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ORIGINAL ARTICLE

Stress induced alterations in pre-pubertal ovarian follicular development in rat

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The objective of the study was to find out whether stress experienced during neo-natal period alters the timing of formation of pre-antral and antral follicles and if so, whether pre-treatment with CRH receptor antagonist prevents these effects in rats. New born rat pups (n= 15) were exposed to maternal separation (6 hours/ day) from post-natal day (PND) 1 to 7 and were killed on PND 8, 11 and 15. The time of exposure was randomly changed every day during light phase (7Am to 7Pm) of the day to avoid habituation. There was a significant increase in serum corticosterone levels on PND 8 and 11 in stress group rats compared to controls indicating stress response in these pups. The ovary of both control and stressed rats contained oocytes and primary follicles on PND 8 and 11 and in showed progress of follicular development upto to pre-antral and early antral follicle formation on PND 11 and 15. However, mean number of healthy oocytes and all categories of follicles at all ages studied were significantly lower in stressed rats compared to controls. Concomitant with these changes, number of atreatic follicles showed an increase over control values in stressed rats. The increase in atresia of follicles was due to apoptosis as shown by increase in the percentage of granulosa cells showing TUNEL positive staining and caspase 3 activity. On the other hand, pre-treatment with CRH- receptor antagonist (CRH $_{9\text{-}41}$) 2ng/ 0.1 ml/ rat prior to undergoing stress regime on PND 1 to 7, prevented alterations in pre- pubertal follicular development thereby indicating that the ovarian changes were due to effects of stress induced activation of HPA axis. The results indicate that, stress during neonatal phase, though does not affect timing of formation of pre-antral and antral follicles, it does enhance atresia of follicles of all categories, including follicular reserve, which may affect the reproductive potential of adults. The results, for the first time reveal that CRF receptor antagonist prevents pre-pubertal ovarian stress response.

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It is reported that the female reproductive system is highly sensitive to stress (Warren and Perloth, 2001) and effect of stress on gonads in adults may be reversible but it may not be true during initial stages of development (Armstrong, 1986). Hence, stress experienced during neonatal or pre-pubertal

phases might have severe adverse effects on the ovarian follicular development. A few studies, reported earlier on influence of neonatal or pre-pubertal stress on reproduction have not focused on ovarian follicular development .For instance, in female rats, delay in puberty following forced swimming exercise (Pellarian- Massecotte *et al.,* 1987) , neonatal handling induced anovulatory cycles (Gomes *et al* 1999) and impaired development of reproductive function (Rhees *et al* , 2002) and neonatal maternal deprivation induced delayed vaginal opening and onset of estrous cycle in rats (Christopher- Lau et al., 1996) have been reported. These studies have not focused on stress induced alterations in pre-pubertal follicular dynamics. It is likely that animals face stressful conditions in their pre-pubertal stage in the wild. Since the pre-antral and antral follicles are first time formed during pre-pubertal phase and complement of follicles at puberty has bearing on adult follicular dynamics (Mazaud *et al.,* 2002) it is imperative to know whether stress during pre-pubertal life affects the ovarian follicular development. In addition it is not known whether treatment with a CRH receptor antagonist can prevent the ovarian stress response. Hence, the present study aims at investigating i) the effects of stress experienced on post-natal days 1-7 on ovarian follicular development and ii) whether pre-treatment with a CRH receptor antagonist, α-helical $CRH_{9-41,}$ prevents ovarian stress response.

MATERIAL 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 for albino rats. The tap water was provided in clean glass bottles. The rats were maintained in 12: 12 light and dark photoperiod (lights on 7AM 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 experimental protocols were approved by the Institutional animal ethics committee (IACE) of University of Mysore.

Experiments:

Experiment 1: The body weight of each pup was recorded immediately after birth on postnatal day (PND) 1 and the pups were randomly segregated into two groups. First group formed controls where in the pups were maintained with their mothers throughout the day without any disturbance. Pups in the second group (stress group) were exposed to maternal separation, 6 hours/ day from PND 1 to 7. The pups in this group were separated from their mothers and transferred to different cages having appropriate bedding. After 6 hours all the pups were shifted back to their original cages with their mothers. Timing of separation was randomly changed every day during light phase (7AM to 7 PM) to avoid habituation. Five pups from both the groups were autopsied on PND 8 ,11 and 15. At each autopsy weight of the body and ovary were recorded, later converted to relative weight (weight per 100 g body weight) of the ovary. The right ovary was fixed in Bouin's fixative and left ovary in buffered formaldehyde gluteraldehyde for histological and immunochemical studies respectively. The blood sample was collected and, serum was separated and stored at -20^{0}C until corticosterone concentration was determined.

Experiment 2: The rat pups on. PND 1 were randomly segregated into three groups. First group

pups served as controls and were maintained with their mothers throughout the day without any disturbance. Each pup in the second and third groups was exposed to maternal separation, 6 hours/ day as described in experiment 1. In the 3 rd group each pup received (ip) CRH antagonist (α- helical CRH_{9-41}), 2ng/ 0.1ml distilled water/day prior to undergoing stress regime. Five pups from all the three groups were autopsied on PND 8 and 11. At autopsy weight of the body and ovaries was recorded. The ovaries were fixed in Bouin's fluid for histological studies.

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). Oocytes within the syncytium were counted from each section. The naked oocytes from every alternate section, the primordial follicles from every 4th section, and primary follicles (type 3a) from every 6th 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 granulosa cells in the largest cross section of the follicle.

TUNEL (Terminal nuceotidyl transferase dUTP nick end labelling) assay:

The follicular atresia, determined by histological criteria was further characterized by conducting TUNEL and caspase- 3 assays. Serial sections (8μm) of the ovary were cut from left ovary fixed in buffered formaldehyde gluteraldehyde and were spread on 3- aminopropyltriethoxy saline coated micro slides. The TUNEL assay was performed in some randomly selected sections using the kit supplied by Roche Diagnostics India Pvt Ltd, Chennai, India, following the procedures of the kit manufacturer. The number of TUNEL positive and negative granulosa cells were counted in randomly selected areas in ovary sections and expressed as percentage of TUNEL positive and negative granulosa cells.

Immunohistochemical localization of caspase-3:

Some randomly selected sections of the left ovary fixed in buffered formaldehyde gluteraldehyde were spread on 3-aminopropyltriethoxy saline coated micro slides. The primary antibody (anti-mouse caspase-3) was purchased from Bi Biotech India Pvt Ltd, R & D Systems, New Delhi, India and a standard immunohistochemistry technique protocol (Feranil *et al.,* 2005) was used. Caspase -3 positive and negative granulosa cells were counted from randomly selected areas and expressed as percentage of caspase-3 positive and negative granulosa cells.

Estimation of serum concentration of corticosterone:

The corticosterone concentration was determined by enzyme linked immuno sorbant assay (ELISA) using the kit purchased from Neogen Corporation, Canada. The corticosterone was extracted from the serum collected at autopsy and stored at – 20^0C, following the procedure of the manufacturer.

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 17.0.

Table 1 Effect of stress experienced during post natal days 1 to7 on body and ovary weight and serum corticosterone levels in rat.

Groups		Body weight (g)	Percent change increase in body weight compared to initial body weight	Relative weight of ovary (mg/ 100g of body weight)	Serum corticosterone level (ng/ml)
PND 8	**Control group**	10.58 ± 0.27^{a}	52.96 ± 1.30^{a}	56.59 ± 0.35^{a}	0.59 ± 0.003^{a}
	Stress group	9.58 ± 0.30^{a}	48.95 ± 0.99^{a}	45.71 ± 0.66^{b}	1.15 ± 0.002^{b}
PND 11	**Control group**	16.70 ± 0.26^{b}	72.40 ± 1.91^{c}	60.23 ± 0.37^{c}	0.77 ± 0.002^{c}
	Stress group	$10.80 \pm 0.38^{a,c}$	59.41 ± 4.24^{d}	48.03 ± 0.60^{d}	1.35 ± 0.002^{d}
PND 15	**Control group**	14.50 ± 1.31^{c}	68.58 ± 0.99^{c}	64.50 ± 1.23^{e}	
	Stress group	12.56 ± 0.39^{d}	60.17 ± 1.15^{d}	54.46 ± 0.68^{f}	
ANOVA F value		**19.29 (df=5,24) P<0.01**	**17.90 (df=5,24) P<0.01**	**99.66 (df=5,24) P<0.01**	**269.62 (df= 3,8) P<0.01**

- df= degrees of freedom. PND, postnatal day.
- All values are Mean ±SE; Mean values with same superscript letters in a given column are not significantly different, whereas those with different superscript letters are significantly ($P<0.05$) different as judged by Duncan's multiple test.

RESULTS

Experiment 1:

Body and ovary weight:

Mean body weight and percentage gain in body weight of the pups in stress group were significantly lower on PND 11 and 15 compared to controls whereas they did not differ on PND 8 compared to controls (Table 1). Relative weight of the ovary in the stress group was significantly lower on PND 8, 11 and 15 compared to controls (Table1).

Serum corticosterone levels:

Mean of corticosterone level was significantly higher in stressed rats on PND 8 and 11 compared to controls (Table- 1).

Histology of the ovary and follicle counts:

On PND 8, oocytes, primordial and primary follicles (both type 3a and 3b) were present in the ovaries of both the control and stress group rats. Pre-antral follicles i.e. type 4, 5a and 5b, and early antral follicles (type 6) were present in the ovaries of control and stress group rats on PND 11 and 15 in addition to primordial and primary follicles, whereas oocytes were not found on PND 15 (Table-2).

Mean number of oocytes (type 1) was

significantly lower in stress group on PND 8 compared to controls (Table-2). Mean number of primordial follicles (type 2) and primary follicles (type 3a) was significantly lower in stress group on PND 8, 11 and 15 compared to those of controls (Table- 2). There was a significant reduction in the mean number of advanced primary (type 3b) and early pre- antral follicles (type 4) in stress group on PND 11 and 15 compared to controls (Table-2). Mean number of pre-antral follicles type 5a was significantly lower in stress group on PND 11 compared to controls where as type 5b was significantly lower in stress group on PND 15 compared to controls (Table-2). Mean number of early antral follicles (type 6) was significantly lower in stress group on PND 15 compared to controls (Table-2).

Atresia:

Mean number of atreatic primary follicles (type 3a) were significantly higher in stressed rats on PND 8, 11 and 15 compared to controls (Table- 3). Mean number of atreatic advanced primary follicles (type 3b) and pre-antral follicles (type 4, 5a and 5b) were significantly higher in stressed rats on PND 11 and 15 compared to controls. Likewise atreatic antral follicles (type 6) number was significantly higher in stressed rats on PND 15 compared to controls (Table-3)

TUNEL assay and caspase-3 localization:

The ovaries of stressed rats revealed the increased percentage of granulosa cells showing TUNEL positive staining on PND 8, 11 and 15 (Fig. 2a & b), and caspase-3 immunolocalization on PND 11 and 15 compared to controls (Table- 4, Fig. 3a &b).

Experiment 2:

Body and ovary weight:

Mean body weight and percentage gain in body weight, mean relative weight of the ovary were significantly reduced on PND 8 and 11 in stressed rats compared to controls and anti-CRF treated stressed rats (Table- 5). Mean relative weight of the ovary in anti-CRF treated stressed rats was significantly reduced compared to controls on PND 8 (Table- 5).

Histology of the ovary and follicular counts:

On PND 8 oocytes, primordial and primary follicles were present in all the three groups and in addition to these, pre- antral follicles (type 4, 5a and 5b) and early antral follicles (type 6) were present in the ovaries of rats in all the three groups on PND 11 (Table- 6). Mean number of oocytes (type 1), primordial follicles (type 2) and primary follicles (type 3a) was significantly lower in stressed rats on PND 8 and 11 compared to controls and anti-CRF treated stressed rats (Table- 6). There was no significant difference in the mean number of advanced primary follicles (type 3b) between controls; anti-CRF treated stressed rats on PND 8 and 11 (Table- 6). Mean number of pre-antral follicles *i.e.* type 4, 5a and 5b and early antral follicles (type 6) was significantly lower in stressed rats compared to controls and anti-CRF treated stressed rats on PND 11 (Table- 6).

Atresia:

Mean number of atreatic primary follicles i.e. both type 3a and 3b was significantly higher in stressed rats on PND 8 on PND 11 compared to controls and anti-CRF treated stressed rats (Table- 7). Mean number of atreatic pre- antral follicles (type 4, 5a and 5b) and early antral follicles (type 6) was significantly higher in stressed rats on PND 11 compared to controls and anti-CRF treated stressed rats (Table-7).

Table 2 Effect of stress on ovarian follicles during pre- pubertal period.

Groups		Mean number of healthy follicles/ ovary ± SE								
		Mean number of oocytes ± SE	Category	Primordial	Primary		Pre-antral			Antral
			Stage	2	3a	3b	4	5a	5b	6
PND 8	Control group	1125.6±49.36^{a}		2251.4± 21.57^{a}	394.8± 16.47^{a}	66.2± 6.06^{a}				
	Stress group	576±32.06^{b}		1973.4± 17.92^{b}	193.6± 3.65^{b}	55.4± 2.83^{a}				
PND 11	Control group	258± 15.15^{c}		2048.2± 7.76^{b}	387.2± 27.81^{a}	145.8± 7.22$^{b, d}$	62± 2.91^{a}	44.6± 2.54^{a}	25.0± 2.14^{a}	5.6± 0.4^{a}
	Stress group	191.6± 14.61^{c}		1469.2± 131.41^{c}	240.8± 6.22^{c}	108.8± 7.75^{c}	30± 2.93^{b}	23± 1.48^{b}	23± 1.48^{a}	2.5± 0.5^{a}
PND 15	Control group			2672.4± 11.83^{d}	449.8± 16.85^{d}	161.4± 9.09^{d}	85.4± 3.66^{c}	64.2± 3.51^{c}	33.2± 1.52^{b}	23.4± 1.28^{b}
	Stress group			1931.2± 37.03^{b}	356.8± 12.15^{a}	129.2± 4.47^{b}	72.4± 3.18^{d}	61± 2.09^{c}	20.6± 1.46^{a}	15± 0.83^{c}
ANOVA F value P<0.01		194.65 (df=3.16)		47.98 (df=5,24)	38.30 (df=3,24)	49.58 (df=5,24)	54.97 (df=3,16)	56.05 (df=3,16)	10.95 (df=3,16)	89.74 (df=3,13)

- df= degrees of freedom. PND, postnatal day.
- All values are Mean ±SE; Mean values with same superscript letters in a given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different as judged by Duncan's multiple test.

Table 3 Mean Number of atreatic follicles in controls and stressed rats.

Groups		Mean number of atreatic follicles/ ovary ± SE					
		Primary		Pre-antral			Antral
		3a	3b	4	5a	5b	6
PND 8	Control group	14.20±0.73^{a}	8.60± 0.50^{a}	-	-	-	-
	Stress group	21.60±0.74^{b}	10.40±0.60^{a}	-	-	-	-
PND 11	Control group	11.60±0.87^{c}	8.60±0.40^{a}	9.00± 0.44^{a}	8.20±0.58^{a}	5.60±0.40^{a}	3.40±0.50^{a}
	Stress group	21.80±0.96^{b}	21.60±0.87^{b}	15.40±0.67^{b}	14.80±0.58^{b}	9.20±0.37^{b}	3.25±1.31^{a}
PND 15	Control group	18.20±0.96^{d}	13.60±0.67^{c}	9.20±0.48^{a}	8.80±0.37^{a}	6.20±0.37^{a}	5.20±0.58^{a}
	Stress group	23.40±0.67^{b}	24.20±1.06^{d}	14.20±0.37^{b}	12.00±0.44^{c}	10.80±0.37^{c}	8.50±0.28^{b}
ANOVA F value		31.62 (df= 5,24) p< 0.01	88.56 (df= 5,24) p< 0.01	42.60 (df=3,16) p< 0.01	36.77 (df=3,16) p< 0.01	42.00 (df=3,16) p< 0.01	10.26 (df=3,16) p< 0.01

- df= degrees of freedom. PND, postnatal day.
- All values are Mean ±SE; Mean values with same superscript letters in a given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different as judged by Duncan's multiple test.

Table 4 Percentage of TUNEL and caspase- 3 positive and negative granulosa cells in control and stressed rats.

		Mean percent of granulosa cells.			
		TUNEL		**Caspase- 3**	
		NEGATIVE	**POSITIVE**	**NEGATIVE**	**POSITIVE**
PND 8	**Control group**	91.00±1.15[a]	9.00±1.15[a]	90.33±0.88[a]	9.67±0.88[a]
	Stress group	77.33±2.02[b]	22.67±2.02[b]	86.66±1.45[a,b]	13.34±1.45[a,b]
PND 11	**Control group**	87.33±0.88[a]	12.67±0.88[a]	88.00±2.08[a,b]	12.00±2.08[a,b]
	Stress group	69.66±0.88[c]	30.34±0.88[c]	83.66±1.76[b,c]	16.34±1.76[b,c]
PND 15	**Control group**	85.66±2.02[a]	14.34±2.02[a]	82.00±1.15[c]	18.00±1.15[c]
	Stress group	63.33±2.33[d]	36.67±2.33[d]	78.66±0.88[d]	21.34±0.88[d]
F value (df=)		42.99 (df= 5,12) (p< 0.05)	42.99 (df= 5,12) (p< 0.05)	8.77 (df= 5,12) (p< 0.05)	8.77 (df= 5,12) (p< 0.05)

- df= degrees of freedom. PND, postnatal day.
- All values are Mean ±SE; Mean values with same superscript letters in a given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different as judged by Duncan's multiple test.

Table-5 Effect of stress and CRF antagonist on body and ovary weight in pre-pubertal rats.

Groups		**Body weight (g)**	**Percentage increase in body weight**	**Relative weight of ovary (mg/ 100g of body weight)**
PND 8	**Control group**	11.47± 0.20[a]	58.18±1.90[a]	58.54±0.48[a]
	Stress group	8.86± 0.20[b]	41.25±1.22[b]	45.83±0.21[b]
	Stress + anti-CRF group	11.40± 0.29[a]	55.25±1.19[a]	55.82±0.49[c]
PND 11	**Control group**	17.09± 0.29[c]	72.47±1.20[c]	62.73±0.54[d]
	Stress group	14.42± 0.45[d]	63.03±1.31[d]	48.30±0.75[e]
	Stress + anti-CRF group	16.92± 0.16[c]	71.61±0.81[c]	61.21±0.55[d]
F value (df=5,24)		**134.04** (p< 0.01)	**77.93** (p< 0.01)	**169.57** (p< 0.01)

- f= degrees of freedom. PND, postnatal day.
- All values are Mean ±SE; Mean values with same superscript letters in a given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different as judged by Duncan's multiple test.

Table- 6 Effect of stress and CRH antagonists on oocytes and ovarian follicles in rat.

Groups		Mean number/ ovary ± SE							
		Mean number of oocytes ± SE	Mean number of follicles ±SE						
			Category: Primordial	Primary		Pre-antral			Antral
			Stage: 2	3a	3b	4	5a	5b	6
PND 8	Control group	1144.25± 57.02[a]	2288.25 ± 35.15[a]	396.75± 11.81[a]	74.75± 4.73[a]				
	Stress group	625.40± 35.32[b]	1925.80 ± 15.46[b]	211.40± 14.95[b]	51.00± 2.96[a]				
	Stress + anti-CRF group	1015.25± 38.58[c]	2050.75 ± 70.48[b]	388.50± 12.03[a]	66.00± 3.10[a]				
PND 11	Control group	260.60± 18.09[d]	2060.60 ± 18.95[b]	333.20± 21.13[a,c]	140.20± 8.62[b]	64.80± 4.8[a]	46.60± 1.43[a]	28.00± 2.12[a]	5.00± 0.31[a]
	Stress group	235.20± 15.12[d]	1500.80 ± 63.93[c]	231.80± 13.47[b]	128.06± 14.87[b]	33.80± 4.11[b]	28.40± 2.80[b]	4.20± 0.37[b]	1.4± 0.60[b]
	Stress + anti-CRF group	230.50± 19.62[d]	2013.00 ± 58.50[b]	299.75± 46.30[c]	134.00± 8.08[b]	61.25± 3.75[a]	40.50± 3.27[a]	25.75± 1.43[a]	4.25± 0.62[a]
F value P<0.01		**156.03 (df=5,21)**	**31.82 (df=5,21)**	**12.39 (df=5,21)**	**20.82 (df=5,21)**	**15.96 (df=2,11)**	**14.43 (df=2,11)**	**78.70 (df=2,11)**	**14.01 (df=2,11)**

- df= degrees of freedom. PND, postnatal day.
- All values are Mean ±SE; Mean values with same superscript letters in a given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different as judged by Duncan's multiple test.

Table-7 Number of atreatic follicles in control, stressed and CRH antagonist treated stressed rats.

Groups		Mean number of atreatic follicles ± SE					
		Primary		Pre-antral			
		3a	3b	4	5a	5b	
PND 8	Control group	13.25±0.62[a]	8.25±0.47[a]	-	-	-	-
	Stress group	22.80±1.24[b]	16.20±1.01[b]	-	-	-	-
	Stress + anti-CRF group	12.75±0.47[a]	9.00±0.40[a,c]	-	-	-	-
PND 11	Control group	13.40±0.74[a]	10.60±0.67[c]	10.20±0.73[a]	8.60±0.50[a]	6.00±0.44[a]	4.20±0.20[a]
	Stress group	21.40±0.81[b]	20.60±0.81[d]	17.60±0.50[b]	15.00±0.44[b]	9.00±0.44[b]	1.00±0.63[b]
	Stress + anti-CRF group	12.25±0.47[a]	10.25±0.47[a,c]	8.75±0.75[a]	8.25±0.62[a]	6.25±0.25[a]	3.00±0.57[a]
F value		**34.43 (df=5,21) P<0.01**	**45.78 (df=5,21) P<0.01**	**51.41 (df=2,11) P<0.01**	**54.56 (df=2,11) P<0.01**	**16.94 (df=2,11) P<0.01**	**11.19 (df=2,11) P<0.01**

- df= degrees of freedom. PND, postnatal day.
- All values are Mean ±SE; Mean values with same superscript letters in a given column are not significantly different, whereas those with different superscript letters are significantly (P<0.05) different as judged by Duncan's multiple test.

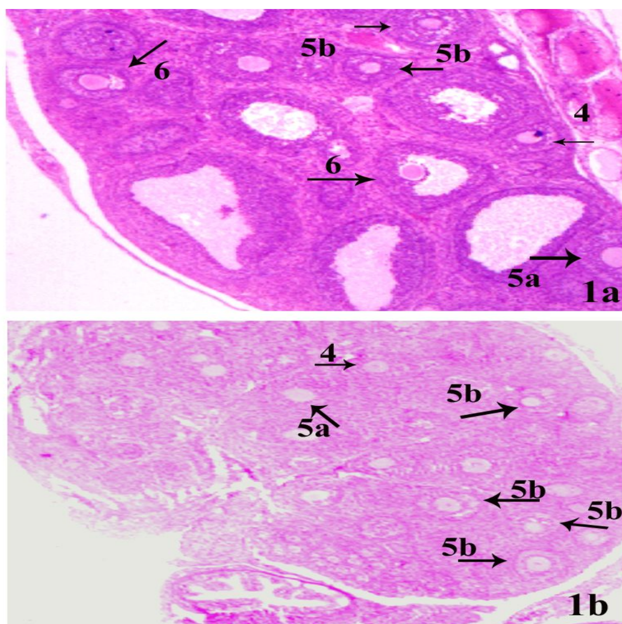

Figure 1 (a & b) Cross sections of the ovary showing pre-antral follicles (type 4, 5a and 5b) and early antral follicles (type 6) on postnatal day 15 in both the control (Fig. 1a) and stressed (Fig. 1b) rats. H7 E, 40X

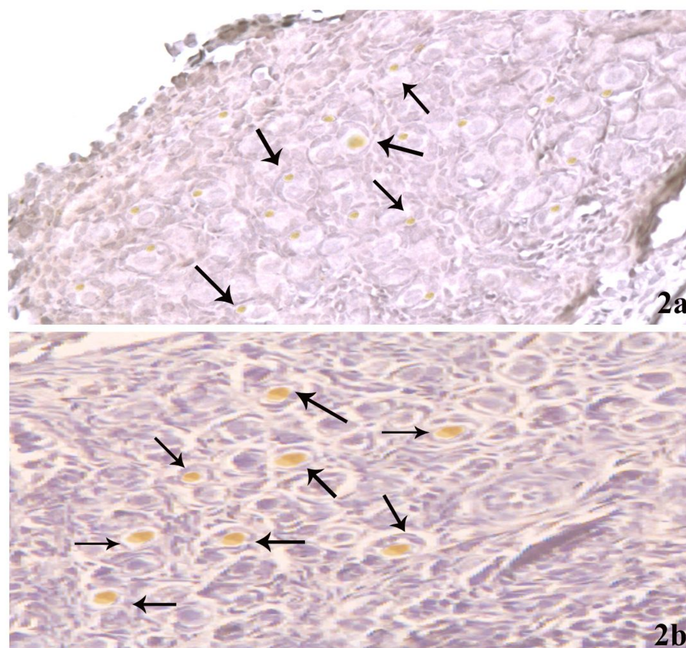

Figure 2 (a & b) Cross section of the ovary showing TUNEL staining in control (Fig. 2a) and stressed (Fig. 2b) rats on PND 11. Note the presence of more number of TUNEL positive oocytes (arrows) in stressed rats compared to controls. 200X.

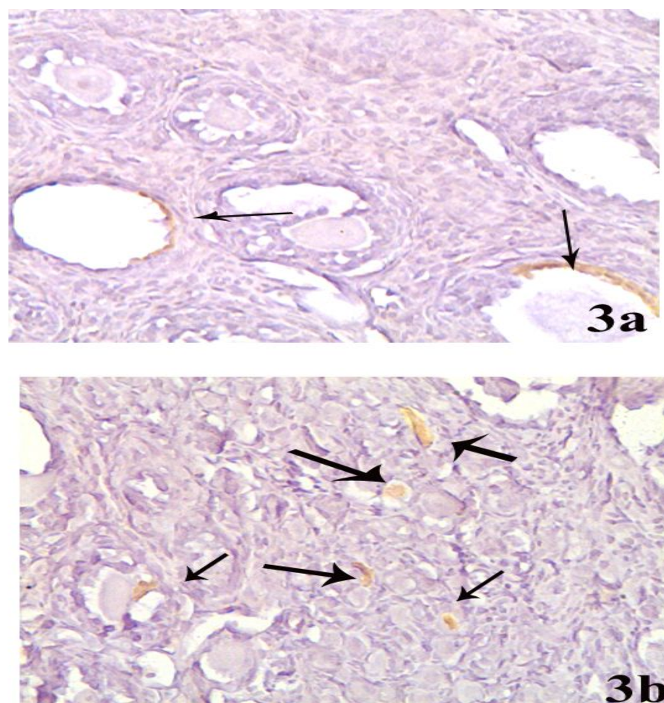

Figure 3 (a & b) Cross sections of the ovary showing caspase- 3 immuno localization on PND 15. Note the presence of more number of granulosa cells in the stressed rats (Fig. 3b) showing caspase-3 immunolocalization compared to controls (Fig. 3a). 100X.

DISCUSSION

Maternal separation is a widely used stressor to study the effects of stress in neonatal rats (Lau *et al.,* 1999; Rhees *et al.,* 2001; Kwak *et al.,* 2009) and an increase in serum levels of corticosterone, which is an indication of stress , has been reported following maternal separation in rats(Wilber *et al.,* 2007). Further, stress is known to inhibit feeding in young animals (Carr 2002; Car and Norris, 2005) leading to loss in body weight. In the present study, maternal separation (6 hours/ day from PND 1 to 7) induced significant increase in serum corticosterone concentration on PND 8 and its prevalence on PND 11 coupled with loss in body weight on PND 11 and 15 indicate that the pups were stressed and hence the ovarian changes were stress responses. Maternal separation from PND 1 to 7, in the present study, resulted in marked alteration in ovarian follicular counts. Since, the follicular compliment at puberty has bearing on adult reproductive life (Mazaud, *et al* 2002), the altered follicular counts induced by maternal separation during pre-pubertal period in the present study may have impact on adult ovarian function.

Several studies revealed stress induced alterations in the follicular development in adults. For instance, stress due to heat alters the efficacy of follicular selection and dominance (Badinga *et al.,* 1993, Jordan *et al.,* 2003) and oocyte development (Hansen, 2009) in cows, first wave of dominant follicle and pre-ovulatory follicles in cattle (Wolfenson *et al.,* 1995) and adversely affects follicular recruitment in goats (Ozawa *et al.,* 2005). Similarly, excessive rain fall or transportation reduces ovulation rate in sheep (Daley, 1999). Present study, for the first time, reveals effects of stress experienced during neo-natal period on pre-pubertal ovarian follicular development. The study gains importance due to the fact that follicular reserve is established and first time different

categories of follicles differentiate during neo-natal and pre-pubertal life respectively. The fact that, controls as well as stressed rats ovaries contained primary follicles on PND 7, pre-antral follicles on PND 11 and antral follicles on PND 15 reveal that stress did not interfere with chronology of differentiation of different category of follicles, as the observations are comparable to those reported earlier in rats (McGee and Hsueh, 2000). However, there was a drastic effect of stress on survival of healthy follicles of different categories in the present study, as maternal separation from PND 1 to 7 , caused a significant reduction in healthy oocytes on PND 8, primordial and primary follicles (type 3a) on PND 8, 11 and 15, pre-antral (4) and antral follicles on PND 15. In addition relative weight of the ovary was also reduced in stressed rats on PND 8, 11 and 15. The results reveal that all categories of follicles in pre-pubertal ovary are sensitive to stress. Stress effects on adult ovary reported earlier (Badinga *et al.,* 1993, Daley, 1999, Wolfenson *et al.,* 1995, Jordan *et al.,* 2003, Ozawa *et al.,* 2005, Hansen, 2009) and in the pre-pubertal ovary in the present study put together demonstrate that irrespective of stage of development stress interferes with ovarian function in mammals.

In the present study, in stressed rats, decrease in the number of healthy follicles of any category was not accompanied by an increase in the number of follicles next in the hierarchy. On the other hand, there was an increase in the number of atreatic follicles concomitant with decrease in number of healthy follicles of each category, thereby suggesting that the decrease in the number of healthy follicles was due to increase in rate of atresia in stressed rats. The degeneration (atresia) of follicles is an apoptotic process (Durlinger *et al.*, 2000), and in the present study molecular markers used to characterize atresia *i.e.* TUNEL assay and Caspase-3 assay also support the increased rate of atresia due to stress. It is reported that caspases execute apoptosis (Hangartner, 2000; Markstrom *et al.,* 2002) and activate DNase (CAD caspase activated DNases), an endonuclease responsible for internucleosomal DNA fragmentation, which is hallmark of apoptosis (Markstrom *et al.,* 2002). Activation of caspase-3 leads to final stages of cellular death, by proteolytic dismantling of large variety of cellular components (Nicholson, 1999; Yakobi *et al.,* 2004) and is required for granulosa cell apoptosis during follicular atresia (Matikainen *et al.,* 2001). The cleavage of genomic DNA during apoptosis may yield double as well as single stranded breaks (nicks), which can be identified by labeling 3^{l}-OH terminal with modified nucleotidyl transferase which catalyses polymerization of labeled nucleotides to 3^{l}-OH DNA ends in the template independent manner. In the present study increase in number of primary, pre-antral and antral atreatic follicles as judged by morphological criteria in stressed rats was accompanied by a significant increase in the percentage of granulosa cells positive to caspase-3 and TUNEL staining thereby, conforming that the loss of follicles is by apoptotic process in stressed rats.

Activation of HPA- axis (Tsigos *et al.,* 1999; Kapoor *et al.,* 2006; Kyrou and Tsigos, 2008) and deleterious effects of HPA axis hormones on gonads in mammals are well documented (Rivest and River, 1995; Ferin, 1999; Tillbrook *et al.,* 2000; Kalantaridou *et al.,* 2004). Activation of HPA axis (Tsigos *et al.,* 1999; Kapoor *et al.,* 2006; Kyrou and Tsigos, 2008) and an increase in the glucocorticoid secretion are the most common stress responses (Maeda and Tsukumara, 2006). Deleterious effects of increased secretion of glucocorticoid are well documented. Glucocorticoids inhibit GnRH and gonadotropin secretion (Dubey and Plant, 1985;

River and Vale, 1985; Rabin *et al.*, 1990; Chatterjee and Chatterjee, 2009; Saketos *et al.,* 1993; O'Conner *et al.,* 2000), reduce sensitivity of gonadotrophin receptors (Rabin *et al.,* 1988; Calegero *et al.,* 1999; O'Conner *et al.,* 2000), reduce expression of acute steroid regulatory protein (STAR) (Haung and Li, 2001) and induce apoptosis of granulosa cells (Sasson *et al.,* 2001; Shimuzu *et al.,* 2005). In the present study, there was a significant increase in serum levels of corticosterone on PND 8 and 11 following exposure to maternal separation from PND 1 to 7 indicating stress response and activation of HPA axis. Concomitant with increased corticosterone level, there was increased rate of follicular atresia (increase in the number of atreatic follicles) in stressed rats. However, pre-treatment with a CRH- antagonist (CRF_{9-41}), prevented stress induced increase in atresia, because number of healthy as well as atreatic follicles *i.e.* primary, pre-antral and antral follicles in CRF_{9-41} treated stressed pups did not significantly differ from controls despite undergoing stress, whereas those of stressed rats without anti-CRF treatment (group 2) showed significant increase in atreatic follicles and decrease in healthy follicles of all categories compared to controls.. Since anti-CRF prevents stress induced increased secretion of corticosterone (Maciag *et al.,* 2002), the altered follicular complement following stress and its prevention due to pre-treatment with anti-CRF in stressed rats in the present study indicate that, stress induced altered follicular development in the present study was due to the activation of HPA- axis. The results for the first time show that, either CRF_{9-41} or similar CRH receptor antagonists can prevent deleterious ovarian stress responses in mammals.

Since many decades, a dogma prevailing in mammalian oocyte biology was that a finite number of primordial follicles are formed (follicular reserve) either pre or post-natally, and this reserve is not renewed in life time (Zuckerman *et al.,* 1951; McLaren, 1984; Anderson and Hirshfeild, 1992; Greenwald and Roy, 1994; McGee and Hsueh, 2000; Guigon *et al.,* 2003; Telfer, 2004; Kerr *et al.,* 2006; Bristol-Gould *et al.,* 2006; Findlay *et al.,* 2009). Gradual depletion of this reserve in adults occurs by atresia and ovulation leading to reproductive senescence (Cohen, 2004). However, in recent years this dogma has been challenged by the concept of neo-oogenesis (Johnson *et al.,* 2004, 2005; Lee *et al.,* 2007). Proliferative large cells purified from neonatal or adult mouse ovaries, maintained *in vitro*, when transplanted into ovaries of chemotheraphy sterilized recipients, generated oocytes, which were fertilized leading to viable offspring (Zou *et al.,* 2009). Pachiarotti *et al.* (2010) also endorsed the neo-oogenesis hypothesis by demonstrating potential germ line stem cells derived from post-natal mouse ovary. These reports revealed that adult mammalian ovary can renew oocytes. However arguments were made against replenishment of oocytes, by statistical analysis of the follicle pool over reproductive period in mice (Bristol-Gould et al., 2006; Faddy and Gosden 2009). A study involving mathematical modeling of the ovarian reserve found no evidence to support the occurrence of neo-oogenesis in humans (Wallace and Kelsey, 2010).

Hence, to date a consensus has yet to emerge regarding the validity of neo-oogenesis in adult female mammals and arguments have been put forth in favour and against the neo-oogenesis (Notarianni, 2011). It is note worthy that except the study of Zou *et al.,* (2009), the oocyte like cells which are source of follicular reserve, described by other workers cited above have not proved the capability of these oocyte like cells to form new individuals after fertilization. Further, thus far there is no

experimental demonstration that despite loss of follicular reserve either by chemotheraphy or other means, the individuals could show normal reproductive life span, comparable to normal individuals, by renewal of lost primordial follicles. The present study provides an evidence for formation of new batch of primordial follicles in pre-pubertal ovary. On PND 15 mean number of healthy primordial follicles were significantly higher in controls as well as in stressed rat ovaries compared to respective groups on PND 11.It is interesting to note that, on PND 15, despite increase in number of follicles of higher categories, i.e. primary, pre-antral and antral, which are derived from primordial follicles, the number of primordial follicles showed a significant increase in contrast to their significant decrease concomitant with increase in number of higher category follicles on PND 11 compared to PND 8 in both the groups. .Hence, increase in number of primordial follicles on PND 15 might be due to formation of new batch of primordial follicles between PND 11 and PND 15.Thus our present study provides a preliminary evidence for possibility of formation of new primordial follicles beyond normal period *i.e.* between PND 1 and 3 (McGee and Hsueh, 2000) reported in rats. However, this phenomenon may be due extended period of formation follicle reserve; rather than neo-oogenesis as it is observed in pre-pubertal rats..

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