, 1988). Since the main factors regulating ovarian activity are gonadotrophin-releasing hormone from hypothalamus and the gonadotrophins, LH and FSH from anterior pituitary gland, several authors have studied the effect of the heat stress on the secretion of these hormones. Similarly, stress like concentrations of glucocorticoids directly inhibit the meiotic maturation of pig oocytes (Yang *et al*., 1999), CRH inhibits the ovarian steroidogenesis (Kalantaridou *et al*., 2004). The assembly of primordial follicles occurs in the later stages of fetal development in human and in the early postnatal period in rodents (Guigon *et al*., 2003; Skinner, 2005 and Felici 2010). Since stress alters secretion of HPA axis hormones and in turn alters the secretion of hormones of HPG axis (Michael and Cook, 1994; Meczekalski and Szymankiewicz, 1999; Tilbrook *et al*., 2000 and Chatterjee *et al.,* 2006),

there are studies revealing the effect of stressors on ovarian follicular development in different groups of mammals. For instance, Stress due to exposure to cold also altered ovarian follicular development as shown by decrease in pre-antral healthy follicles without compensatory increase in atresia in rats (Dorfman *et al.,* 2003). However, studies conducted on post-natal stress thus far have not focused on this aspect and deal with other aspects of reproduction (Herrenkohl, 1979; Pellerin-Massicotte *et al.,* 1987; Kinsley and Bridges, 1988; Gutierrez *et al.,* 1989; Christopher *et al.,* 1996 and Rhees *et al.,* 2001). The view held earlier was that each species produces specific number (finite) of follicles at birth, majority of which under go degeneration (atresia) while others ovulation. Hence, once the stock is exhausted, there is no renewal of follicles to replace the lost follicles. However, recently renewal of primordial follicles (Johnson *et al.,* 2004, 2005 and Kerr *et al.,* 2006) and presence of extra ovarian germ cells, capable of forming primordial follicles have been demonstrated (Canning *et al.,* 2003 and Johnson *et al.,* 2005). Heat stress decreased ovarian function in cattle (Wolfenson *et al*., 1995), suggesting a differential inhibitory effect of heat stress on the functions of granulosa and theca cells by concurrent and delayed effects on the steroidogenic capacity of ovarian follicles. Changes in reproductive hormone secretion represent the final sequence in the neuron-endocrine pathway leading to the diminished reproductive performance associated with stress. The purpose of the present study was to determine the effect of stress hormone on follicular dynamics in the pre-pubertal rats.

## MATERIALS AND METHODS

**Animals and their maintenance:**

Wistar albino rats bred and maintained by the central animal facility of University of Mysore were used. The rats were maintained in polypropylene cages containing a bed of paddy husk and had free access to food and water throughout the day. The food was standard rat chow pallets of which nutritional contents were according to recommended standard diet for albino rats. The rats were maintained in 12:12 light and dark photoperiod (light on 7 am to 7 pm). Animal care, treatment and anesthesia were according to the guidelines of the committee for purpose of control and supervision of experiments on animals (CPCSEA). All experiment protocols were approved by the institutional animal ethics committee (IACE) of University of Mysore.

**Experimental protocols:**

Pre-pubertal female Wister albino rat were exposed to stress from post-natal day (PND) 15 to 21. Rats weighting 15 to 20g were procured from central animal facility, Mysore University and were segregated into seven groups and each group consisted of five rats. The first group was the initial control, and the rats of this group were sacrificed on day 1 of the experiment i.e. post-natal day 15. Rats in second group (treatment control) received administration of the vehicle (0.1 ml saline/rat/day), where in the rats were maintained with their mothers throughout the without any disturbance. Rats in the third group (stress group) were exposed to maternal separation from mothers 6 hours/day. The fourth group received 5µg Naltrexone/stress/day (Sigma Chemical, St. Louis, MO); fifth group received 1µg Mifepristone/ stress/day (Sigma Chemical, St. Louis, MO); sixth

group received 5µg Naltrexone plus 1µg Mifepristone/ stress/day and seventh group was received 10 IU FSH/ stress/day (Manufactured in India by: Bharat serum and vaccines limited), respectively. The rats in this groups (third, fourth, fifth, sixth, seventh) were separated from their mothers and transferred to different cages having appropriate bedding whereas the injections (ip) were given daily for 7 days. After 6 hours all the rats were shifted back to their original cages with their mothers. Timing of separation was randomly changed every day during light phase (7 AM to 7 PM) to avoid habituation. The rats were autopsied 24 h after the last injection. At each autopsy, weight of the body, the ovary, the uterus and fallopian tube were recorded, later converted into relative weight [weight (mg)/100g/body weight] of the organs. The right ovary was fixed in Bouin's fixative for histological studies respectively. The blood sample was collected, and serum was separated and stored -20˚C until 17β-estradiol concentration was determined.

**Histology and follicle counts:**

The ovaries fixed in Bouin's fluid were processed according to the standard histological method and 5µm thick serial paraffin sections were cut and stained with hematoxylin and eosin. Different categories of follicles were identified and classified according to Pederson and Peters (1968). The primordial follicles were counted from every $4^{th}$ section, and primary follicles (type 3a) from every $6^{th}$ section were counted. A different counting procedure was followed for advanced primary follicles (type 3b) and pre-antral and antral follicles. Each section of the ovary was observed and only the follicles showing full size oocyte was included in counts of respective category and care was taken not to repeat the counting of the same follicle more than once.

**Follicular atresia:**

Atreatic follicles were identified following morphological criteria described by Greenwald and Roy (1994) in hematoxylin-eosin stained serial sections the ovary. The earliest sign of atresia was presence of 5% pyknotic granulose cells in the largest cross section of the follicle.

**Estimation of serum concentration of 17β-estradiol:**

The 17β-estradiol concentration was determined by enzyme linked immuno sorbant assay (ELISA) using the kit purchased from DRG Instruments GmbH, Germany and DRG International Inc., USA. The 17β-estradiol was extracted from the serum collected at autopsy and stored at -20˚C, following the procedure of the manufactured.

**Statistical analysis:**

The mean values of each parameter were computed using data on a minimum of five animals in each group and expressed as mean ± SE. the mean values were compared by one way analysis of variance followed by Duncan's multiple range test and judged significant if $P<0.05$. All statistical analysis was carried out using SPSS 11.5.

## RESULTS

**Weight of the body and organs:**

The rats in all the experimental groups showed a significant gain in the body weight during the period of experimentation. However, percent gain in the body weight of rats exposed to stress-treated was significantly lower compared to that of respective control, and that of stress with drugs

groups was significantly higher than rats exposed to stress, whereas it did not significantly differ from the respective control group. There was a significant increase in the relative weight of the ovary, the uterus, and the Fallopian tubes in treatment controls compared to initial controls The mean relative weight of the ovary, uterus, and fallopian tube in this group of stress-treated rats showed significantly decrease compared to those of treatment control, whereas relative weight of the ovary did not significantly variation among treatment controls, stress group and stress with drugs groups. The relative weight of the uterus and Fallopian tube of stress group (day 15 to 21) and stress with drugs group showed a significant decrease compared to respective controls. The body weight and different organs significantly decreased at the end of stressed (Table 1, fig. a & b).

**Histology of the ovary and follicle counts:**

The ovary of the initial control of the post-natal day 15 exhibited normal histomorphology and consisted of primordial (type 2), primary (type 3a, 3b), pre-antral (type 4, 5a, 5b) and antral (type 6) follicles whereas, antral (type 7) and pre-ovulatory (type 8) follicles were not developed. The follicles were found embedded in stromal tissue and the ovary was covered by a surface epithelium. Microscopic observation of the ovary of the treated group did not reveal any marked variation in gross histomorphology when compared to controls. However, quantification of different category of follicles differed in their numbers. There were healthy follicles of all the categories from primordial to large antral stage and also atretic follicles of these categories in various stages of atretia judged by the presence of pyknotic granulosa cells. The atreitic follicles were seen in all groups. The ovaries of initial controls contained highest number of primordial (type2) follicles compared to all other groups.

There was a significant decrease in mean number of healthy follicles belonging to 3a, 3b, 4, 5a, 5b, 6 and 7 whereas a significant increase in 3b follicles in treatment controls compared to initial controls. The ovaries of stress, the mean number of healthy follicles of all categories except pre-ovulatory follicles was significantly decrease in treatment controls compared to initial control, whereas stressed rats combine with (Naltrexone, Mifepristone and FSH) groups, the mean number of all categories of healthy follicles accept pre-ovulatory were significantly increase in treatment groups compared to controls. Mean number of type 3b healthy follicles was significantly higher in stress combine with drugs groups compared to stress group, whereas there was no significant change in the mean number of other category follicles of these two groups. The stressed rats showed a significant decrease in mean number of healthy primary (type 3a, 3b), pre-antral (type 4, 5a, 5b) and antral (type 6, 7) follicles compared to treatment controls, whereas the mean number of other categories of follicles although reduced in stress group was not statistically significant. The mean number of healthy antral follicles although lower than controls, the difference was not statistically significant (Table 2).

**Follicular atresia:**

The atretic follicles belonging to stages 3a to 7 showed a significant increase in their number in treatment controls compared to initial controls, whereas in stressed rats the mean number of atretic follicles belonging to primary (type 3a, 3b), pre-antral (type 4, 5a, 5b) and antral (type 6, 7)

follicles higher compared to treatment controls, there was no significant change in the mean number of type 7 atretic follicles. On the other hand, there was a significant increase in mean number of atretic in stressed rats. The atreitic follicle was a significant decrease in the mean number of primary (type 3a and 3b), pre-antral (type 4, 5a, 5b) and antral (type 6, 7) follicles in stressed rats combine with drugs groups compared to treatment controls, whereas other categories of follicles did not show significant variation in their number (Table 3).

**Serum 17β-estradiol levels:**

In analysis hormone, the serum 17β-estradiol hormone showed a significant high level in the initial control of the post-natal day 15. There were significant decrease in the serum 17β-estradiol hormone of controls and treatment groups compared to the initial control while the group of stressed rats showed a significant decrease in serum 17β-estradiol hormone when compared to control, whereas the serum 17β-estradiol hormone of stress combine with drugs groups was significant increase compared to controls (Table 1, fig.c).

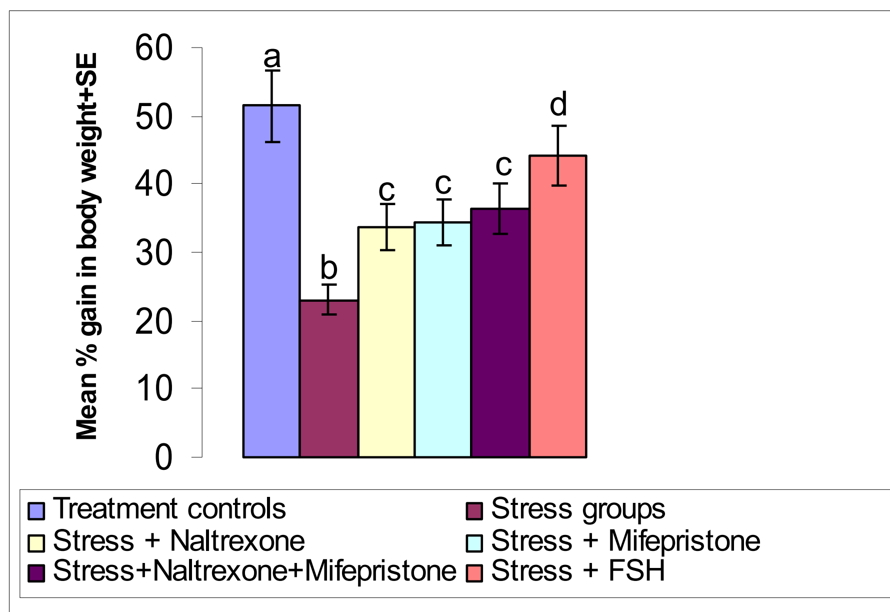


**Figure a.** Vertical bars showing percentage gain in body weight of rats in different groups; ANOVA was conducted after transforming the values using arc sine transformation; of rats in different groups. Groups with same superscript letters are not significantly different, whereas those with different superscript letters are significantly ($P<0.05$) different. Lines above the bars indicate standard error (SE).

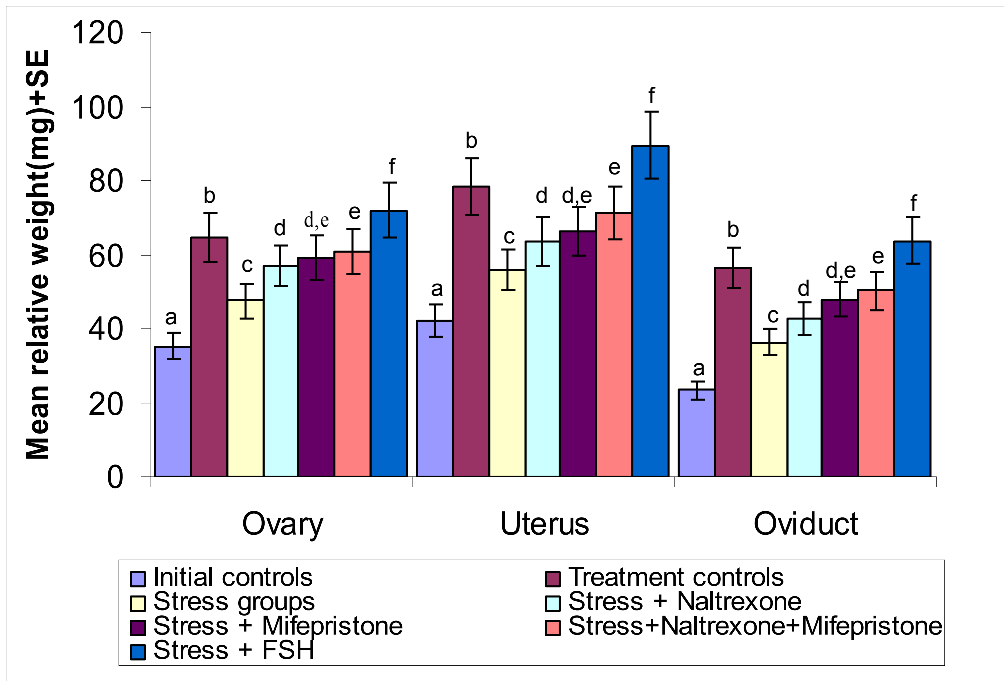


**Figure b.** Vertical bars showing mean relative weights of the ovary, the uterus and the oviduct of rats in different groups; ANOVA was conducted after transforming the values using arc sine transformation; of rats in different groups. Groups with same superscript letters are not significantly different, whereas those with different superscript letters are significantly ($P<0.05$) different. Lines above the bars indicate standard error (SE).

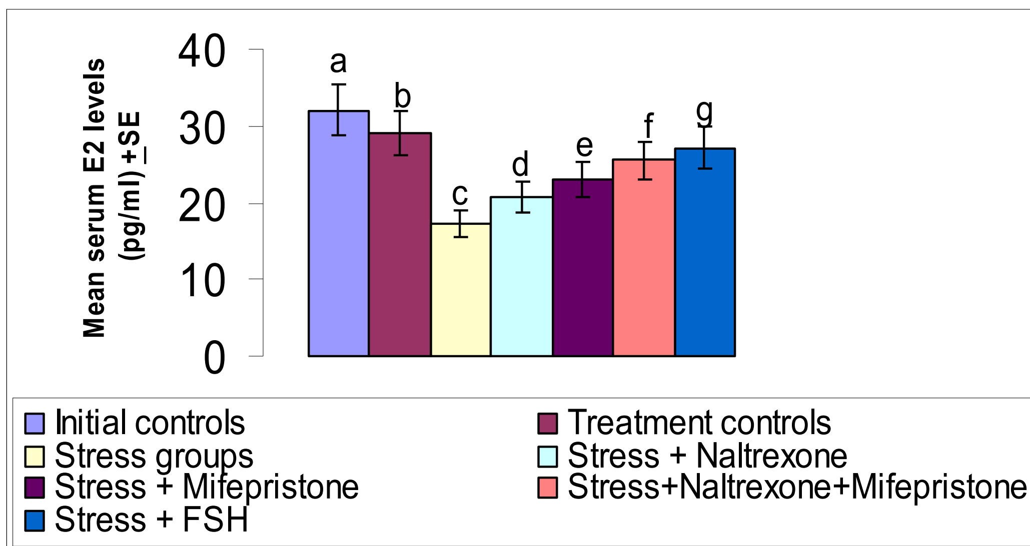


**Figure c.** Survival of wheat coleoptiles (%) after damaging heating. *1* – control, *2* – 4-HBA (10 µM), *3* – BHT (5 µM), *4* – 4-HBA (10 µM) + BHT (5 µM), *5* – α-naphthol (1 µM), *6* – 4-HBA (10 µM) + α-naphthol (1 µM), *7* – salicylhydroxamic acid (500 µM), *8* – 4-HBA (10 µM) + salicylhydroxamic acid (500 µM).

**Table.1 Effect of stress hormone antagonists on body weight, reproductive organs and serum $E_2$ levels in Pre-pubertal rats**

| Groups and Treatment | Body weight (g) | % gain in body weight | Weight (mg) per 100g body weight | | | Serum $E_2$ levels (pg/ml) |
|---|---|---|---|---|---|---|
| | | | Ovary | Uterus | Fallopian tube | |
| 1. Initial controls | - | - | 35.20±1.06$^{a}$ | 42.26±2.51$^{a}$ | 23.34±0.76$^{a}$ | 32.06±0.70$^{a}$ |
| 2. Treatment controls | 32.08±1.78$^{a}$ | 51.42±1.90$^{a}$ | 64.78±1.45$^{b}$ | 78.38±3.11$^{b}$ | 56.38±0.20$^{b}$ | 29.14±0.20$^{b}$ |
| 3. Stress groups | 21.02±0.42$^{b}$ | 23.06±1.90$^{b}$ | 47.40±1.16$^{c}$ | 55.86±2.32$^{c}$ | 36.34±1.36$^{c}$ | 17.14±0.20$^{c}$ |
| 4. Stress + Naltrexone | 23.48±0.48$^{c}$ | 33.80±2.37$^{c}$ | 57.02±1.51$^{d}$ | 63.60±1.79$^{d}$ | 42.82±0.65$^{d}$ | 20.78±0.49$^{d}$ |
| 5. Stress + Mifepristone | 25.66±0.51$^{c,d}$ | 34.46±4.44$^{c}$ | 59.36±0.19$^{d,e}$ | 66.16±1.65$^{d,e}$ | 47.92±1.34$^{d,e}$ | 23.14±0.23$^{e}$ |
| 6. Stress+Naltrexone+Mifepristone | 27.88±0.79$^{d,e}$ | 36.46±1.41$^{c}$ | 60.90±1.10$^{e}$ | 71.26±2.46$^{e}$ | 50.14±4.43$^{e}$ | 25.48±0.16$^{f}$ |
| 7. Stress + FSH | 29.46±0.46$^{e}$ | 44.06±1.60$^{d}$ | 72.00±1.73$^{f}$ | 89.52±0.53$^{f}$ | 63.68±2.41$^{f}$ | 27.16±0.28$^{g}$ |
| ANOVA F-Value df=(6,28) | 170.69 P<0.001 | 51.89 P<0.001 | 91.17 P<0.001 | 48.51 P<0.001 | 40.79 P<0.001 | 183.16 P<0.001 |

**Note.** Mean values in each column were compared by one way ANOVA followed by Duncan's multiple tests. Mean values with same superscript letters are not significantly different, whereas those with different superscript letters are significantly ($P<0.05$) different. All values are mean ± SE; df= degree of freedom.

**Table-2 Effect of stress hormone antagonists on ovarian follicular development in Pre-pubertal rats**

| Groups and treatment | Mean number of healthy follicles per ovary/stages ±SE | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Types of Follicles | Primordial | Primary follicles | | Pre-antral follicles | | | Antral follicles | |
| Stages | (T2) | (3a) | (3b) | (4) | (5a) | (5b) | (6) | (7) |
| 1. Initial controls | 4137.6±8.64$^{a}$ | 173.2±1.82$^{a}$ | 334.20±4.72$^{a}$ | 27.20±1.77$^{a}$ | 5.20±0.58$^{a}$ | 6.20±0.66$^{a}$ | 6.0±0.83$^{a}$ | - |
| 2. Treatment controls | 315.2±4.04$^{b}$ | 817.2±2.80$^{b}$ | 1938.2±16.34$^{b}$ | 500.0±4.43$^{b}$ | 221.6±2.42$^{b}$ | 105.2±1.71$^{b}$ | 18.20±0.58$^{b}$ | 11.60±0.74$^{a}$ |
| 3. Stress groups | 215.2±2.59$^{c}$ | 605.2±4.88$^{c}$ | 1535.2±16.86$^{c}$ | 367.6±5.92$^{c}$ | 147.8±1.95$^{c}$ | 60.8±2.65$^{c}$ | 8.20±0.66$^{c}$ | 5.60±0.40$^{b}$ |
| 4. Stress + Naltrexone | 238.8±5.55$^{d}$ | 712.6±5.64$^{d}$ | 1622.0±15.64$^{d}$ | 420.2±7.57$^{d}$ | 172.2±2.70$^{d}$ | 77.4±2.67$^{d}$ | 11.0±0.54$^{d}$ | 13.60±0.50$^{c}$ |
| 5. Stress + Mifepristone | 266.8±3.38$^{e}$ | 740.2±7.33$^{e}$ | 1681.80±8.63$^{e}$ | 453.0±6.95$^{e}$ | 182.6±2.13$^{e}$ | 89.2±2.31$^{e}$ | 15.0±0.44$^{e}$ | 15.80±0.37$^{d}$ |
| 6. Stress+Naltrexone+Mifepristone | 292.6±2.33$^{f}$ | 778.2±5.21$^{f}$ | 1842.2±14.91$^{f}$ | 472.6±4.56$^{f}$ | 190.4±2.61$^{f}$ | 94.6±2.52$^{e,f}$ | 16.2±0.37$^{e}$ | 19.00±0.83$^{e}$ |
| 7. Stress + FSH | 299.2±5.66$^{f}$ | 802.2±4.78$^{g}$ | 1869.0±9.01$^{f}$ | 489.6±2.54$^{g}$ | 208.6±2.69$^{g}$ | 98.0±1.51$^{f}$ | 21.6±0.81$^{f}$ | 22.20±1.15$^{f}$ |
| ANOVA F-Value df=(6,28) | 84207.23 P<0.001 | 2104.29 P<0.001 | 1792.53 P<0.001 | 988.76 P<0.001 | 1030.20 P<0.001 | 257.19 P<0.001 | 78.65 P<0.001 | 130.36 P<0.001 |

**Note.** Mean values in each column were compared by one way ANOVA followed by Duncan's multiple tests. Mean values with same superscript letters are not significantly different, whereas those with different superscript letters are significantly ($P<0.05$) different. All values are mean ± SE; df= degree of freedom.

**Table-3 Effect of stress hormone antagonists on ovarian follicular development in Pre-pubertal rats**

| Groups and Treatment | Mean number of atreitic follicles per ovary/stages±SE | | | | | | |
|---|---|---|---|---|---|---|---|
| Types of Follicles | Primary follicles | | Pre-antral follicles | | Antral follicles | | |
| Stages | (3a) | (3b) | (4) | (5a) | (5b) | (6) | (7) |
| 1. Initial control | 21.60±1.80$^{a}$ | 38.40±0.92$^{a}$ | 5.80±0.66$^{a}$ | 3.20±0.48$^{a}$ | 6.4±0.50$^{a}$ | 2.00±0.0$^{a}$ | - |
| 2. Treatment controls | 295.20±4.31$^{b}$ | 669.8±10.15$^{b}$ | 80.60±1.91$^{b}$ | 33.40±1.02$^{b}$ | 25.60±0.67$^{b}$ | 6.80±0.37$^{b}$ | 2.20±0.20$^{a}$ |
| 3. Stress groups | 349.20±2.39$^{c}$ | 747.80±4.88$^{c}$ | 126.40±2.06$^{c}$ | 95.20±4.06$^{c}$ | 47.00±3.06$^{c}$ | 9.40±0.92$^{c}$ | 4.80±0.37$^{b}$ |
| 4. Stress + Naltrexone | 316.60±2.02$^{d}$ | 725.80±2.13$^{d}$ | 102.40±1.63$^{d}$ | 78.20±1.90$^{d}$ | 34.00±0.83$^{d}$ | 4.80±0.37$^{d}$ | 3.60±0.24$^{c}$ |
| 5. Stress + Mifepristone | 313.60±1.80$^{de}$ | 724.20±2.08$^{d}$ | 91.80±1.39$^{e}$ | 73.00±2.00$^{d,e}$ | 33.6±1.02$^{d}$ | 4.00±0.44$^{d}$ | 3.60±0.40$^{c}$ |
| 6. Stress + Naltrexone + Mifepristone | 307.20±3.02$^{e,f}$ | 714.60±1.96$^{d}$ | 88.80±2.31$^{e,f}$ | 69.60±1.50$^{e}$ | 32.80±0.66$^{d}$ | 3.80±0.48$^{d}$ | 3.40±0.50$^{c}$ |
| 7. Stress + FSH | 304.00±2.34$^{f}$ | 686.40±5.81$^{e}$ | 86.00±1.58$^{f}$ | 41.60±1.63$^{f}$ | 30.20±0.66$^{d}$ | 3.60±0.24$^{d}$ | 3.40±0.50$^{c}$ |
| ANOVA F-Value | 1765.67 | 2625.40 | 468.09 | 230.59 | 82.48 | 25.60 | 17.65 |
| df=(6,28) | P<0.001 | P<0.001 | P<0.001 | P<0.001 | P<0.001 | P<0.001 | P<0.001 |

**Note.** Mean values in each column were compared by one way ANOVA followed by Duncan's multiple tests. Mean values with same superscript letters are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different. All values are mean ± SE; df= degree of freedom.

**DISCUSSION**

In the present study, separate rat pups from mother is used to induce stress in rats, as this procedure is widely used to study the effects of stress on different physiological process including reproduction (Endo, *et al*., 2001; Dayas, *et al.,* 2004; Kim *et al.,* 2004; Hesketh, *et al*., 2005; Uysal, *et al.,* 2005 and Cui, *et al*., 2008). This effect is in accordance to other reports in chronic exercise (Jain and Stevenson, 1991; Marti *et al*., 1993). In the present study demonstrated failure of stressed pre-pubertal rats to gain body weight similar to control, as the groups of (pre-pubertal) rats showed a significantly lower percent gain in the body weight. Similarly, decrease in body weight of rats due to pre-natal stress (Cabrera *et al.,* 1999) and neo-natal stress (Pellerin-Massicotte *et al*., 1987) has been reported. For instance, altered reproductive functions and impaired sexual behavior were found in offspring due to pre-natal stress exposure (Herrenkohl, 1979; Kinsley and Bridges, 1988 and Gutierrez *et al.,* 1989). In the present study percent gain in body weight of stressed rats was lower than controls. Since follicle number can provide important information about function of the ovary (Myers, *et al.,* 2004) the counts of different categories of follicles in the ovary following different stress treatments was carried out in the present study. Exposure of rats from day 15 to 22 to stress in the present study did not interfere with recruitment of primary to antral follicles all these were found in the ovaries on day 22 i.e., after completion of treatment, both in control and stress group rats. However, marked variations in the number of follicles were observed. There was a depletion of approximately 20% to 50% (follicle category wise) healthy follicles, in stressed rats compared to controls. The fact that all the categories of follicles were differentiated despite stressful condition, but the number of follicles was altered in all categories indicates impaired ovarian function. Observations on the other category follicles also support this view i.e., though there was no significant difference in other categories of

healthy follicles. Although, earlier workers, Pellerin-Massicotte *et al.* (1987), Christopher *et al.* (1996) and Rhees *et al.* (2001) studied the reproductive parameters following stress, they did not focus on stress induced alterations in follicular development. However, altered follicular development in 3 week old rats exposed to heat stress is reported by Shimizu *et al.* (2005). Reduction in number of ova ovulated in response to gonadotrophins, decrease in number of healthy pre-antral follicles and increase in atretic follicles were found in 3 week old rats exposed to heat stress (Shimizu *et al.,* 2005). Stress is also known to affect follicular development in mammals. Studies on stress induced alterations in follicular development have been conducted in cattle, sheep and rodents. Influence of heat stress on ovarian follicular kinetics has been studied in dairy cattles because it was a common experience that reproductive efficiency diminishes during summer. Experimentally induced that heat stressed cows tended to have increased number of follicles (≥10mm) during first wave, fewer small follicles (3 to 5mm) and medium sized follicles (6 to 9 mm) during second wave and lower plasma estrogen (Wolfenson *et al.,* 1995). Wilson *et al.,* (1998) reported altered growth and development of follicles, decreased serum level of estradiol and delay in luteolysis, resulting in failure of ovulation of second wave dominant follicles due to heat stress in cows. Similarly heat stress inhibited the development of dominant follicle during pre-ovulatory period as a consequence serum level of estradiol was reduced in cows (Wilson *et al.,* 1998). Trout *et al.,* (1998) reported increased number of small follicles (2 to 5mm) from day 11 to 15 of the estrous cycle and an increased progesterone levels due to heat stress in Holstein cows. Roth *et al.* (2000) reported increase in number of medium sized follicles (6 to 9mm) during the second follicular wave which was accompanied by higher plasma FSH levels and decrease in inhibin levels due to heat stress in cows. Further, exposure of cows to heat stress resulted in impaired steroidogenesis in medium and pre-ovulatory follicles (Roth, *et al.,* 2000). Heat stress delayed the timing of recruitment of ovulatory follicles and decreased LH receptor level and estradiol synthesis activity in the follicle (Ozawa *et al.,* 2005). Similarly infusion of stress like concentration of cortisol suppressed follicular development and LH surge in sheep (Macfarlane *et al.,* 2000). Chronic intermittent cold stress for 3 to 4 weeks in adult rats resulted in a decrease in the number of pre-antral healthy follicles without compensatory increase in atretic follicles and appearance of new category of follicles with hypertrophied thecal cell layers after 4 weeks of stress (Dorfman *et al.,* 2003). In addition to cold and heat stress, separated pups rat from mother induced stress also alters follicular dynamics in rodents. Stress due to electric foot shock in mice treated with gonadotrophins decreased number of ova ovulated *in vivo* and also rate of *in vitro* fertilization and serum levels of estradiol in rats (Kim *et al.,* 2004). The present study which involved exposure of pre-pubertal rats to stressor, indicates that pre-pubertal ovary is more vulnerable to disruptive action of stress, as the rate of atretia as judged by number of atretic follicles in each category in pre-pubertal rats (day 15 to 22) exposure. Regarding the mechanism of stress induced alterations in follicular development, it is well known that follicular recruitment and survival depend on gonadotrophic hormone (Greenwald and Roy, 1994 and McGee and Hsueh, 2000), Heat stress strongly inhibited FSH receptor level and aromatase activity in granulosa cells and estradiol

levels in follicular fluid of early antral, antral and pre-ovulatory follicles and increase in apoptosis of granulosa cells (Shimizu *et al.,* 2005). Based on these observations Shimizu *et al.,* (2005) hypothesized that heat stress inhibits function of follicular granulosa cells and suppress the follicular development. Since stress induced alterations in physiological processes are mediated by stress related hormones, especially hormones of HPA-axis, these might have role in altered ovarian activity. Glucocorticoid receptors have been shown in theca / granulosa cells of ovary and role of glucocorticoid in normal functioning of ovary and deleterious effect of high levels of glucocorticoid on reproduction have been reported (Michael and Cook, 1994; Tilbrook *et al.,* 2000 and Smith and Waddell, 2000). Glucocorticoid induced inhibition of steroidiogenic enzymes (Michael and Cook, 1994) and Suppression of hypothalamo-pituitary-gonadal axis due to activation of HPA axis under stressful condition is well known and reduction in GnRH and gonadotrophin secretion due to stress is documented (Tilbrook *et al.,* 2000). Hence, these changes might be due to impaired gonadotrophin secretion (Ferin, 1999 and Tilbrook *et al.,* 2000) gonadotrophin action (Shimizu *et al.,* 2005) or direct action of stress hormones (glucocorticoid) on follicular cell functions. The mechanism suggested above might be causing degeneration follicles under stressful conditions. Stage dependent hormone and growth factor regulation of follicular atretia is shown (Markström *et al.,* 2002). In primordial follicles oocyte apoptosis is responsible for subsequent follicular degeneration (Markström *et al.,* 2002). Locally produced growth factors are important for survival of pre-antral follicles (Markström *et al.,* 2002) where as early antral stage onwards FSH is the important survival factor for follicles (Hirshfield, 1991). It remains to be investigated whether stress related hormones interfere with these factors to induce apoptosis and there by cause degeneration of follicles. Since fluctuation in gonadotrophin levels during rodent estrous cycles affect pre-antral follicles which are known to have functional FSH and LH receptors (McGee and Hsush, 2000), it is possible that stress induced alterations in gonadotrophin secretion or action might have caused these degenerative changes in pre-antral follicles. In experiment, studies on stress effect on ovarian follicular development. Exposure of 15 days old rat pups to stress 6hrs per day, from day 15 to 22 resulted in a significant decrease in healthy follicles of all categories viz., primordial, primary, and pre-antral and a concomitant increase in mean numbers of atretic follicles of these categories, whereas similar treatment with drugs from day 15 to 22 resulted in significant an increase in healthy follicles of primordial, primary, and pre-antral follicles and decreased atretic of all categories of follicles.

The results indicate massive loss of follicles as healthy follicles of different categories were 20% to 50% less than in controls. Overall, the results of the study reveal that pre-pubertal follicular development is more vulnerable to stress effect and stress induced altered follicular complement at puberty results in early reproductive senescence.

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