Long-term chronic stress exposure induces PCO phenotype in rat

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Abstract

Thus far the effects of chronic stress on the ovary were studied for shorter durations. However, responses of the ovary may vary with durations of exposure to stress. Hence, we investigated the responses of the ovary following exposure to different durations of chronic stress. Exposure of rats to restraint (1 h) and after a gap of 4 h to forced swimming (15 min) daily for 4 or 8 weeks resulted in significant decreases in the activities of the ovarian antioxidant enzymes, 3 β -hydroxysteroid dehydrogenase and percentage of healthy granulosa cells with concomitant increases in the number of atretic follicles, the percentage of apoptotic granulosa cells and ovarian malondialdehyde concentration. However, the response of the ovary to similar stress regime for 12 weeks was paradoxical as there were increases in the activities of ovarian antioxidant enzymes and 3 β -hydroxysteroid dehydrogenase, the number of healthy antral follicles, and decreases in ovarian malondialdehyde concentration and percentage of apoptotic granulosa cells. These changes were accompanied by hyperglycaemia and an increase in the serum levels of insulin, testosterone and oestradiol. In addition the cystic follicles were found in the ovaries of these rats. However, the number of oestrous cycles and active corpora lutea showed significant decrease in all the durations of stress exposure. The results demonstrate a differential response of ovary to short- and long-term exposure to chronic stress.

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Introduction

The ovarian functions are influenced by several factors viz., light (Campbell & Schwartz 1980), temperature (Roth *et al.* 2000, Saraswathi *et al.* 2010), radiation (Said *et al.* 2012), endocrine-disrupting chemicals (Patel *et al.* 2015), etc. In recent years, stress has emerged as one of the major factors interfering with female reproduction. Stress activates hypothalamus–pituitary–adrenal (HPA) axis (Chrousos *et al.* 1998). The activated HPA axis suppress the hypothalamus–pituitary–ovarian (HPO) axis (Whirledge & Cidlowski 2010). Several studies have shown altered ovarian functions due to stress. For instance, stress-induced impairment in the follicular development (Roth *et al.* 2000, Ozawa *et al.* 2005, Bhat & Yajurvedi 2011, Wu *et al.* 2011) and the developmental potential of oocytes (Zhang *et al.* 2011, Lian *et al.* 2013, Gao *et al.* 2016); blockage of ovulation (Daley *et al.* 1999); reduction in steroidogenesis (Whirledge & Cidlowski 2010) and irregular oestrus cyclicity (Saraswathi *et al.* 2010) have been reported. Stress also results in oxidative damage due to increased production of free radicals (Spiers *et al.* 2015). Several studies have shown the role of reactive oxygen species (ROS) in the folliculogenesis, oocyte maturation and steroidogenesis (Agarwal *et al.* 2005, Gupta *et al.* 2008, Ruder *et al.* 2009). However an increase in ROS

content in the follicular fluid environment results in early ovarian senescence (Gupta *et al.* 2008). Although a number of studies have shown chemical oxidants (Devine *et al.* 2012) and pathology-induced oxidative damage in the ovary (Agarwal *et al.* 2005), thus far there are only a couple of studies that demonstrate the stress-induced oxidative damage in the ovary (Saraswathi *et al.* 2012, Lian *et al.* 2013). In addition, whether the ovarian stress response, including antioxidant defence, differs according to duration of stress exposure is not known. It is possible that the stress effects on ovarian follicular development and antioxidant defence might be more severe with an increase in exposure period, or might remain in the same state, or the ovary may cease to respond due to habituation. Hence, this study tests the hypothesis that ovarian stress response with reference to antioxidant defence, follicular development and steroidogenic activity varies with durations of exposure.

Materials and methods

Animals and experimental design

Adult female Wistar rats in the weight range of 170–190 g and exhibiting regular estrous cycles were obtained from the central animal house, University of Mysore, Mysuru. The rats were provided food and water *ad libitum* and maintained

under 12-h light/12-h darkness photoperiod. This experiment was approved by the Institutional Animal Ethical Committee of University of Mysore and followed the guidelines of the committee for treatments and maintenance of animals. Three experiments were conducted and in each experiment, rats were divided into 2 groups viz., control and stress groups. Control rats were maintained without any disturbance, whereas each rat in stress group was exposed to two stressors according to the procedure of [Grissom et al. \(2008\)](#): restraint (1 h) in an open-ended cylindrical glass restrainer measuring 6.7 cm in diameter and 22.3 cm in length and then after a gap of 4 h to forced swimming (15 min) in a chromatographic jar of 45.72 cm height and 22.23 cm outer diameter, filled with 2/3rd of water at room temperature ($27 \pm 2^\circ\text{C}$). The rats were exposed to this stress regime every day for 4, 8 and 12 weeks in experiments 1, 2 and 3 respectively. In each experiment, stage of the estrous cycle of each rat was recorded every day by the vaginal smear method ([Cooper et al. 1993](#)). The average number of estrous cycles per 4 weeks per group was determined at the end of each experiment. After completion of each experiment rats in control and stress groups were autopsied. At each autopsy, blood sample, the ovaries and the adrenal glands were collected and processed for different analyses as described in the following section.

Fasting blood glucose concentration

Fasting blood glucose concentration was estimated in all the rats at weekly intervals throughout the experimentation by collecting blood samples from tail vein and using GOD-POD kit ([Barham & Trinder 1972](#)).

Biochemical analyses

The ovary was homogenised in 0.32M sucrose solution, centrifuged and the supernatant was used for assays to quantify the activities of different enzymes viz., 3β -hydroxysteroid dehydrogenase (3β -HSDH) ([Shivanandappa & Venkatesh 1997](#)), superoxide dismutase (SOD) ([Marklund & Marklund 1974](#)), catalase (CAT) ([Aebi 1984](#)), glutathione S-transferase (GST) ([Habig et al. 1974](#)) and glutathione peroxidase (GPx) ([Tappel 1978](#)). The ferric reducing antioxidant power (FRAP) ([Benzie & Strain 1996](#)) and malondialdehyde concentration (MDA) ([Ohkawa et al. 1979](#)) were also determined in the ovary.

Hormone assays

Serum concentrations of estradiol, testosterone, insulin (Calbiotech, Spring Valley, CA, USA) and corticosterone (Demeditec diagnostics GmbH, Germany) were estimated using ELISA kits and procedures provided by the respective manufacturer.

Histological study

Serial paraffin sections ($5\mu\text{m}$) of the ovary were cut and stained with haematoxylin and eosin. The antral follicles were identified according to the description of [Pedersen and Peters \(1968\)](#). Each section of the ovary was focused and the

number of antral follicles of $380\text{--}740\mu\text{m}$ size showing full-size oocytes was recorded. Care was taken to avoid counting the same follicle more than once and healthy and atretic follicles were separately counted. The aggregate of follicles in all the sections was expressed as the number of antral follicles/ovary/rat. The criteria for identification of atretic follicles were as per the description of [Greenwald and Roy \(1994\)](#).

The number of active corpora lutea was counted focussing each section of the ovary under $40\times$, and avoiding counting the same corpora lutea more than once. The active (newly formed) corpora lutea were identified based on the presence of active luteal cells and angiogenesis between them, whereas regressing (previously formed) corpora lutea showed vacuolated and apoptotic cells with fibrosis ([Yoshida et al. 2009](#)).

Apoptosis

Granulosa cells were isolated from the ovary ([Campbell 1979](#)) and analysed for apoptosis by using acridine orange (AO) and ethidium bromide (EB) stains (1:1 0.01% AO:EB) ([Kasibhatla et al. 2006](#)). The apoptotic cells emitted yellow or orange fluorescence whereas healthy cells emitted green fluorescence. One hundred randomly selected cells per ovary were scanned and the number of apoptotic and healthy cells was recorded and percentage of apoptotic cells was computed.

DNA ladder assay

The DNA was extracted from the ovary following the method of [Compton \(1992\)](#). The DNA samples were subjected to agarose gel (2%) electrophoresis and changes were documented using gel doc.

Statistical analyses

The mean value of each parameter was computed considering data on 5 rats/group. The mean values of control and stress group were compared using Student's *t*-test and judged significant if $P < 0.05$. Since rats in 1st, 2nd and 3rd experiments belonged to different age groups, Student's *t*-test was preferred to test the significance between control and stress groups in each experiment, rather application of ANOVA considering all the groups in 3 experiments.

Results

Stress markers

Weight of the body and the adrenal gland

There was a significant increase in body weight of controls in each experiment as shown by percent increase in body weight compared with initial body weight, whereas that of rats in stress groups in all the exposure periods was significantly lower than the respective controls ([Table 1](#)). The weight of the adrenal gland was significantly increased in rats of stress group in each duration compared with the respective controls ([Table 1](#)).

Table 1 Effects of chronic stress on weight of the body and adrenal gland, the adrenal 3 β -HSDH activity and the serum corticosterone concentration of rats.

Groups	% change in body weight compared to initial body weight (n = 8)	Relative weight of the adrenal gland (mg/100 g body weight) (n = 8)	Adrenal 3 β -HSDH activity (nmol/mg/min) (n = 5)	Serum concentration of corticosterone (ng/mL) (n = 5)
4-week exposure				
Control	14.35 $\pm$ 1.22	26.88 $\pm$ 1.11	0.48 $\pm$ 0.02	127.50 $\pm$ 19.20
Stress	6.84 $\pm$ 1.62	31.60 $\pm$ 0.68	0.73 $\pm$ 0.08	457.50 $\pm$ 12.50
P	<0.003	<0.05	<0.05	<0.001
8-week exposure				
Control	6.67 $\pm$ 1.81	28.38 $\pm$ 1.03	0.37 $\pm$ 0.01	210.00 $\pm$ 26.22
Stress	-3.19 $\pm$ 2.04	37.71 $\pm$ 0.81	0.48 $\pm$ 0.01	456.25 $\pm$ 3.75
P	<0.05	<0.001	<0.001	<0.001
12-week exposure				
Control	5.69 $\pm$ 0.24	27.75 $\pm$ 0.84	0.23 $\pm$ 0.01	162.50 $\pm$ 17.02
Stress	1.58 $\pm$ 0.11	37.00 $\pm$ 1.09	0.34 $\pm$ 0.01	405.00 $\pm$ 31.75
P	<0.001	<0.001	<0.001	<0.003

All the values are mean $\pm$ S.E.

Mean values of control and stress groups were compared using Student's *t*-test and judged significant if *P* < 0.05.

Adrenal 3 β -HSDH activity and serum concentration of corticosterone

The activity of the adrenal 3 β -HSDH and the serum concentration of corticosterone were significantly increased in rats exposed to stressors in each duration compared with the respective controls (Table 1).

Fasting blood glucose concentration

Fasting blood glucose concentration estimated at weekly intervals showed a significant increase from 2nd week through 12th week (except 6th week) in stress group rats compared with controls in 12-week exposure experiment (Fig. 1). The fasting blood glucose concentrations of rats in 1st and 2nd experiments wherein the rats were exposed to stress for 4 and 8 weeks, respectively, resembled those of 4- and 8-week exposure in the third experiment.

Figure 1 Fasting blood glucose concentrations in control and rats exposed to stress for 12 weeks. Note the higher levels of glucose in stressed rats compared with controls. The data on blood glucose levels of rats exposed to stress for 4 and 8 weeks (i.e., 1st and 2nd experiment) are not included, as these did not significantly differ from the values following 4- and 8-week exposure in 12-week exposure experiment. *significantly (*P* < 0.05) different compared with controls as judged by Student's *t*-test.

Hence, data of the 1st and 2nd experiments were not presented to avoid repetition.

Estrous cyclicity

The average number of estrous cycles/4 weeks was significantly lower in stress group rats of all the exposure periods compared with the respective controls (Table 2).

Hormone profile

The serum estradiol concentration did not differ in rats after 4-week stress exposure compared with controls, whereas it was significantly increased after 8 or 12 weeks of stress exposure compared with the respective controls (Table 2). The serum levels of testosterone did not differ after 4- or 8-week exposure to stressors, whereas it was significantly increased after 12-week exposure compared with the respective controls. The serum insulin concentration was significantly decreased after 4-week exposure to stressors, whereas it was significantly increased after 8- or 12-week exposure compared with controls (Table 2).

Ovarian changes

Weight of the ovary

The weight of the ovary was significantly lower in rats exposed to stressors for 4 and 8 weeks, whereas it was significantly higher in rats exposed to stress for 12 weeks compared with the respective controls (Table 2).

3 β -HSDH activity

The ovarian 3 β -HSDH activity was significantly decreased in 4- and 8-week stressed rats, whereas it was significantly increased after 12 weeks of stress exposure compared with the respective controls (Table 2).

Table 2 Effects of chronic stress on oestrous cyclicity, ovarian weight and serum hormone concentrations in rats.

Groups	Relative weight of ovary (mg/100 g body weight) (n=8)	Ovarian 3 β -HSDH activity (nmol/mg/min) (n=5)	Average number of oestrous cycles/month (n=8)	Serum oestrogen concentration (pg/mL) (n=5)	Serum testosterone concentration (ng/mL) (n=5)	Serum insulin concentration (μ U/mL) (n=5)
4-week exposure						
Control	43.50 $\pm$ 1.04	0.62 $\pm$ 0.08	6.14 $\pm$ 0.26	17.50 $\pm$ 3.23	0.75 $\pm$ 0.010	13.63 $\pm$ 1.07
Stress	33.80 $\pm$ 2.33	0.34 $\pm$ 0.03	4.00 $\pm$ 0.31	13.33 $\pm$ 1.67	0.58 $\pm$ 0.08	3.17 $\pm$ 1.48
P	<0.003	<0.05	<0.001	NS	NS	<0.003
8-week exposure						
Control	50.25 $\pm$ 2.01	0.39 $\pm$ 0.03	5.64 $\pm$ 0.32	19.17 $\pm$ 5.69	1.25 $\pm$ 0.08	8.60 $\pm$ 1.17
Stress	41.00 $\pm$ 0.89	0.26 $\pm$ 0.01	4.42 $\pm$ 0.15	61.67 $\pm$ 3.33	1.00 $\pm$ 0.14	13.33 $\pm$ 0.60
P	<0.05	<0.05	<0.05	<0.001	NS	<0.05
12-week exposure						
Control	38.17 $\pm$ 3.52	0.19 $\pm$ 0.01	5.55 $\pm$ 0.33	23.33 $\pm$ 2.79	0.83 $\pm$ 0.15	6.40 $\pm$ 1.04
Stress	51.00 $\pm$ 2.65	0.30 $\pm$ 0.00	4.13 $\pm$ 0.20	38.33 $\pm$ 1.67	1.56 $\pm$ 0.12	25.25 $\pm$ 4.71
P	<0.05	<0.001	<0.05	<0.003	<0.05	<0.05

All the values are mean $\pm$ S.E.M.

Mean values of control and stress groups were compared using Student's *t*-test and judged significant if *P* < 0.05.

Counts of antral follicles and corpora lutea

The mean number of healthy antral follicles was significantly lower and higher in rats exposed to stressors for 4 and 12 weeks respectively compared with controls, whereas it did not differ significantly from controls following 8-week stress exposure (Table 3). However, the number of atretic antral follicles showed significant increase in stress-exposed rats of each duration compared with controls (Table 3).

The mean number of active corpora lutea was significantly lower in stress group rats in all the durations compared with the respective controls (Table 3). In addition, the ovaries of rats exposed to stress for 12 weeks were conspicuous by the presence of cystic follicles in a range of 2–5/ovary with a mean of 3.4 $\pm$ 0.51. The cystic follicles contained fluid-filled cavity surrounded by granulosa and theca layers and the thickness of the thecal layer was more than that of antral follicles of controls (Fig. 2). However, cystic follicles were not found in the ovaries of rats exposed to stress for 4 or 8 weeks.

Apoptosis of granulosa cells

The percentage of healthy granulosa cells was significantly decreased in rats exposed to stressors for 4 and 8 weeks and increased significantly after 12-week exposure to stressors. There was a significant increase in the percentage of apoptotic granulosa cells after 4 (Fig. 3) and 8 weeks of stress regime, but it was decreased after 12-week exposure to stressors compared with the respective controls (Table 3).

DNA ladder assay

The breaks in the DNA of the ovary as shown by the formation of the ladder was observed in rats exposed to stress for 8 weeks, whereas after 4 or 12 weeks of exposure to stress DNA of the ovary did not show such breaks (Fig. 4).

Activity of antioxidant enzymes

The activities of antioxidant enzymes SOD, GST and GPx in the ovary were significantly decreased in rats

Table 3 Effects of chronic stress on follicle development and apoptosis of the granulosa cells in rat.

Groups	Mean number of antral follicle $\pm$ S.E.		Mean number of active corpora lutea $\pm$ S.E. (n=5)	Percentage of granulosa cells	
	Healthy (n=5)	Atretic (n=5)		Healthy (n=5)	Apoptotic (n=5)
4 weeks					
Control	21.25 $\pm$ 0.48	11.25 $\pm$ 0.63	7.25 $\pm$ 0.48	30.32 $\pm$ 3.58	69.33 $\pm$ 1.32
Stress	16.00 $\pm$ 0.41	15.25 $\pm$ 0.85	2.25 $\pm$ 0.63	18.64 $\pm$ 2.24	80.79 $\pm$ 4.83
P	<0.001	<0.05	<0.003	<0.05	<0.05
8 weeks					
Control	21.50 $\pm$ 0.96	8.25 $\pm$ 0.95	9.00 $\pm$ 0.71	16.88 $\pm$ 0.87	82.54 $\pm$ 0.66
Stress	24.00 $\pm$ 1.22	16.50 $\pm$ 0.65	3.25 $\pm$ 0.48	12.95 $\pm$ 0.33	87.83 $\pm$ 0.49
P	(NS)	<0.001	<0.003	<0.05	<0.05
12 weeks					
Control	10.67 $\pm$ 1.20	5.75 $\pm$ 1.25	6.25 $\pm$ 0.63	11.30 $\pm$ 2.03	88.83 $\pm$ 2.01
Stress	17.75 $\pm$ 1.31	12.00 $\pm$ 1.08	1.25 $\pm$ 0.25	17.43 $\pm$ 0.69	82.41 $\pm$ 1.09
P	<0.05	<0.05	<0.001	<0.05	<0.05

All the values are mean $\pm$ S.E.M.

Mean values of control and stress groups were compared using Student's *t*-test and judged significant if *P* < 0.05.

Figure 2 (A–D) Photomicrographs of the cross sections of the ovary of control (A & B) and rats exposed to stress for 12 weeks (C & D). B and D are the enlarged views of a portion of follicular wall of the ovaries in A and B respectively. Note the presence of cystic follicle (CF, C) and an increase in the thickness of theca layer in cystic follicle (D) in stressed rat compared with that of the antral follicle of control rat (B) (H&E, A & B, 40 $\times$; C & D, 200 $\times$). AF, antral follicle; CL, corpus luteum; G, granulosa layer; T, theca layer.

Figure 3 (A and B) Photomicrographs of the ovarian granulosa cells stained with acridine orange and ethidium bromide in control (A) and stress group (B) rats after 4-week stress. Green colour represents the healthy, and yellow or orange colour represents the apoptotic cells. Note the presence of more number of healthy cells in control group whereas more number of apoptotic cells in stressed rats.

exposed to stressors for 4 or 8 weeks, whereas there was a significant increase in activities of these enzymes following 12-week exposure to stressors, compared with the respective controls (Table 4). However, the activity of CAT and FRAP were significantly decreased in all the durations of stress exposure (Table 4).

Malondialdehyde concentration

The malondialdehyde concentration in the ovaries of the rats exposed to stressors for 4 or 8 weeks showed a significantly higher concentration whereas that in the ovaries of rats exposed to stress for 12 weeks was significantly lower compared with the respective controls (Table 5).

Discussion

The immediate response of mammals to stress is an activation of the HPA axis (Chrousos *et al.* 1998). In this study, an increase in the adrenocortical activity as shown by an increase in the relative weight and

Figure 4 Photomicrographs of gel electrophoretic profile of DNA from the ovaries of rats exposed to stress for 4, 8 and 12 weeks and control rats. Note the presence of DNA breaks only in rats exposed to stress for 8 weeks whereas no such breaks in rats exposed to stress for 4 or 12 weeks. C, control lane; M, marker lane; S, stress lane.

Table 4 Effects of chronic stress on the activities of antioxidant enzymes and ferrous reducing antioxidant power (FRAP) of ovary in rat.

Groups	SOD (U/mg protein) (n=5)	CAT (nmol/mg/min) (n=5)	GST (μ mol/mg/min) (n=5)	GPx (μ mol/mg/min) (n=5)	FRAP (μ mol/L FeSO ₄) (n=5)
4-week exposure					
Control	0.090 $\pm$ 0.00	0.0017 $\pm$ 0.00	698.61 $\pm$ 40.93	0.176 $\pm$ 0.04	546.47 $\pm$ 104.11
Stress	0.04 $\pm$ 0.00	0.0005 $\pm$ 0.00	267.07 $\pm$ 53.37	0.079 $\pm$ 0.01	129.22 $\pm$ 14.81
P	<0.001	<0.003	<0.001	<0.05	<0.05
8-week exposure					
Control	0.126 $\pm$ 0.01	0.0025 $\pm$ 0.00	333.60 $\pm$ 3.69	0.064 $\pm$ 0.00	495.68 $\pm$ 39.91
Stress	0.078 $\pm$ 0.01	0.0014 $\pm$ 0.00	183.04 $\pm$ 6.30	0.017 $\pm$ 0.00	161.08 $\pm$ 12.81
P	<0.003	<0.05	<0.001	<0.001	<0.001
12-week exposure					
Control	0.075 $\pm$ 0.01	0.0023 $\pm$ 0.00	252.97 $\pm$ 16.25	0.011 $\pm$ 0.00	882.59 $\pm$ 34.66
Stress	0.114 $\pm$ 0.01	0.0014 $\pm$ 0.00	452.60 $\pm$ 9.93	0.064 $\pm$ 0.00	510.15 $\pm$ 133.14
P	<0.05	<0.05	<0.001	<0.001	<0.05

All the values are mean $\pm$ S.E.M.

Mean values of control and stress groups were compared using Student's *t*-test and judged significant if *P* < 0.05.

3 β -HSDH activity of the adrenal gland and serum corticosterone concentration in rats subjected to restraint and forced swimming in all the experiments reveals that the rats were under stress. It is further supported by a decrease in percentage gain in body weight of rats in all the durations of exposure, because loss of body weight is associated with stress (Jeong *et al.* 2013). Since stress induces hyperglycaemia (Nirupama *et al.* 2010), an increase in fasting blood glucose concentration observed in stressed rats throughout the experiment from 2nd week of stress exposure in this study also suggests that rats were under stress. It is remarkable that even after 12 weeks of everyday exposure to stressors, the rats showed an adrenal stress response, thereby indicating that rats were not habituated to stress regime. Concomitant with activation of the adrenocortical activity in stressed rats, ovarian functions viz., follicle development, steroidogenesis and antioxidant status were altered. Hence, these changes were ovarian stress responses.

Several earlier studies reported stress-induced alterations in ovarian functions. For instance, irregular estrous cyclicity in rats (Sarawathi *et al.* 2010,

Mourlon *et al.* 2011) and altered ovarian follicular development in cows (*Bos taurus*) (Roth *et al.* 2000), rats (Dorfman *et al.* 2003, Bhat & Yajurvedi 2011, Barra *et al.* 2014), goat (*Capra hircus*) (Ozawa *et al.* 2005) and mice (Wu *et al.* 2011) have been reported. Further, stress-induced impairment in developmental potential of oocyte (Zhang *et al.* 2011, Lian *et al.* 2013, Gao *et al.* 2016) and reduction in ovulatory rate (Zhang *et al.* 2011) have been observed in mice. Similarly, changes in secretions of reproductive hormones are also observed due to stress (Roth *et al.* 2000). The results of this study, i.e., irregular estrous cyclicity, reduction in the number of antral follicles and active corpora lutea as well as a decrease in ovarian 3 β -HSDH activity and antioxidant potential after 4- or 8-weeks exposure to stress indicate impaired ovarian functions and are in agreement with earlier studies. However, the maximum duration of stress exposure in the above studies was 30 days, whereas this study demonstrates that the ovary continues to show stress response beyond 30 days. Further the study reveals for the first time a paradoxical response of the ovary after 12-week exposure compared with 4- or 8-week exposure, as there was an increase in the number of antral follicles, serum oestrogen and testosterone levels coupled with an increase in antioxidant status of the ovary. On the contrary, Paredes *et al.* (1998) reported formation of precystic follicles following 3-week cold-restraint stress exposure, whereas the ovary resembled controls excepting a decrease in the total number of healthy pre-antral and antral follicles after 11-week stress exposure. The differences between the study by Paredes *et al.* (1998) and this study will be further discussed.

Exposure to stressors activates the HPA axis resulting in increased secretion of corticotrophin-releasing hormone and adrenocorticotrophic hormone leading to the activation of the adrenal cortex and results in an increase in secretion of glucocorticoids (Daley *et al.* 1999, Levy & Tasker 2012). Corticotrophin-releasing hormone suppresses GnRH secretion (Kyrou & Tsigos 2008) whereas glucocorticoids inhibit gonadotrophin

Table 5 Durational effects of chronic stress on the concentration of malondialdehyde (MDA) in the ovary of rat.

Groups	MDA concentration (nmol/mg protein) (n=5)
4-week exposure	
Control	328.10 $\pm$ 17.19
Stress	876.45 $\pm$ 62.41
P	<0.001
8-week exposure	
Control	194.70 $\pm$ 10.92
Stress	372.99 $\pm$ 58.79
P	<0.05
12-week exposure	
Control	285.66 $\pm$ 21.06
Stress	177.69 $\pm$ 4.76
P	<0.003

All the values are mean $\pm$ S.E.M.

Mean values of control and stress groups were compared using Student's *t*-test and judged significant if *P* < 0.05.

secretion (Breen *et al.* 2004, Levy & Tasker 2012) and also directly interfere with ovarian functions by triggering apoptosis of ovarian granulosa and cumulus cells (Whirledge & Cidlowski 2010, Yuan *et al.* 2016). Since gonadotrophins are key regulating hormones, impairment in their secretion/action results in altered ovarian activities under stressful conditions. In this study, there was an activation of the adrenal cortex coupled with higher levels of corticosterone in all the durations of stress exposure. Hence, alterations in the ovarian activity might be due to suppressive actions of HPA axis hormones.

In addition to stress-induced hormonal imbalance, compromise in antioxidant status of the ovary might also adversely affect ovarian activities because stress (Nayanatara *et al.* 2005) and high levels of glucocorticoids (Spiers *et al.* 2015) cause an increase in the generation of ROS due to accelerated metabolic reactions (Liu & Mori 1999) resulting in oxidative damage in tissues. In addition, restraint-induced oxidative stress impaired developmental potential of oocytes, whereas administration of antioxidants overcame this detrimental effect (Lian *et al.* 2013). Hence, this study determined whether stress-induced hormonal imbalance was accompanied by oxidative stress in the ovary. Indeed, ovaries of rats after 4 or 8 weeks of stress exposure showed an increase in the concentration of MDA concomitant with a decrease in the activity levels of antioxidant enzymes (SOD, CAT, GST and GPx) and thereby indicated a state of oxidative stress. Increased concentration of MDA suggests high level of ROS (Mittal *et al.* 2014), which is known to induce granulosa cell apoptosis (Sinha *et al.* 2013). Hence an increase in atresia of antral follicles and apoptosis of granulosa cells determined by acridine orange and ethidium bromide staining in this study were due to damage caused by ROS. The fact that though oxidative stress was found after 4- as well as 8-week exposure, the DNA breaks in ovarian tissue was observed only after 8-week exposure, indicating the severity of stress effects on the DNA to increase with duration of exposure. However, despite longer duration of exposure, DNA breaks were not observed in rats exposed to stress for 12 weeks. In these rats the antioxidant potential of the ovary was higher than the controls as shown by an increase in the antioxidant enzyme activities and decrease in the MDA levels in the ovary. A decrease in the oxidative stress due to altered ovarian functions, discussed in the later section, may be the reason for the absence of DNA breaks in rats exposed to stress for 12 weeks.

Thus far studies have reported inhibitory effects of stress on ovarian functions (Whirledge & Cidlowski 2010). However, this study demonstrates the paradoxical response of the ovary following exposure to longer duration (12 weeks), as shown by increases in the number of healthy follicles, steroidogenic activity and antioxidant potential in contrast to decrease in these

parameters following 4- or 8-week stress exposure. The altered response of the ovary might be due to the prevalence of hyperandrogenism after 12-week exposure. The testosterone stimulates follicular growth (Murray *et al.* 1998, Sen *et al.* 2014); promotes theca and granulosa cell proliferation and inhibits apoptosis (Das *et al.* 2008); promotes gonadotrophin responsiveness and steroidogenesis in granulosa cells (Hillier 1994); augments follicle FSH receptor expression and growth (Weil *et al.* 1999, Sen *et al.* 2014) and induces IGF and IGF1 receptor expression in the ovary (Vendola *et al.* 1999). These actions put together increase the number of healthy follicles, which consequently results in increases in other parameters. The fact that hyperandrogenism after 12-week stress was accompanied by an increase in the number of follicles, whereas low levels of androgen following 4- or 8-week stress were accompanied by a decrease in the number of follicles, further supports hyperandrogenism-induced follicular excess. One of the features of the polycystic ovary (PCO) is hyperandrogenism (Gambineri *et al.* 2002). Indeed, in this study there was development of cystic follicles in the ovaries of rats exposed to stress for 12 weeks concomitant with an increase in serum testosterone levels. In addition, these rats also exhibited other characteristics of PCO phenotype viz., irregular oestrous cyclicity, hyperestrogenism, hyperinsulinaemia, follicular excess and follicular arrest. In addition androgen (Mancini *et al.* 2008) and oestrogen (Borras *et al.* 2010) are known to stimulate antioxidant system. In this study on rats exposed to stress for 12 weeks, there was an increase in antioxidant potential of ovary as shown by elevated activities of antioxidant enzymes and decrease in lipid peroxidation concomitant with higher serum levels of testosterone and estrogen. Thus the altered paradoxical response of the ovary after 12 weeks of stress was due to pathophysiological development i.e., polycystic ovarian condition.

Interestingly, Paredes *et al.* (1998) observed the formation of precystic follicles following 3-week stress due to cold-restraint exposure, but not after a similar stress regime for 11 weeks, though there was a decrease in the total number of healthy pre-antral and antral follicles in both the durations of exposure. However, in the present study, cysts were not observed in the ovary after 4- or 8-week stress exposure, whereas these were formed after 12 weeks of stress due to restraint and forced swimming. The difference in these two studies may be due to the fact that hyperandrogenism, which causes cyst formation in the ovary, was found after 3-week exposure but not after 11 weeks in the study by Paredes *et al.* (1998), whereas in this study it was found after 12-week exposure. Further, Paredes *et al.* (1998) suggested that a decrease in the total number of healthy pre-antral and antral follicles in their study was due to increased rate of follicular development caused by cold-restraint-induced sympathetic overstimulation

of the ovarian activity. On the contrary, in the present study the number of healthy antral follicles was increased after 12-week exposure concomitant with hyperandrogenism. Thus, follicular excess was due to hyperandrogenism in this study in contrast to stress-induced increased rate of follicular development in that of Paredes *et al.* (1998).

Several factors viz., genetic and environmental (prenatal and postnatal) (de Melo *et al.* 2015), obesity (Gambineri *et al.* 2002) and stress (Paredes *et al.* 1998, Dorfman *et al.* 2003, Greiner *et al.* 2005, Saraswathi *et al.* 2012) are known to cause polycystic ovary phenotype. In earlier studies, it was shown that sympathetic activation induced by stress due to chronic cold exposure resulted in PCO phenotype (Paredes *et al.* 1998, Dorfman *et al.* 2003, Greiner *et al.* 2005) whereas involvement of HPA axis was yet to be demonstrated. This study is the first to demonstrate occurrence of PCO condition due to chronic stress-induced activation of the HPA axis in normally cycling non-obese rats. Moreover, this study also reveals the pathogenesis of the PCO condition as mentioned below.

Chronic stress is known to cause hyperglycaemia due to dysregulation of carbohydrate metabolism (Zardooz *et al.* 2006, Nirupama *et al.* 2010), which consequently leads to hyperinsulinaemia (Tomas *et al.* 2002). In this study, in rats a consistent hyperglycaemia was observed after 2-week stress exposure, which was accompanied by a decrease in serum insulin concentration following 4-week exposure and hyperinsulinaemia after 8- or 12-week exposure. High concentration of corticosterone is known to increase insulin degrading enzyme (IDE) expression in the liver under stress (Zardooz *et al.* 2006). Since there was a consistent increase in the serum concentration of corticosterone in all the durations of exposure in this study, a decrease in the serum insulin levels after 4-week exposure may be due to activation of IDE. However, after longer duration of stress exposure, insulin resistance might have developed due to consistent hyperglycaemia resulting in hyperinsulinaemia despite the degradation of insulin. It is known that increased insulin levels stimulate ovarian theca cells to secrete enormous amount of androgen (Ehrmann *et al.* 1995, Allahbadia & Merchant 2011) and increase LH pulse frequency (Allahbadia & Merchant 2011), both of these lead to hyperandrogenism. Therefore, hyperinsulinaemia in this study induced the hyperandrogenism, which is the cause of follicular excess as discussed earlier. Further, simultaneously estradiol levels were also higher in stressed rats that developed PCO condition. It is known that a high estradiol concentration in PCO modifies the normal feedback regulation of FSH that results in arrest of maturation of large antral follicles (Franks & Hardy 2010). Indeed, in this study despite an increase in the number of antral follicles, there was a decrease in the number of active corpora lutea, indicating a reduced rate of ovulation due to failure

of some of antral follicles to undergo maturation and ovulation. Thus, this study clearly demonstrates that stress-induced hyperinsulinaemia sets in the sequence of events leading to PCO phenotype in normal cycling rats. To conclude, this study reveals that the ovary responds differentially to the stress depending on the duration of exposure; shorter periods exert suppressive action, whereas a longer exposure results in pathophysiological development resulting in reduced ovulation despite excessive follicular recruitment. Further, the mechanism underlying differential response of the ovary to different stress regimes, despite a similar increase in corticosterone concentration needs to be investigated, focussing on the expression of glucocorticoid receptors and enzymes following different exposure durations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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