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A Study on the Estrus Cycle and Superovulation of the Mouse

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= Abstract =Pregnant Mare's Serum Gonadotropin (PMSG) has been used for superovulation in animal models by similar techniques used in human *in vitro* fertilization and embryo transfer (IVF & ET). Intraperitoneal injection of PMSG and human chorionic gonadotropin (HCG) 48 hours apart will result in ovulation approximately 14 to 19 hours later. The number of oocytes ovulated is affected by age, strain, dose of gonadotropin, timing of injections, and environmental factors.

This study was undertaken to evaluate the effect of estrus cycle on the retrieval of mouse oocytes in conjunction with mouse *in vitro* fertilization as a quality control testing for Seoul IVF & ET program. Vaginal smears were made by lavage of the vagina with saline solution using an eyedropper. We observed the cell types on the Papanicolaou stained smear to confirm the stage of the mouse estrus cycle.

Superovulation was induced with intraperitoneal injection of 5.0 I.U. PMSG followed by 5.0 I.U. HCG 48 hours later. Oocytes retrieval was done 16 hours after the intraperitoneal injection of HCG.

Oocytes retrieval rates according to the stage of estrus cycle were 14.9 ± 2.6 in proestrus stage(N=16), 15.8 ± 3.4 in estrus stage(N=24), 15.7 ± 3.8 in metestrus stage(N=16), and 15.1 ± 4.6 in diestrus stage(N=18). These results revealed that the stage of estrus cycle in mice did not affect the oocyte retrieval rate.

Key Words: ***Superovulation of mice, Estrus cycle, Mouse oocyte***

INTRODUCTION

Superovulation through the administration of exogenous gonadotropins has been widely used in mice for experimental purposes (Edwards & Fowler 1959; Maudlin & Fraser 1977; Bo *et al.* 1983; Seidel 1983) and the mouse *in vitro* fertilization system is a valuable research model because direct observations of the earliest preimplanted developmental stage are possible (Brinster 1963; Kaufman & Whittingham 1972; Hafez & Semm 1982).

After the pioneering work of Brinster (1963), numerous modifications of mouse IVF system about strain, PMSG doses, media, timing of injection, sperm sources and concentration, preparation of oocytes, effects of additive to fertilization media were tried (Mastroianni & Biggers 1981).

This study was undertaken to evaluate the effect of estrus cycle on the mouse oocyte retrieval rate in conjunction with mouse IVF as a quality control testing in Seoul IVF & ET program.

MATERIALS AND METHODS

1. Experimental Animal

Female ICR strain mice aged 6 to 8 weeks were housed in a room maintained at 20°C to 21°C and were exposed to 14 hours of light per day.

2. Media

Ham's F-10 nutrient mixtures (Gibco Laboratories, Grand Island, New York) was prepared as stock solution with 9.81 gm of powdered Hams F-10(x4) medium with glutamine, 75 mg of penicillin G (Sigma Chemical Co., St. Louis, Missouri), 75 mg of streptomycin (Calbiochem, La Jolla, California), and 250 ml of distilled water (5x) through

Table 1. Classification of stages of estrus cycle by cell morphology in vaginal smears

Stage of cycle	Cell type*			Smear density
	Leukocytes	Nucleated epithelia	Cornified epithelia	
Proestrus	0 to † Often degenerating	† to ††† Well-formed (predominant)	0 to †	Medium
Estrus	0	0	†† to ††† **	Medium to heavy
Metestrus I	0 to ††	0	†† to ††† ***	Medium to heavy
Metestrus II	†† to †††	† to ††	† to ††	Medium to heavy
Diestrus	† to †††	†	0	Thin

* Cell density: 0=none, †=few, ††=moderate, †††=heavy

** Relatively small cells (predominant)

*** Larger, more flat and clumped than in estrus (predominant)

passing Millipore with positive pressure, and it was stored in the refrigerator at 4°C. Fresh culture media were prepared on the day before the experiment. This was adjusted to pH 7.3 and 280 mOsm and incubated at 37°C under 5% CO_2 in air for 18 to 24 hours prior to use.

3. Superovulation

Superovulation was induced by intraperitoneal injections of 5 I.U. PMSG (Sigma Chemical Co., St. Louis, Missouri) and HCG (Serono Diagnostics Ltd., London) 48 hours apart after selecting mature female mice at random respecting their estrus cycle.

4. Determination of the stage of estrus

Vaginal smears were made by lavage with saline using an eyedropper or cotton-tipped applicator before superovulation.

Vaginal smear specimens were stained with Papanicolaou stain solution, and it was examined under the microscope.

Classification of the estrus cycle was adopted according to following criteria (Nelson *et al.* 1982: Table 1).

5. Oocytes retrieval

Oocytes-cumulus complex (OCC) was retrieved from the ampullary portion of the oviduct through flushing by insertion of the blunted tip of a 30-gauge needle attached to a 1 ml tuberculin syringe containing medium into the fimbria of the oviduct. Oocytes-cumulus complex was dispersed in 1.0 ml Ham's F-10 medium in 60×15 mm tissue culture Falcon dishes. The number of oocytes in cumulus masses were counted under the inverted phase contrast stereomicroscope.

RESULTS

1. The distribution of various estrus cycle

The distribution of various estrus cycle among 74 experimental female mice were proestrus stage 16(21.6%), estrus stage 24(32.5%), metestrus stage 16(21.6%), and diestrus stage 18(24.3%).

2. The oocytes retrieval rate

The oocytes retrieval rate respecting the estrus cycle were 14.9 ± 2.6 in the proestrus stage, 15.8 ± 3.4 in the estrus stage, 15.7 ± 3.8 in the metestrus stage, and 15.1 ± 4.6 in the diestrus stage (Table 2). These differences of the oocytes retrieval rate respecting the estrus cycle were not statistically significant.

DISCUSSION

One of the most revealing and readily measurable markers of reproductive milieu is the vaginal estrus cycle and cytologic approach reflecting the hormonal milieu that maintains ovulatory function. Despite its accessibility and information values, vaginal cyclicity has not been extensively exploited as a research tool in the study of reproductive physiology (Nelson *et al.* 1982). Extensive leukocytosis might reflect insufficient estradiol secretion due to reduced numbers of maturing follicles or a delay in their development. This explanation is consistent

Table 2. Oocytes retrieval rates of ICR strain mice superovulated in various stages of estrus cycle

	Proestrus	Estrus	Metestrus	Diestrus
Number of Animals	16	24	16	18
Oocytes Retrieval				
Mean	14.9	15.8	15.7	15.1
S.E.M	2.6	3.4	3.8	4.6

with delayed preovulatory rise of plasma estradiol level observed during the initial prolongation of cycles in aging mice (Nelson *et al.* 1981).

The mouse can be induced to superovulate by the administration of gonadotropin. Intraperitoneal injections of PMSG and HCG 48 hours apart will result in ovulation which is affected by age, strain, dose of gonadotropin, timing of injections and environmental factors (Ackerman *et al.* 1983). The highest percentage (82.7%) of oocyte nuclear maturation was obtained when oocytes were collected 48 hours after the injection of PMSG (Unnithan 1974). PMSG did not increase the number of large follicles, but it changed the balance between healthy and atretic follicles by preventing or delaying atresia. The number of follicles beginning to grow is not altered by blocking the secretion of the endogenous gonadotropins or by injection of exogenous gonadotropins (Peters *et al.*1975). Blood levels of PMSG decreased below the threshold for the maintenance of follicles 72 hours after pretreatment, so ovulation did not occur regularly in the case of HCG injection after 72 hours following pretreatment with PMSG (Sasamoto 1972).

Female mice undergo cyclic changes in their reproductive system every 4 to 5 days throughout the year (Nelson *et al.* 1981), so they are considered as polyestrus. On the other hand, the populations of primordial and growing follicles were nearly exhausted by postnatal 13 to 14 months (Gosden *et al.* 1983). Since aged mice still have ova in their ovaries, this would indicate that the loss of reproductive ability in mice is due to reduced ovarian response to gonadotropins (Nelson *et al.* 1982). The estrus cycle of mice differs from the menstrual cycle of humans in that there is no menstrual phase and the mouse is receptive to copulation during a short period of the cycle.

Like the human menstrual cycle the estrus cycle is a combination of related secretory, anatomical, and behavioral cycles resulting from hypothalamic-pituitary activity and gonadal hormone secretion. Proestrus preceeds the estrus and involves involution of the corpus luteum and swelling of the follicles due to renewed FSH secretion. In mice during induced cycles, LH reached a mean of about 40 ng/ ml between 16 and 17 hours of proestrus, and levels of FSH reached a peak of around 2800 ng/ml about 2 hours later, between 19 and 20 hours (Murr *et al.* 1973). Estrus lasts for 9 to 15 hours. Healthy follicles measuring 100 to 149 μm in diameter continue to grow to attain 200 to 400 um at estrus, so a period of about 10 days, *i.e.,* two successive estrus cycles, is required for small follicles of about 100 to 149 um in diameter to grow to reach the ovulatory size (Numazawa & Kawashima 1982). Metestrus follows ovulation and lasts for 10 to 14 hours. The ovaries contain corpus luteum and small follicles. Diestrus makes up most of the cycle as it lasts for 60 to 70 hours. During this stage the corpus luteum regress in preparation to start the cylce over again.

The determination of the various stages of the estrus cycle of the mouse can be easily done by the observation of the appearance of the vagina (Champlin *et al.* 1973). They pointed out that the frequent vaginal cytologic smearing could result in cornification of the vaginal epithelium and induce pseudopregnancy or abnormally long cycles. They identified the stage of estrus cycle by the observation of the degree of vaginal swelling, particularly with respect to the dorsal lip, the color and moistness of the tissues, the size of the vaginal opening, and the presence or absence of visible cellular debris in the vagina.

The induction of ovulation in mammals by administration of exogenous gonadotropin may be responsible for an increased incidence of chromosomal abnormalities in the resulting embryos or increasing fetal mortality (Maudlin and Fraser 1977: Edwards and Fowler 1959). Superovulation techniques using gonadotropins had been tried extensively in attempts to increase the number of young born to domestic animals in spite of the high rate of resorption resulting in little or no increase in the

number of offspring. In the case of mice, the number of young born alive was not increased, even though approximately double the number of living fetuses found in controls were present immediately before birth.

Metestrus mice ovulated more eggs than other estrus cycle mice after 1 I.U. PMSG, and diestrus mice more commonly had no implanted embryos (Edwards and Fowler 1959).

Since the superovulation was performed on the weekly basis in this study, the phase in which the mice were by the luck of the draw. The facts that the mouse is in the diestrus stage for over 60 percent of its cycle and the estrus stage tended to give a more definite smear must be taken into consideration in analysis of data. Our data revealed that the stage of estrus cycle in mice did not affect the oocyte retrieval rate.

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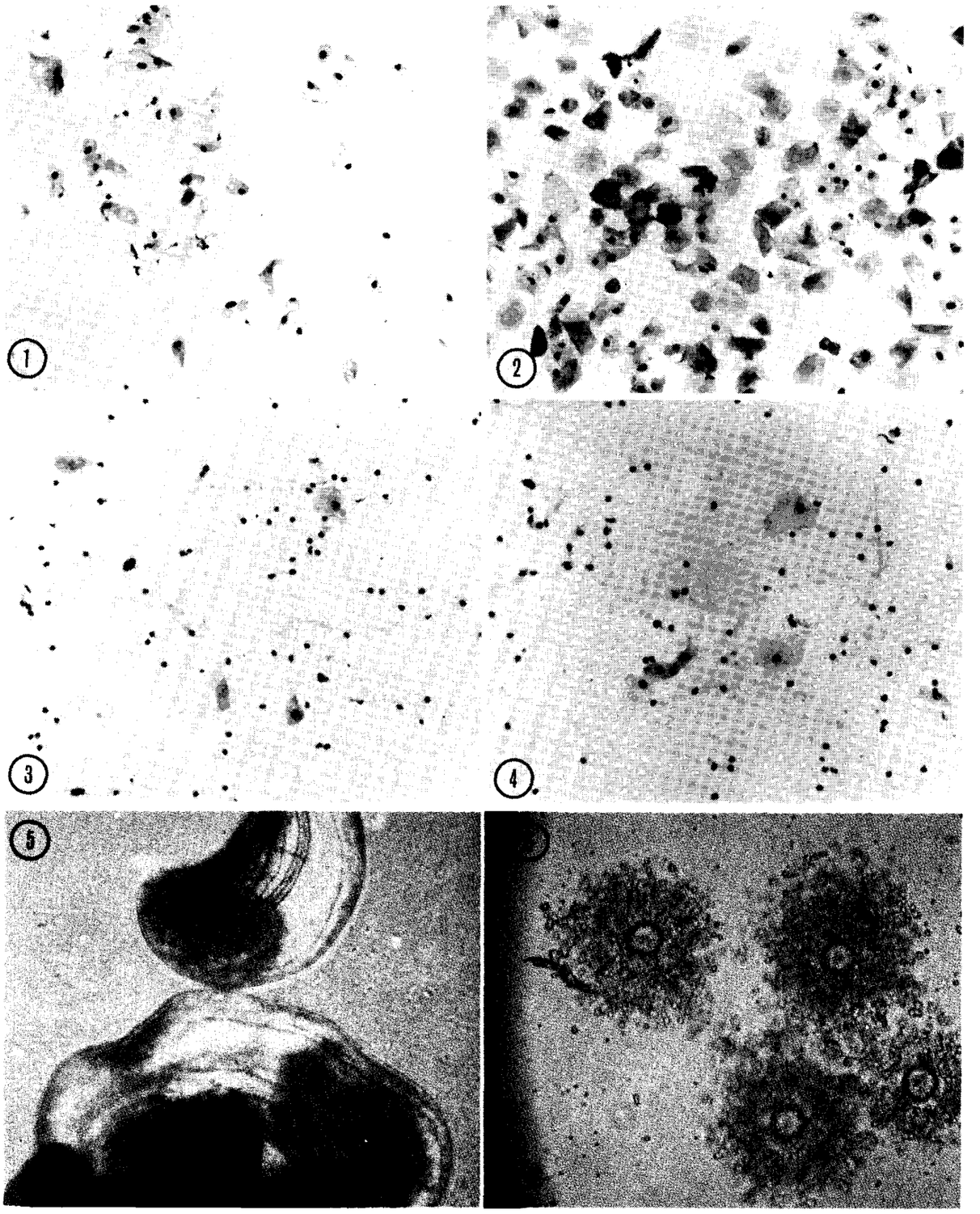

Fig. 1. Proestrus stage Predominant nucleated epithelia with few leukocytes and cornified cell (x200).
Fig. 2. Estrus stage-Predominant cornified epithelia with absent leukocyte and nucleated epithelia (x200).
Fig. 3. Metestrus stage-Predominant leukocytes with few nucleated and cornified epithelia (x200).
Fig. 4. Diestrus stage-Predominant leukocytes with few irregulary shaped nucleated epithelia (x200).
Fig. 5. Oocyte-cumulus complex (OCC) in the ampullary portion of the oviduct (x100).
Fig. 6. Dispersed oocytes-cumulus complex (x100).

＝국문초록＝

마우스의 發精週期 및 排卵誘發에 關한 實驗

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人間의 體外受精 및 胚兒移植術과 비슷한 技法을 利用하여 妊娠馬血淸性腺刺戟호르몬으로 動物에서 排卵誘導가 施行되었으며 48時間의 間隔을 두고서 妊娠馬血淸性腺刺戟호르몬 및 人융모성性腺刺戟호르몬을 投與하면 14～19時間 後에 排卵이 있게 된다. 排卵되는 卵子의 個數는 마우스의 年齡, strain, 性腺刺戟호르몬의 投與用量 및 投與時期가 環境因子 等에 따라 左右된다.

本實驗은 本敎室에서위 體外受精 및 胚兒移植術에 對한 quality control 方法으로 施行中인 마우스 卵子의 體外受精實驗의 一環으로 마우스의 發精週期가 마우스 卵子 獲得에 미치는 影響을 檢討하기 爲하여 實施하였다. 마우스 膣細胞塗抹은 生理的 食鹽水로 洗滌하여 만든 후 Papanicolaou 染色法을 利用하여 製作하였으며 塗抹標本에서 細胞種類에 따라 마우스 發精週期를 決定하였다.

PMSG 5.0 IU 및 HCG 5.0 IU를 48時間 間隔으로 腹腔內 注入한 後, HCG 腹腔內 注入 16時間 以後에 卵子獲得을 實施하였다.

마우스의 發精週期에 따란 卵子獲得率은 發精前期(N＝16), 發精期(N＝24), 發精中間期(N＝16) 및 發精後期(N＝18)의 各 期에서 各各 14.9±2.6個, 15.8±3.4 個, 15.7±3.8 個 및 15.1±4.6 個로 이들 間에는 統計的으로 有意한 差異가 없었다. 마우스의 發精週期는 마우스 卵子獲得率에 影響을 미치지 않는 것을 觀察할 수 있었다.