

**EFFECT OF CHRONIC COLD RESTRAINT AND IMMOBILIZATION STRESS  
ON ESTROUS CYCLE IN RATS.**

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**Summary**

Chronic stress is known to produce a state of dyshomeostasis (allostasis) that involves significant behavioral, endocrinological and neurobiological changes. The underlying mechanisms and complications of which are not clearly understood. The present study aims at investigating the effect of chronic cold restraint (a moderate physical/metabolic stressor) and immobilization (physical and psychological stress) on estrous cycle in rats. It was found that stress produces a significant increase in proestrous phase indicating the non maturation of follicles, supported by histopathological studies. The serum levels of glucose, cholesterol and triglycerides were decreased. Corticosterone levels were increased. Ovary and uterus weight was decreased due to the non availability of hormones. Adrenal gland weight was increased indicating hypertrophy. All the above observations shows stress produces a significant change in the estrous cycle.

**Key words:** Chronic stress, immobilization, chronic cold restraint, estrous cycle

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## **Introduction**

Stress has become an integral part of the human condition worldwide and every individual is likely to face stressful situations in day to day life which seriously perturb physiological and psychological homeostasis [1]. Stress produces significant behavioral, endocrinological, neurobiological and immunological changes; and the underlying mechanisms and complications are not clearly understood [2]. Chronic stress represents a state of dyshomeostasis (allostasis) resulting from the inability of the adaptive stress response to cope with the intensity or the frequency of various stressors[3]. Stress activates hypothalamic-pituitary-adrenal (HPA) axis and the sympathoadrenomedullary system, the degree of activation depends on stress duration, type and intensity[4].

Reproductive functions are suppressed under various stress conditions like restraint, strenuous exercise, malnutrition, infection and surgical trauma [5]. This is emerging as a serious problem with modern day life style. Prolonged or chronic stress causes anovulation which results in infertility due to suppression of gonadotrophic hormones. Also high stress perception is a risk factor for severe premenstrual pain, pregnancy outcomes including preterm delivery and low birth weight, as well as postpartum depression and early onset of perimenopause [6].

The aim of the present study was to identify relevant and reliable stress paradigms so that prospective investigations of neuro-endocrinology can be initiated. Chronic cold restraint (a moderate physical/metabolic stressor) and immobilization stress (physical and psychological stress) were chosen as stress inducers to know their effects on estrous cycle in rats.

## **Methods**

### **Animals**

Female Wistar rats (170-200g) were procured from Drugs Testing Laboratory, Bangalore. All animals were housed in a group of 6 in polyethylene cages under standard housing conditions (12:12 h light and dark cycle, temperature  $22\pm 2^{\circ}\text{C}$  and humidity  $50\pm 5\%$ ) with standard feed pellet and free access to water *ad libitum*. Standard hygiene conditions were maintained. The animal experiments were performed in accordance with our Institutional Animal Ethics Committee (IAEC/NCP/15/09) and by the animal regulatory body of the government.

### **Chronic cold restraint stress[7]**

Animals were individually placed in a cold chamber at  $4^{\circ}\text{C}$  inside plastic cylinders (21cm in length x 6 cm in diameter). Both ends of the cylinders were closed with ventilated sliding doors.

**Immobilization stress [8]**

Animals were individually placed inside plastic cylinders (21cm in length x 6 cm in diameter) at ambient temperature. Both ends of the cylinders were closed with ventilated sliding doors.

Female rats having regular estrous cycle were selected. Rats were divided into three groups containing six animals in each group.

Group I- Control

Group II- Chronic cold restraint stress (4°C) 3h daily for 28days.

Group III- Immobilization stress 3h daily for 28days.

Every day immediately after the stress session, vaginal smears were examined in all the groups. One hour after the last stress session both control and experimental rats were sacrificed by cervical dislocation. Blood was collected and serum was separated. The serum levels of corticosterone, cholesterol, glucose and triglycerides were estimated using kits supplied by SPAN Diagnostic Ltd and determined using semi auto analyzer. Ovaries, uteri, and adrenal glands were isolated and weighed. One each of ovary, uterus, and adrenal gland were isolated and preserved in 10% buffered formalin solution and were further subjected to histopathological studies.

**Statistical analysis**

Data are expressed as mean  $\pm$  SEM. The values obtained were analyzed by one way ANOVA followed by Dunnett's *t* tests.  $P < 0.05$  were considered statistically significant.

**Results**

Chronic cold restraint and immobilization stress caused a significant ( $P < 0.001$ ) decrease in the duration of the estrous and metestrous phase, no change in the duration of the diestrous phase and a significant ( $P < 0.001$ ) increase in duration of proestrous phase when compared with the control group. (Table-1)

**Table: 1 Effect of chronic cold restraint and restraint stress on duration of different phases of estrous cycle in female rats for 28 days.**

Groups	Mean days of Proestrous	Mean days of Estrous	Mean days of Metestrous	Mean days of Diestrous
Vehicle control	4.16 $\pm$ 1.07	5.8 $\pm$ 0.54	7 $\pm$ 0.5	11.16 $\pm$ 0.7
Chronic cold restraint stress	14.16 $\pm$ 1.04***	2.5 $\pm$ 0.56***	2.83 $\pm$ 0.4***	10.33 $\pm$ 0.95 <sup>ns</sup>
Immobilization stress	13.16 $\pm$ 1.85***	1.51 $\pm$ 0.61***	1.36 $\pm$ 0.55***	12.5 $\pm$ 0.80 <sup>ns</sup>

Values represent mean  $\pm$  SEM. Data were analyzed by one way ANOVA followed by Dunnett's *t* test.

Number of animals in each group  $n = 6$ . Comparison made with control group.

\*\*\*  $P < 0.001$ , ns non significant.

Chronic cold restraint and immobilization stress resulted in significant decrease in liver, ovary and uterus weight ( $P < 0.001$ ) and increased in adrenal gland weight ( $P < 0.001$ ) when compared to control group.(Table-2)

**Table 2: Effect of chronic cold restraint and Immobilization stress on different organ weights (mg/100g) in female rats.**

Groups	Ovary	Uterus	Adrenal gland
Vehicle control	0.04±0.001	0.24±0.01	0.030±0.0004
Chronic cold restraint stress	0.018±0.001***	0.09±0.007***	0.056±0.003***
Immobilization stress	0.017±0.001***	0.15±0.009***	0.054±0.002***

Values represent mean ± SEM. Data were analyzed by one way ANOVA followed by Dunnett's *t* test. Number of animals in each group n = 6. Comparison made with vehicle control group.  
\*\*\*  $P < 0.001$ .

Chronic cold restraint and immobilization stress resulted in significant decrease in serum triglycerides, cholesterol and glucose levels ( $P < 0.001$ ) and significant increase in corticosterone level when compared with control group.(Table-3)

**Table 3: Effect of chronic cold restraint and immobilization stress on serum triglycerides, cholesterol, glucose and corticosterone levels.**

Groups	Triglycerides mg/dl	Cholesterol mg/dl	Glucose mg/dl	Corticosterone ng/ml
Vehicle control	40.61±0.51	59.92±0.59	134.3±0.39	150.45±0.72
Chronic cold restraint	24.06±0.36***	40.24±0.51***	87.43±0.43***	712.67±0.72
Immobilization stress	26.65±0.44 ***	39.36±0.60***	79.35±0.37***	489.89±0.72

Values represent mean ± SEM. Data were analyzed by one way ANOVA followed by Dunnett's *t* test. Number of animals in each group n = 6. Comparison made with Vehicle control group.  
\*\*\*  $P < 0.001$ .

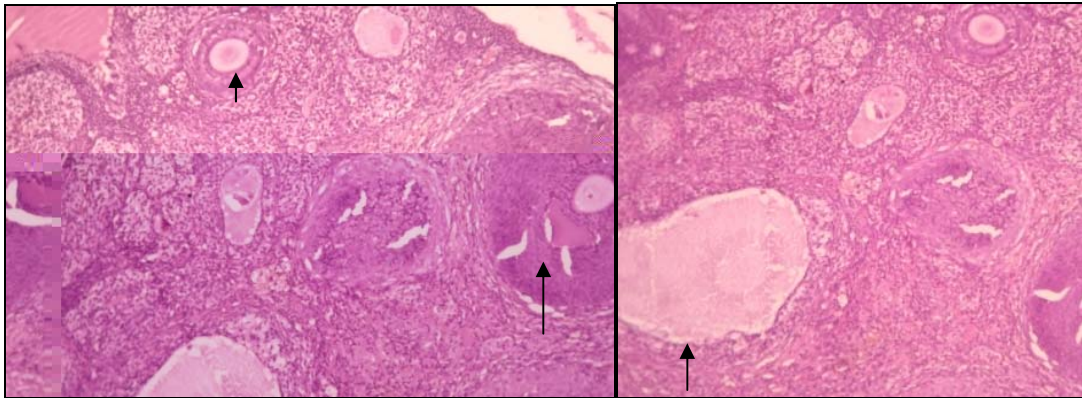


Fig:1 Section of the control rat ovary shows normal stroma, developing follicles and matured graffian follicle. H&E 200x

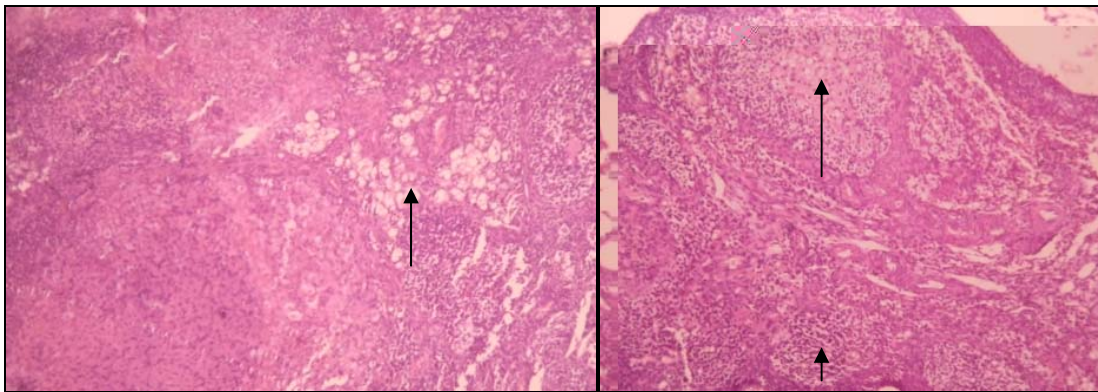


Fig:2 Section of the (chronic cold restraint stress) rat ovary shows hyperchromatic nucleus, many primary follicles and corpus fibrosum H&E 200x

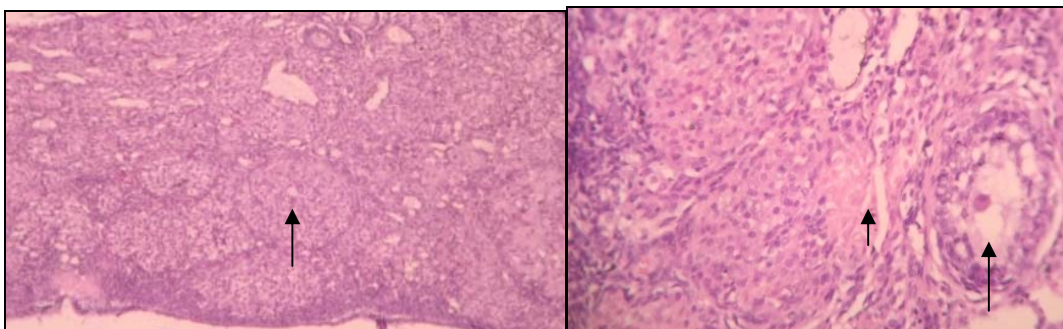


Fig:3 Section of the (Immobilisation stress) rat ovary shows changes in stroma, primordial follicles and atretic changes. H&E 200x

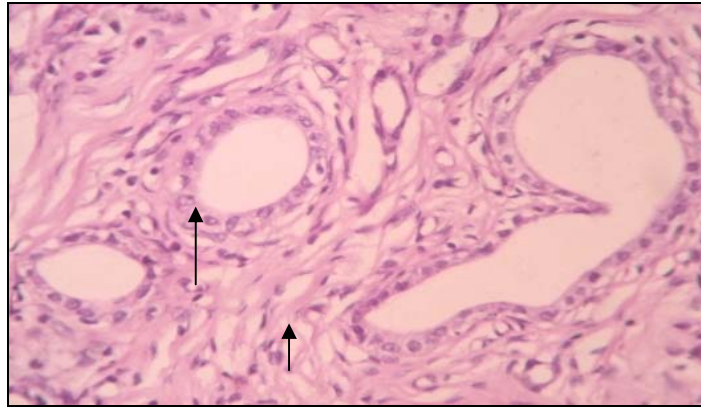


Fig:4 Section of the control rat uterus shows normal endometrium and stroma. (endometrial glands are short, straight and tubular (Figure, Long-arrow) lined by low columnar epithelium with no features of proliferation. The stroma appears compact comprising of stromal cells with vascular spaces (Figure, Short-arrow). H&E 400x

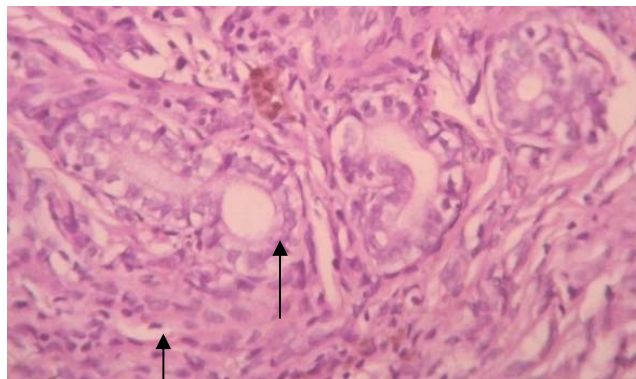


Fig:5 Section of the (chronic cold restraint stress) rat uterus shows changes in the endometrium and stroma. (endometrial glands are tortuous and tubular lined by tall columnar epithelium with subnuclear vacuolations (Figure, Long-arrow). The stroma appears compact comprising of stromal cells with vascular spaces and mononuclear inflammatory cell infiltrations (Figure, Short-arrow). H&E 400x

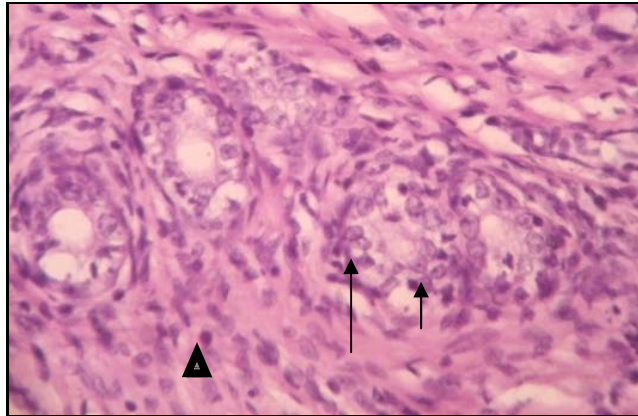


Fig:6 Section of the (Immobilisation stress) rat uterus shows changes in the endometrium and stroma. (endometrial glands are short, straight and tubular lined by stratified low columnar epithelium with subnuclear vacuolations (Figure, Long-arrow). Some of the lining epithelial cells show apoptotic changes (Figure, Short-arrow), while few show clear cell change. The stroma appears compact comprising of stromal cells with vascular spaces and mononuclear inflammatory cell infiltrations (Figure, Arrow-head).H&E 400x

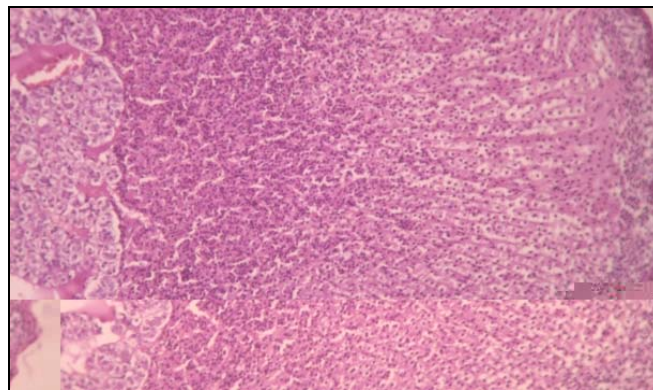


Fig:7 Section of the control rat adrenal gland shows normal cortex and medullary regions H&E 200x

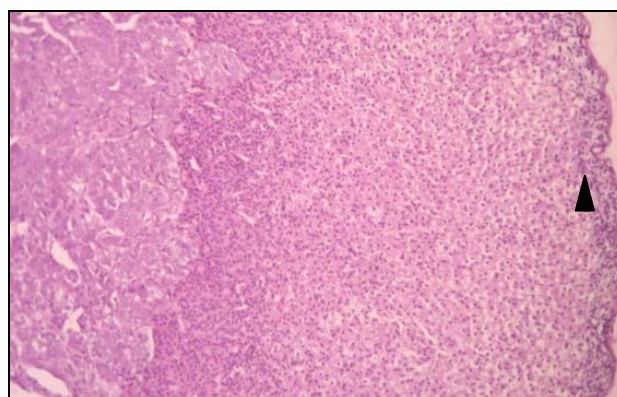


Fig:8 Section of the (chronic cold restraint stress) rat adrenal gland shows hypertrophy in the cortex. H&E 200x

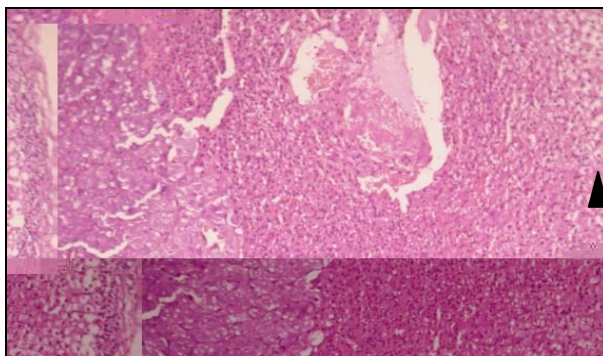


Fig:9 Section of the (Immobilisation stress) rat adrenal gland shows hypertrophy in the cortex region. The zona glomerulosa shows congested and dilated vascular spaces along with areas of hemorrhage. H&E 200x

### **Discussion**

The present study was undertaken to study the effect of cold restraint and immobilization stress on estrous cycle in female rats. The estrous cycle in females involves many histological, physiological, morphological and biochemical changes within the ovary. During the estrous cycle the maturation and ovulation of preovulatory follicles takes place under the combine and balance influence of ovarian and extra ovarian hormones [9]. Any imbalance in these hormones leads to irregularity in the function of ovary and irregular changes in the duration of estrous cycle. Both the stressors produced a significant increase in the proestrous phase when compared to control indicating the arrest of follicular development at the initial stages, presence of atretic follicles supported by the histopathological studies (Fig 1,2,3). Atretic follicles are degenerating preovulatory follicles, the degeneration of preovulatory follicles takes place when their growth and differentiation becomes disrupted [10]. The disruption in the growth and differentiation of preovulatory follicles takes place either due to non availability of steroidal hormones, which are essential for their maturation and differentiation [11], or due to non availability of local estrogen produced by granulosa cells [12], or due to availability of imbalanced endogeneous steroid and protein hormones [13].

Ovary can be considered to be an aggregate of three endocrine tissue, the stroma, the follicle and the corpus luteum the weight of these tissues constitute the net weight of ovary. During the estrous cycle the weight of the ovarian tissue increases under the influence of gonadotrophic and steroidal hormones [10]. The decrease in weight of ovaries in stressed rats indicates the decrease in activity of stroma, follicle and corpus luteum in the ovary. This decrease is due to non availability of either gonadotrophic or steroidal hormones or both or due to oxidative stress. Weight of the uterus was also significantly reduced in stressed rats indicating the non availability of the hormones required for the development of uterus. The endometrium showed a change in the histoarchitecture of endometrial glands.



Most of the endometrial glands are tortuous and tubular lined by tall columnar epithelium (cold stress) and tubular lined by stratified low columnar epithelium (immobilization) with subnuclear vacuolations . The stroma appears compact comprising of stromal cells (congestion) with vascular spaces and mononuclear inflammatory cell infiltrations. (Fig 4, 5)

The increase in adrenal weight caused due to stress, thus inhibiting the basic signs of stress response. Hypertrophy of the adrenal glands are seen indicating the active involvement of the hypothalamic-pituitary-adrenal (HPA) axis, which is highly responsive to stress [14]. The adrenal hypertrophy takes place in response to the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary for increased corticosterone from cortical cells to combat stress [15].

Exposure to chronic cold restraint and immobilization stress showed a significant decrease in serum glucose, cholesterol and triglycerides and increase in corticosterone levels. Acute stress produces an increase in serum glucose, cholesterol and triglycerides [16] whereas during chronic stress the decrease in glucose levels is due to redirection of energy substrates to the specific stress demanding sites[17].The depletion of stored glycogen during stress initiates gluconeogenesis and utilizes reserve fats as a secondary substrate in response to corticosterone for which the level of triglycerides and cholesterol were decreased [18].The reduction in plasma triglycerides in stressed rats may be secondary to the effect of catecholamines on the triglyceride lipase activity in the adipose tissue[19]. In conclusion, significant changes in estrous cycle were seen when exposed to various stressors. Further studies have to be carried out to elucidate the role of different hormones in stressful conditions.

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