

Early Induction of Estrous Cycle Experimentally in Laboratory Mice

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Abstract

Background: The early induction of heat in laboratory animals like mice is so essential for reproductive performance, and detection of estrus could be achieved by many ways like visual evaluation, vaginal cytology. Mice could be artificially induced to heat period to get them receptive for male.

Aims: To induce estrous cycle in mice early than normal puberty

15 young mice age 4 weeks grouped to three groups, first group treated as control, second group received 0.05 ug estradiol benzoate while third group treated with 0.05 ug GnRH s/c., the same dose for two groups 2 and 3 repeated after three days. Vaginal cytology smears were taken 48 hours after last injection for animals of this study. The results of the study revealed significant showing of estrus behavior in group 2 compared with other groups that used hormones accelerate signs of estrus in mice in early stages.

Conclusion: Setting of the scope and period of the heat cycle and the creating of the heat are helpful in the learning, examine and assessment the impacts of hormone on the reproduction performance.

Keywords: Mice, Estradiol Benzoate, GnRH, Heat cycle, Reproductive cycle Lab



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Introduction

determining the level of estrous considers so helpful for choosing mouse that would be mated when coupled with the male (to investigate the time of pregnancy or the pseudopregnancy) or following level of heat as variable agent that could affect the impact of the research. The heat cycle in mice is categories into four levels (proestrus, heat, metestrus, and diestrus) and every four-five days repeat except interrupted by gestation, false gestation, or anestrus (Tables 1, 2). The heat cycle qualified and discussed in detail by many previous studies [Kurita, 2010, Merkwitz et. al. 2016]. All the changes that happening in mice heat cycle is the evident in animal's anatomy and physiology. The alteration of heat cycle could be easily detected by depending many different methods to mark the level of heat and comprise assessment the cytological sample of vagina in the mice [Byers et. Al.,2012, Ajayi and Akhigbe, 2020], calculating electrical resistance [Ramos et. Al. 2001], biochemical tests for the urine [Achiraman et al 2011], and visual watching for external genital organs Figure 1 [Merkwitz et. al. 2016]. The assessment of the vaginal epithelia considers satisfied as the common accurate way for determine the levels of heat cycle anyway, it considers labor dense demanding vaginal epithelia cells to be got, transmitted for glass slide, also air dried of vaginal sample, stained, and seeing for evaluating.

The cytological sample obtained from vagina of rodents considers one of the most common ways to determine any phase of four stages of estrous cycle depended on the evaluation of the epithelia cells of sample under hormonal control. In place of, the electrical resistance of the vaginal samples of the epithelial cells during the period before estrus is huger than any other phases and that may be calculated depended a probe to determine the mouse that is in the proestrus. One the other hand this method cannot depend to identify the others phases of estrous cycle. The way for identifying the phases of heat cycle by depending visual watching revised by Champlin, et al [1973] considers faster than cytological and electrical resistance ways. Depending on training staff, this method could check more than one

hundred mice within ten minutes, no need more tools and the checking may be done without any leaving vivarium.

Moreover, depending the visual way for the evaluation of heat cycle, a few risks of stimulating the pseudopregnancy or hurt vaginal cells that associated could be with inserting a stick swab for obtaining epithelia cells or using a probe for calculating electrical resistance (Ajayi and Akhigbe, 2020). Investigation process to identify the mice which being in the proestrus or heat were usually to be attractive to males leading this way good suited for determining mouse likely to couple, easing timed coupling. In Jackson Laboratory's Reproduction Science series higher than 92% of mouse "selection" as being in the period before estrus or heat successfully coupling during the night. The pseudo gestation outbred albino mouse or crossbreed agouti mouse is usually depending as fetus recipients and discovering the phases of the proestrus/heat in these types considers straight forward (Kurita, 2010). Learning and depending visual and cytological ways for identification the phase of estrous cycle needs practice (Ajayi and Akhigbe, 2020). Depending the visual watching and cytological samples ways consider the most suitable for determining mice that in proestrus or heat for induce the animals for coupling or pseudo pregnant mouse. For this reason, accurate checking all phases of the heat cycle is needed, the cytological sample from vagina of mice is recommended and depended on the type and proportion of vaginal cells in the sample smear. There are few studies about using the reproductive hormones to induce estrus in mice early, so the current study conducted to evaluate many hormones to accelerate the estrus in young mice.

Table 1: Length of various phase of estrus cycle in mice.

Estrus stage	Cycle length (hours)
Proestrus	<24
Estrus	12-48
Metestrus	8-24
Diestrus	48-72

Table 2: Appearance of the vagina at different phases of the estrous cycle

Estrous stage	features
Proestrus	Vagina of mice are gaping, wet, moist and pink. Many longitudinal folds are visible on ventral and dorsal lips.
Estrus	Vaginal shows same seen in proestrus stage, but vagina is lesser pink and less wet.
Metestrus	Vagina shows dry and pale appearance. The dorsal lips are not oedematous like in heat phase
Diestrus	Vagina tissue is wet and had tiny opening and bluish-purple color.

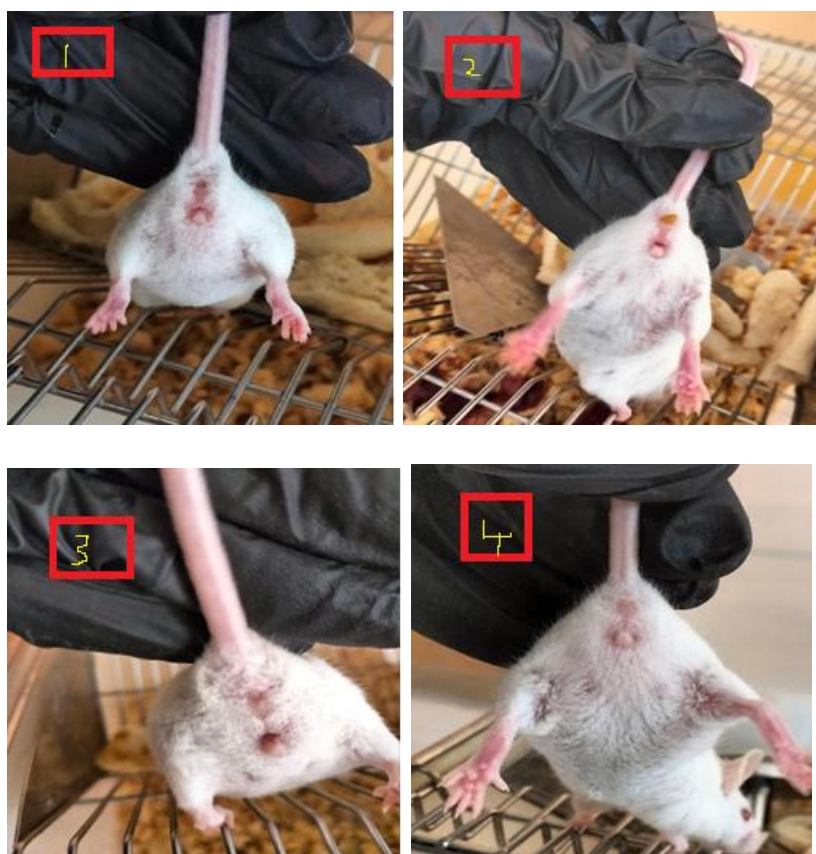


Fig.1 The external genitalia of mice in different phase of estrus cycle of mouse
1-Proestrus 2-Estrus c-3-Metestrus 4-Diestrus

Materials and Methods

Ethics Approval

The Committee of animal care in faculty of veterinary medicine accepted all the procedures depended in the current study and all mice were maintained at Laboratory in accordance with all institutional protocols and the Guide for the Care and Use of Laboratory Animals under the No. 3327 dated 28/10/2024.

Mice

Total 15 mice were depending in this study aged 3-4 weeks and got housed in non-barrier facility These mice supplied with photoperiod of fourteen hrs of using light and ten hrs of darkness. Every five mice housed in one cage and supplied with available food and water.

Vaginal Cytological sample

The vaginal swabs were got using a cotton tipped swab (Puritan Medical Products Company, LLC Guilford, ME) wetted with ambient temperature physiological saline and inserted into vagina of the restrained mouse. Thees swabs were gently turned and rolled against the vaginal wall and then removed. Cells were transferred to a dry glass slide by rolling the swab across the slide. The slide was air dried and then stained with Giemzia stain 5% (Accustain, Sigma-Aldrich, St. Louis, MO) for 5-10 minutes. The slides were rinsed with water, overlaid with a coverslip, and viewed immediately at 10^x and 40^x magnifications under bright field illumination. The phase of the heat cycle evaluated depended on the presence or lack of WBC, cornified of epithelial cells, and nucleated cells depending of study by Merkwitz, et al (2016).

When the mice are in proestrus stage, usually cornified and nucleated cells are existing. Many leukocytes could be existent if the mice are in early stage of proestrus phase. When the phase of the cycle progress and advances to heat, usually cornified squamous vaginal cells are existent. When cycle in mice is not interrupted by gestation, false pregnancy, and phenomena, metestrus phase will start. Metestrus stage is the shortest phase and corpora lutea (CL) shape form but this CL fails to complete

luteinize due to deficiency of progesterone hormone. The vaginal lining will start to slough and proof of the estrus stage, cornified vaginal epithelial cells and neutrophils are present in vaginal sample swabs. Many nucleated vaginal epithelia cells are present in late stage of metestrus phase. Diestrus phase considers longest of the phases constant two days and may be more. Vaginal sample swabs during the stage of diestrus shows primarily neutrophils and few epithelial vaginal cells during ending of diestrus. WBC are remaining predominant type of cell removed cellular debris.

Experimental Procedure:

All 15 mice divided into three groups, each group five mice. first group treated as control and the second group received 0.05 ug estradiol benzoate s/c while third group treated with 0.05 ug GnRH s/c the same dose for two groups 2 and 3 repeated after three days. Vaginal cytology smears were taken 48 hours after last injection for the animal of the study.

Anatomical samples

After dividing the mice into groups and taking the vaginal swab separately, the samples are dissected separately as well, where the mouse is anesthetized with (0.1 cc) of ketamine 5% IM, (MKO Melt Dose Pack, MKO Troche) which begins to take effect on the mouse after about a (1min) , then it is exposed on the dorsal side **Figure 2** and an incision is made using scissors and the free part is pulled up wards by using thumb forceps **Figure 3**, then we also remove the viscera gently and carefully, then we go down to where the ovary and uterus are there and the bladder which is considered a guide. After that, the ovary, the uterine horns and the uterus are separated gently and placed on a clean slide to see the changes according each group.

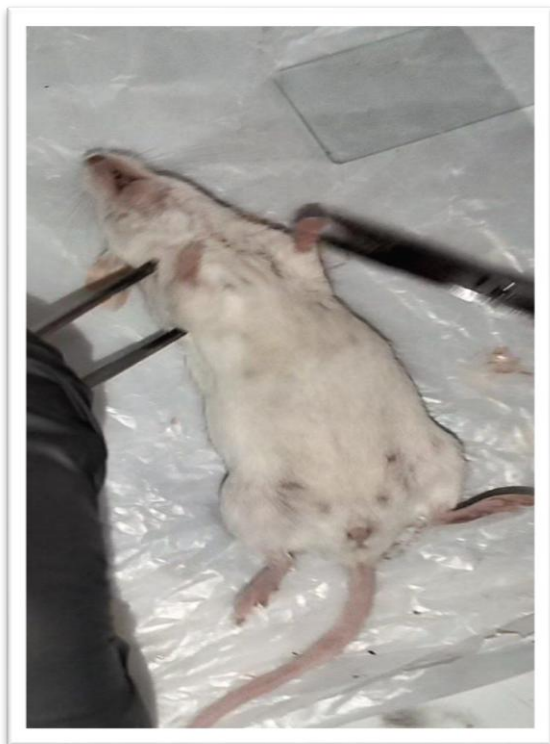


Figure 2: The mouse in dorsal site



Figure 3: Site of incision

Results:

The cytological vaginal sample contains three types of epithelia cells. These cells include leucocytes, cornified cells and nucleated epithelial. Evaluation of the stage of heat cycle is based on percentage of cells in the cytological vaginal sample.

The study showed in the first group, which was given estradiol and involved five mice, 98% of the mice were in the estrous phase, as when the swab sample was placed under the microscope 10^{\times} we see the large, blue or deep purple stained spots/clumps of cells appear. Often, the cornified cells appear as large clumps or sheets, and the nucleus may not be visible or may be very faint. These signs indicated that mice in estrus phase, at this stage, most of the cells are cornified.

The cell nucleus is often not visible (or very atrophied) because the cell has lost most of its cellular content. There are almost no (or very few) white blood cells in the smear. Under the microscope, the smear appears to be covered with cell plates or flakes (broad, flat cells). **Figure 4**

This study recorded in second group which was given GnRH and involved five mice, under microscopic examination, the sample contained cells of different shapes, with areas rich in small cells and purple spots, which may indicate the presence of white blood cells or cellular debris. This indicates that the mice in this group, at a rate of 65%, are in the (Diestrus) stage, as we find in the microscopic Numerous rounds nucleated cells which are uniform in size and appearance characterize the proestrus phase. They appear in clusters or individually. There are also few a nucleated cornified epithelial cells. Some white blood cells may be present in the female in early proestrus **Figure 5 and 6.**

Finally, the least group is control and it contain from 5 mice after take the swab from vagina showed different phases from each cycle.

The anatomical results of this study showed increasing the ovarian activity of the group that involved administration of GnRH comparison with other groups, increasing the number of follicle and corpus luteum in ovaries in this group. Anyway, ordinary preovulatory follicles developed and progress CL formation were seen in ovary harvested from spontaneously mated mouse.

In the ovaries of immature mice, sequential treatment with GnRH and estradiol induced an increase in organ weight, stimulated follicular maturation and brought about multiple ovulations. Corpora lutea were formed at the site of the ovulated follicles. Overall, the coordinated structural changes in the stimulated immature ovaries were comparable with those seen in the ovaries of spontaneously cycling adult mice **Figure 7.**

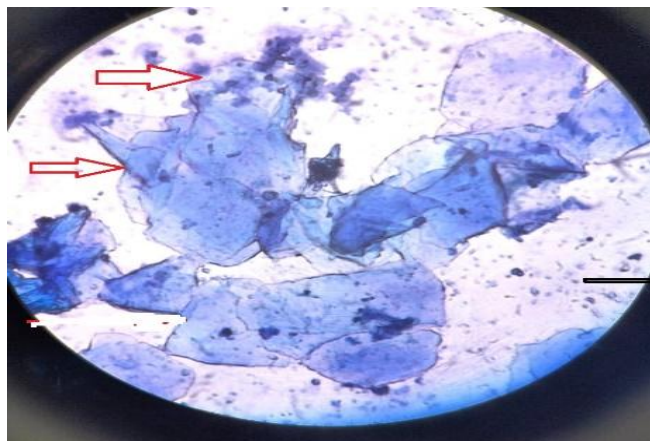


Figure 4: Vaginal cytology representing estrus phase, red arrow indicated for cornified cells under microscopic power 40^x

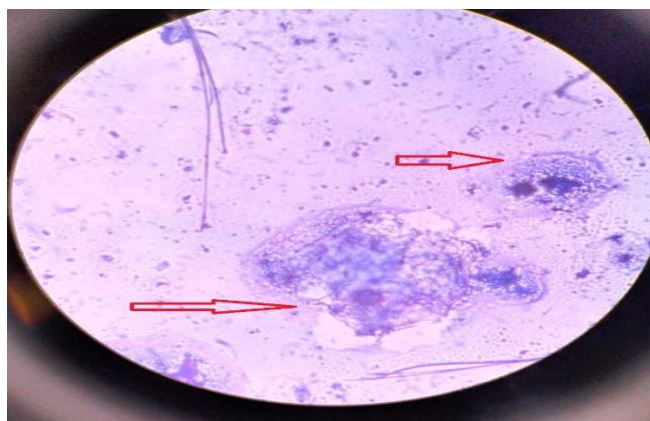


Figure 5: Vaginal cytology representing proestrus phase, red arrow indicated for epithelial cells under microscopic power 10^x

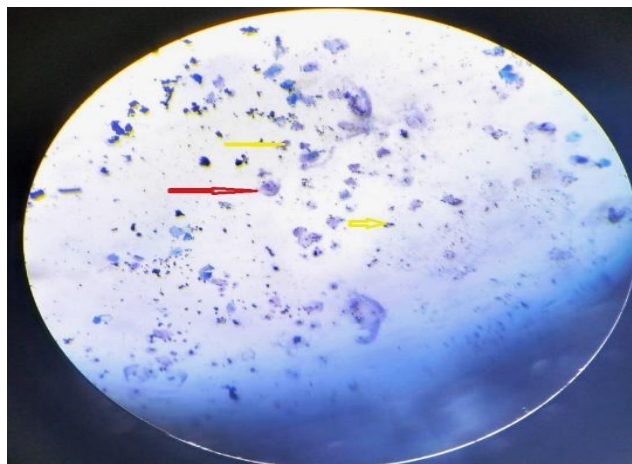


Figure 6: Vaginal cytology representing diestrus phase, red arrow indicated for epithelial cells and yellow arrow indicated to neutrophils under microscopic power 10^x

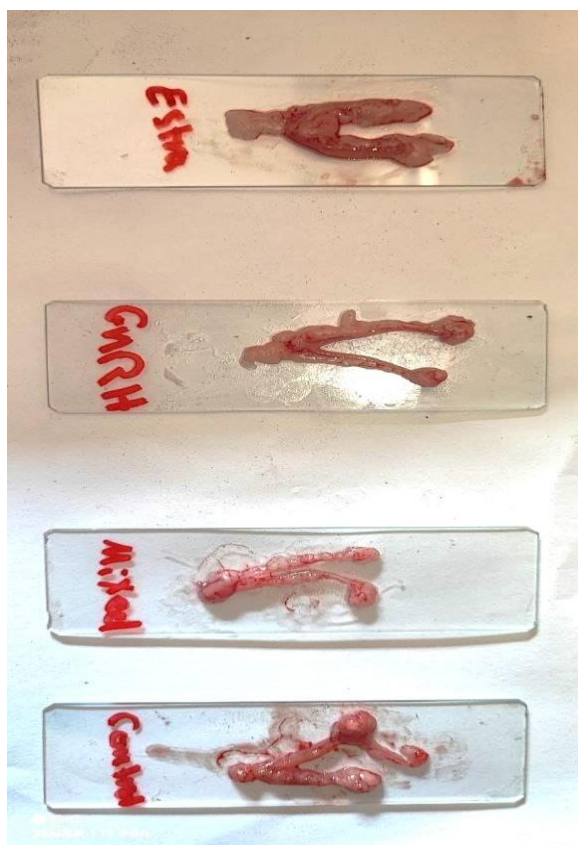


Figure 7: Female genitalia after many hormonal treatments
Discussion

Identifying the phase of the heat cycle by visual watching is soft and simple to learn. The visual discovering way is best to investigate proestrus and heat mice. It may be hard to distinguish the others phases of estrous cycle by depending this way alone. If all phases of cycle must be evaluated accurately, the vaginal sample by cytological way depending the heat cycle investigation tool is still recommended yet (Merkwitz et. al., 2016).

The regulation of hormones in the heat cycle in the rodent (especially in rats) has recorded in detail. All Mullerian tube-derived organs such as salpinx, uterine horns and vagina afford cyclical morphological changes under impact of steroid hormones by ovaries like progesterone and estrogen (Merkwitz et. al., 2016, Silveira et. al., 2017). In certain, the cyclical shedding of the superficial mucoid and cornified stratum in the epithelium of vagina is noteworthy, the exfoliative vaginal cytological and histological sample are depended worldwide for evaluation and categories of the heat cycle phases in rats (Scudamore, 2014). Anyway, the vaginal epithelium cell of the rat has depended to evaluate estrogens (Long et. al., 2000). Unfortunately, mouse shows irregular heat cycle more much than the rats according to continuous stress of bad animal housing (McLean et al., 2012). Moreover, the acceptable exfoliative vaginal epithelium cytology is hard to get in mouse than other rodents, and vaginal sample swab assign significant stress element for mice (McLean et al., 2012). thus, it was widely understood that in mouse, different than rats. For this reason, it is widely believed that in mouse, then rats, the specific phase of the heat cycle is well evaluated by planning and analysis of appropriate histological division post-mortem of vagina of mice. Modern research by Gal et al. (2014) has recorded that neither exfoliative vaginal sample cytological nor gonadotropin factors and sex hormone plasma concentrations could be predicting the heat cycle phase of mice (Gal et al., 2014). There are necessity for investigate specific phase of the heat cycle in mice post-mortem, this entails the depending huge numbers of mice in any study.

The standard for diestrus phase was the presence of tiny, stratified, squamous, non-cornified vaginal cells with many proliferating in base layers of tissue. The proestrus phase is known by stratified cell, non-cornified and, one to two widely superficial stratum of cuboidal cells and may be cylindrical mucin cells. The heat phase was distinguished by the existence of a extended squamous, stratified epithelium vaginal cell with notable cornification of superficial stratum. Finally, the standard applied for distinguished the metestrus phase I and II are the existence of a stratified vaginal epithelium cells with little to heavily infesting and transmigrating neutrophils and complete of the cornified stratum (Scudamore, 2014, Merkwitz et. al., 2016).

Preovulatory GnRH and LH surges are essential for successful reproduction in most species. The GnRH surge is initiated by high physiologic estradiol levels caused by a switch in feedback action of this steroid from negative to positive feedback on both the GnRH network and pituitary. The importance of estradiol in the control of ovulation led to the development of models in which estradiol levels were modified in ovariectomized animals to induce the positive feedback response while reducing other ovarian variables (Silveira et al., 2017).

The current study showed increasing the ovarian activity of mice that deal with GnRH and this also agree with many previous studies that recorded plain preovulatory follicles progressed and CL foundation was known in the ovary harvested from cycling mouse (Merkwitz et. al., 2016, Silveira et al., 2017). Many other studies will need and depended histological changes in female genitalia in mice (Ahmed and Samarraa, 2024), and rat (Haichal, and Towfik, 2025).

In conclusion, determination of the length and phases of the estrous cycle and induction of estrus are useful in teaching and research and evaluating the effects of drugs/chemicals on the reproductive functions.

Conclusion: determination of the length and phases of the heat cycle and make induction of heat are so helpful in learning, research and evaluating the impact of many hormones on reproductive performance.

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