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A STUDY ON THE EFFECT OF RANDOMIZED CHRONIC STRESS ON ESTROUS CYCLE AND OVARIAN CYST IN *RATTUS NORVEGICUS*

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ABSTRACT

Psychological stress is the body's response for stressors. Fear, anxiety and depression are widely attributed as a potent causative stress factors to instill PCOS in non-obese women with oligo/anovulation being the symptom. Recent reports have shown more than 90% of the patients are under moderate to high psychological stress. The study aims to examine the effect of randomized chronic stress on the variation of estrous cycle in rats to deepen our understanding of the effect of psychological stress in women leading to oligo/anovulation and hence manifesting into PCOS. A total of 20 *Rattus norvegicus* strains were grouped as control and stress groups with 10 animals each. Stress group of animals were exposed to different parameters of stressors for a study of 30 days with control group of animals being kept undisturbed for the same number of days. Vaginal fluid from all the grouped animals was collected, smeared on a clean glass slide, stained and observed under a microscope. At the end of the study, animals were euthanized and the ovarian morphology was examined against the observed variation in estrous cycle via vaginal smear analysis to the cystic ovaries. A significant difference with a value of p=0.05 for the estrous cycle, i.e., 4.3±0.46 estrous cycles in control group animals and 2.5±0.5 estrous cycles in stress group of animals during the study period were observed. Further a significant difference with p=0.001 for number of days to complete one estrous cycle, i.e., 5.8±0.98 days/estrous cycle in control and 8.5±1.28 days/estrous cycle in stress group animals were noted and the morphological change in the ovaries of stress group animals to the control group of animals was further correlated and the result established. Variations were significantly observed across different phases of estrous cycle correlating with the effect of chronic stress induction. The study identifies the effect of randomised chronic stress induction in altering the estrous cell cycle of rat, effect in the formation of cyst in the animal ovaries and hence establishing the possible link between chronic stress exposure and PCOS induction *in vivo*.

KEY WORDS:
Stress, Polycystic ovary syndrome, Anovulation, Non-obese individual, *Rattus norvegicus*, Estrous cycle, Vaginal smear, Ovarian morphology.

INTRODUCTION:

The global epidemiological data have been reported the prevalence of the disease in the reproductive age group women ranging up to 25% [1]. Study of menstrual cycle in human takes around 25-28 days to complete posing a daunting task ahead to study chronic stress induced variations of menstrual cycle in humans. A numerous number of animal studies on the disease occurrence and prognosis have been done, which have shown the different route of cause for the disease [2]. Body's non-specific response to the psychological stressors activates hypothalamus-pituitary-adrenal (HPA) axis [3], wherein, cortisol is produced as a primary stress hormone in humans and corticosterone in case of rodents, [4]. The current study explores variations occurring during estrus cycle in rat models due to the induction of chronic psychological stress and its influence over the formation of ovarian cysts.

Menstrual cycle:

The menstrual cycle in humans comprises of the ovarian cycle and the uterine cycle. The ovarian cycle comprises of follicular phase, ovulatory phase and luteal phase, whereas, uterine cycle comprises of proliferative phase, secretory phase, premenstrual phase and menstrual phase [5], taking around 25-28 days to complete one single menstrual cycle [5]. To overcome this difficulty, study of the menstrual cycle/sexual cycle in the animal models having a shorter period of cycle with a well-defined and easily noticeable different phases in their sexual cycle [6,7] is preferred choice in the study.

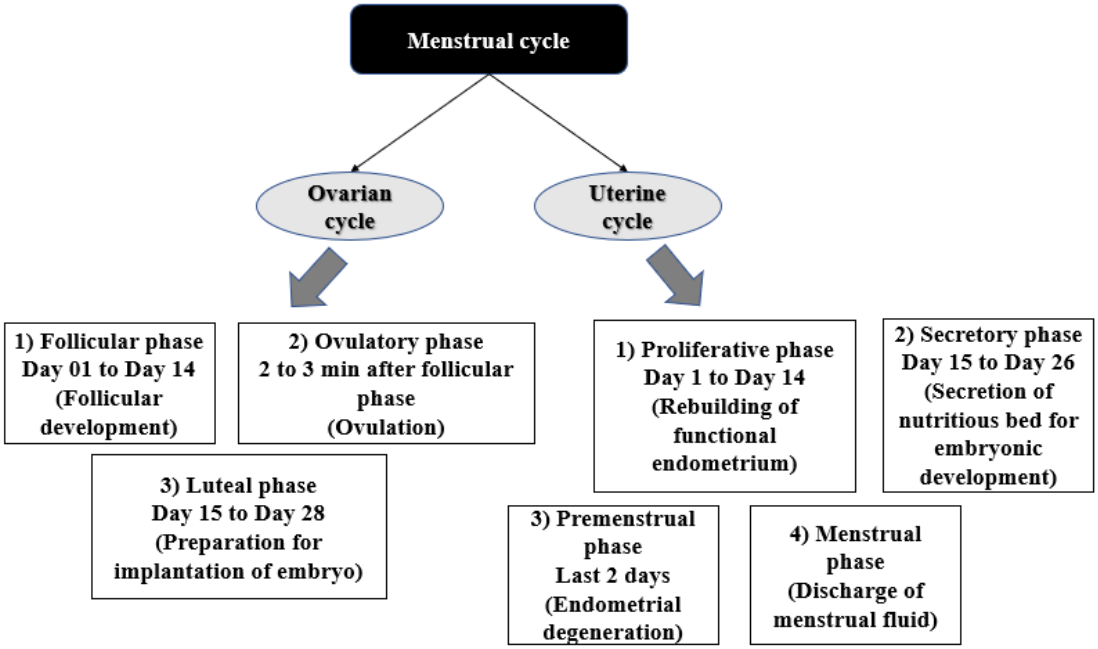

Fig 01: Flow diagram showing different phases of menstrual cycle.

Estrous cycle:

Sexual cycle in non-primates is termed as estrous cycle with the periodicity of the estrous cycle being highly variable among the species [6-9]. Estrous cycle comprises of four stages, viz., Metestrus [Diestrus I] (Fig. 08A) and Diestrus [Diestrus II] (Fig. 08B), Proestrus (Fig. 08C), Estrous (Fig. 08D) [10].

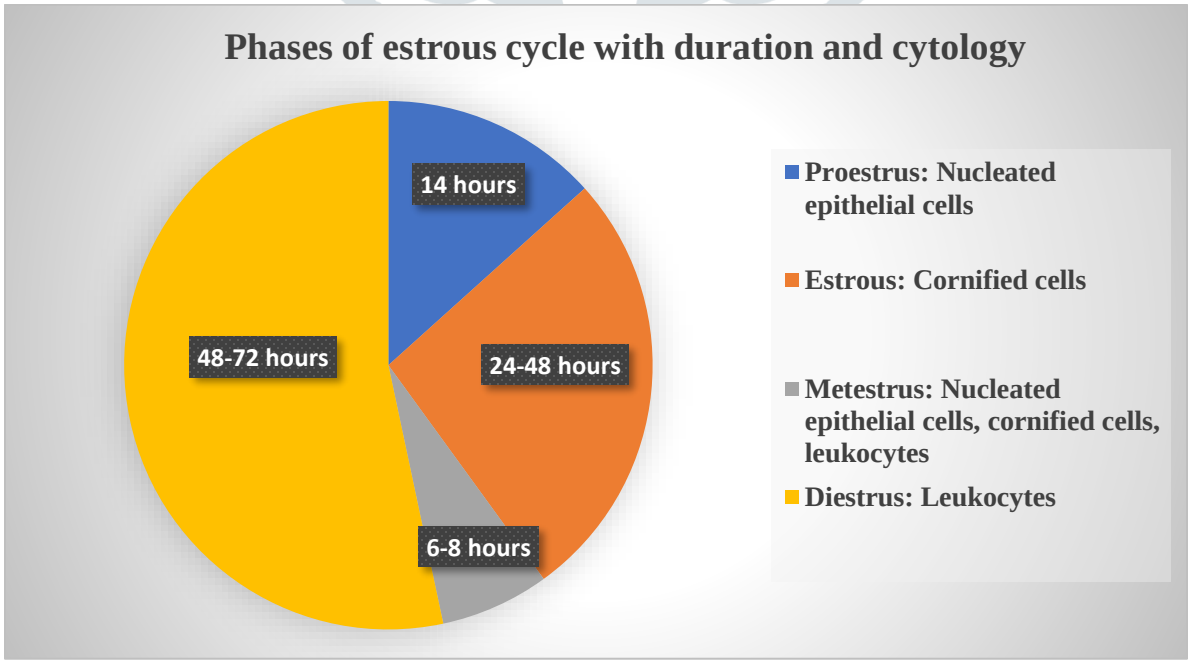

Fig. 02: Graphical representation of Estrous cycle

A brief comparison between the estrous cycle and the menstrual cycle is given in Table 01.

AIM OF THE STUDY: The study aims to explore the effect of randomized chronic stress on the estrous cycle of rats invitro and its implications in understanding the effect of psychological stress in women leading to oligo/anovulation and PCOS manifestation.

Table 01: Comparison between estrous and menstrual cycle

Sl. No.	Estrous cycle	Menstrual cycle
1	Non-primates	Primates
2	Requires shorter time period to complete an estrus cycle, i.e., approximately 4-5 days	Requires longer time period to complete a menstrual cycle, i.e., approximately 25-28 days
3	Includes 4 phases, i.e., proestrus, estrous, metestrus and diestrus	Includes 2 major sub cycles, i.e., ovarian cycle and the uterine/menstrual cycle
4	Vaginal fluid cytology of different stages: Proestrus with predominant nucleated epithelial cells, estrous with cornified cells, metestrus with approximately same amount of nucleated epithelial cells, cornified cells and leukocytes, diestrus with predominant leukocytes	Ovarian cycle comprises of follicular phase, ovulation, luteal phase, and uterine cycle/menstrual cycle comprises of proliferative phase, secretory phase, premenstrual phase and menstrua phase
5	No bleeding	Bleeding

MATERIALS AND METHODS:

Study venue: Central Animal Facility, JSS Medical College, JSS Academy of Higher Education and Research, Mysuru.

Study time: February 2022 to March 2022.

Grouping: Control adult female wistar rats (No. 20), weighing 180 g to 200 g were obtained from the animal facility of JSS AHER, Mysuru. Animals were fed with food pellets and RO water *ad libitum* and were maintained under the standard conditions of 12 h light and 12 h dark shift and the temperature 18°C-22°C, humidity of 40%-60% with air circulation of 12 to 15 air changes per hour and illumination level of 130 to 325 Lux. Corn cob was used as the bedding material for the animals which was changed every alternative day.

Study design: Total animals procured were divided into two groups i.e., control group and stress group with 10 animals in each. The stress group of animals were exposed to different stress parameters with necessary modifications to the standard protocol [10,11], and the control group of animals were kept undisturbed in cage.

Methods:

Stress parameter 01: Restraining: The animals were restrained for 45 min in an iron wire mesh restrainer [L-13 cm (Adjustable lid – 2 cm) × W-5 cm × H-6 cm] (Fig. 03). Animals inside the restrainer were kept constantly on an active phase by gentle touch at regular intervals using a micropipette tip.

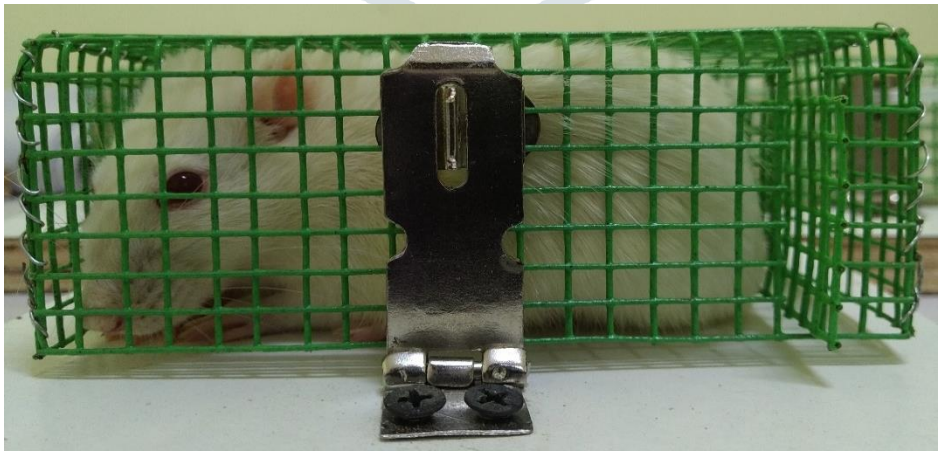

Fig 03: Rat restrained in a wire mesh restrainer.

Stress parameter 02: Dark shift: Immediately after restraining, the animals were shifted to a dark chamber for 2 h (Fig. 04) to induce the effect of upstreaming reproductive functions [12].

Fig 04: Rats kept under dark condition.

Stress parameter 03: Forced swimming: Immediately after dark shift, the animals were forced to swim for about 15 min in a transparent glass jar [H-60 cm × L-25 cm × W-25 cm] filled with 2/3rd of tap water having the temperature of 25±2^{0}C (Fig. 05). After swimming stress induction, all the animals were dried by using a clean cotton towel.

Stress parameter 04: Acute heat: Followed by the swimming stress, animals were exposed to an acute heat stress for about 3-5 min by using SalonDry Compact 1000 W Philips hair dryer after once the animals were dried with cotton towel (Fig. 06).

Stress parameter 05: Social isolation: Animals were kept under social isolation for about 24 h randomly twice a week (Fig. 07).

Fig 05: Rat under forced swim stress.

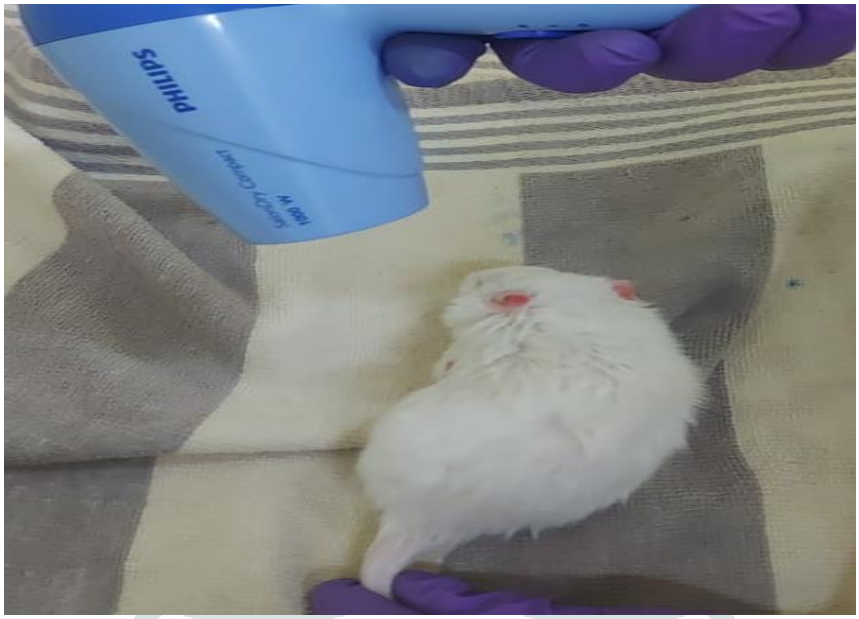

Fig 06: Rat under heat stress.

The restraining stress induction regime was carried out for 30 days at 6 days/week exposure. At the end of the restraining duration, the animals were euthanized and the ovaries were collected for further study.

A B

Fig 07: (A) Rats in group. (B) Rat under social isolation.

Vaginal smear preparation and analysis: Frosted micro slides (Blue star®) were sterilized using distilled water and 70% methanol wash and approximately 200 µl of physiological saline (0.9%) pre-collected in a 1ml pasture pipette was gently inserted into the vagina of restrained rat for about 5sec until the vaginal secretions got collected. The collected vaginal secretion was dropped on a pre-sterilized glass slide, smear prepared and air dried.

Staining: Giemsa's stain was prepared as per the standard protocol [13] and the working standard was prepared from the stock with a 1:1 dilution using distilled water. The air-dried slides were stained with the Giemsa's stain for about 15 min, washed with distilled water and kept for air dry until further microscopic observations.

Statistical analysis: Student's t test was applied to check the significance of the difference in average number of days/estrous cycle, no. of estrous cycles/study period in control and stress groups. Statistical results were established (Table 02) and the data under study was found to be significant with a calculated student's t test value was 5 for average no. of days/estrous cycle to a tabulated t value of 3.922 at a Degree of freedom 18 with confidence level 99.9% and the calculated t value for estrous cycle/study period was 1.74 to a tabulated t value of 1.73 at a Degree of freedom 18 with confidence level of 90% against $p<0.05$.

Animal ethical clearance: Ethical clearance for the current research work has been approved by the Institutional Animal Ethics Committee (IAEC), JSS AHER, Mysuru, Karnataka, India. The study was approved and signed on 27.11.2021 with project proposal number JSSAHER/CPT/IAEC/084/2021.

RESULTS:

Effect of stress on estrous cycle: During the entire study period of 30 days, control group of animals had completed around 4.3±0.46 cycles whereas, stress group animals completed only 2.5±0.5 cycles. The stress group animals showed diestrus or metestrus phase more frequently during their estrous cycle.

Effect of stress on ovarian morphology: The ovarian morphology has shown considerable level of morphological variations between control and stress group animals. The stress group animals have developed poly cyst-like formations on the ovaries, whereas a smooth surfaced ovary has been observed in case of control group animals (Fig. 09). This poly-cyst formation in stress group animals confirms the effect of active participation of random chronic stress inductions in animal model system.

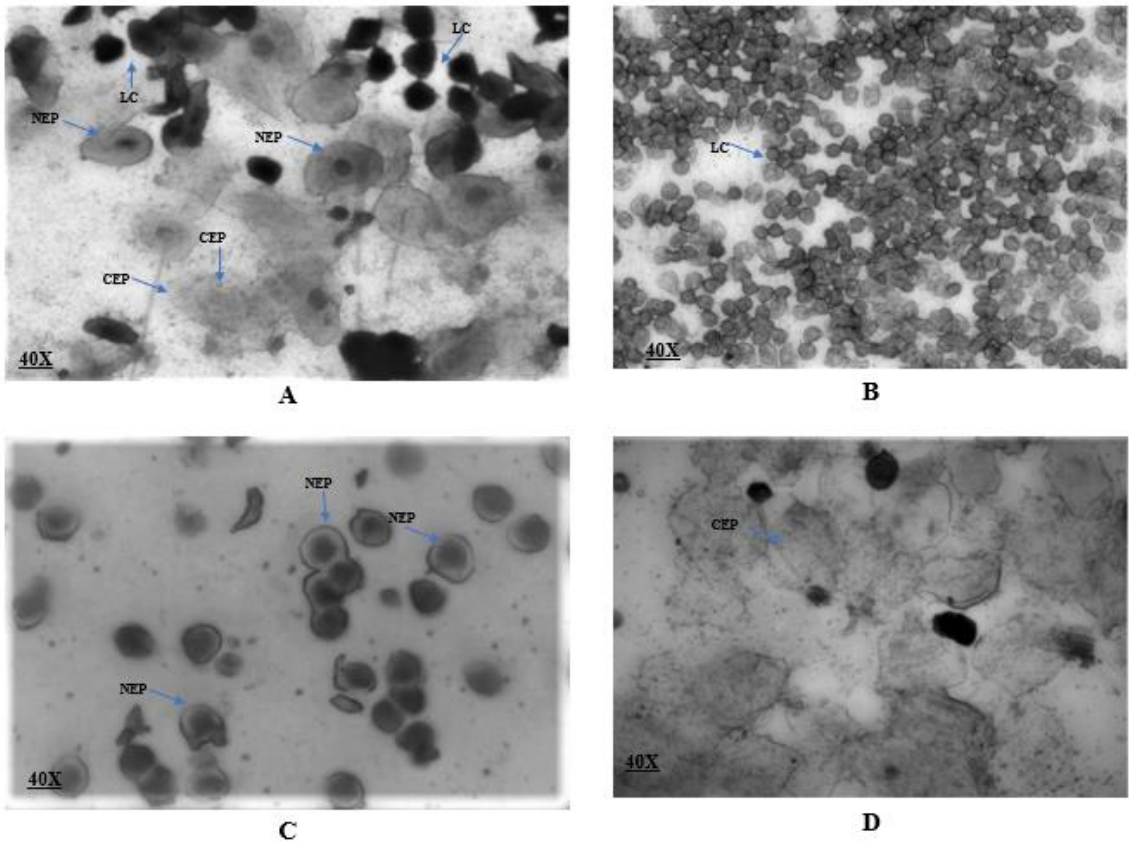

Fig 08: (A) Equal amount of cornified epithelial cells, nucleated epithelial cells and Leukocytes, indicating metestrus phase. (B) Predominant Leukocytes, indicating diestrus phase. (C) Predominant nucleated epithelial cells, indicating proestrus phase. (D) Predominant cornified epithelial cells, indicating estrus phase. [NEP – Nucleated epithelial, CEP – Cornified epithelial, LC – Leukocytes]

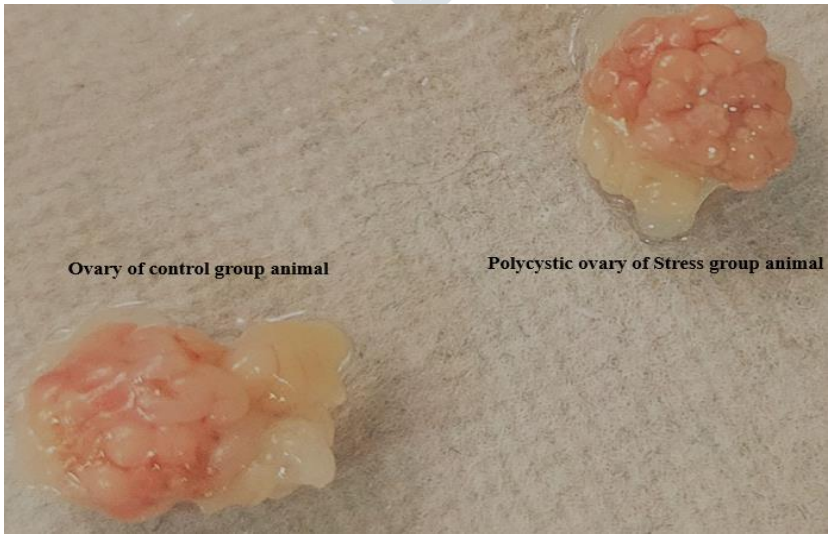

Fig 09: Ovaries of control and stress group animals.

Table 02: Statistical analysis report

Group	No. of days/estrous cycle (n=10)	No. of estrous cycles during experimental duration (n=10)
Control (*Rattus norvegicus*)	5.8±0.98 p=0.0005	4.3±0.46 p=0.05
Stress (*Rattus norvegicus*)	8.5±1.28 p=0.0005	2.5±0.5 p=0.05

Discussion:

Randomized chronic stress parameters have been adopted to observe the early variations in estrous cycle and their impact in ovarian cyst morphology. PCOS induced animal model development in most of the research has been studied using androgen injections or the high fat diet [14,15], where the current study targets the stress induced PCOS in non-obese/lean individual and hence used different stress parameters for PCOS induction. Earlier animal studies have revealed the potential role of chronic stress interference in follicular development [10,11,16]. Rats have always been the study specimen of choice to address variable time period of different stressors to observe the early symptoms of the PCOS and disease prognosis [10,16,17]. A 3-week cold restraint stress exposure has reported to induce PCOS in rats [17]. A report on 3 different kinds of expressions in rats exposed to 3 different time period of stress exposure for a shorter duration of 4 weeks showed a suppressive action and a long period of 8–12-weeks exposure to stress exhibited a pathophysiological effect on the ovaries [10]. In the current study, significant difference in estrous cycle among the stress group animals were observed, where, the control group animals had completed around 4.5±0.46 cycles and stress group animals had completed around 2.5±0.5 cycles during the experimental period and the stress group animals were stayed most of time in diestrus or metestrus phase in their estrous cycle, which is concordance with the earlier reports cited. A decreased number of estrous cycles per 4 weeks, i.e., 6.14±0.26, 5.64±0.32, 5.55±0.33 in control animals and 4.00±0.31, 4.24±0.15, 4.13±0.20 in stress group animals have been reported during 4, 8 and 12 weeks of study respectively [10]. Another report with a study period of 3 weeks has noticed a same percentage time for the different stages of estrous cycle among the rats, with authors identifying a significant difference among the regularity in transition from proestrus to estrous showing 27% among stress group animals and 70% among the control group [17]. Apart from the significant changes in estrous cycles, poly-cysts formation in the ovaries of stress group animals were observed showing the identical morphological resemblance to the earlier study reports [18, 19]. Variations across estrous cell cycle and a concordant change in the ovarian morphology are in compliance of the 2 out of 3 Rotterdam criteria's and hence establishing the induction of PCOS in the rat models studied [20].

CONCLUSION: The current study has established the significant effect of random chronic stress in inducing PCOS (according to Rotterdam criteria) in lab rat models. Further, the study forms as a baseline work to explore the molecular reasoning behind chronic stress induced estrous cycle variations and histopathological dissection of the cysts formed in the ovaries.

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Conflict of Interest: The authors hereby declare no conflict of interest related to this publication.

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