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Original article

Superovulation at a specific stage of the estrous cycle determines the reproductive performance in mice

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ABSTRACT

Inconsistent reproductive performance has been reported in superovulated mice. Hence, the aim of this study was to analyze the effect and possible mechanism of superovulation timing on mouse reproductive performance. The results showed that mice superovulated at the metestrous (23.08 ± 6.08%) and diestrous stages (33.33 ± 11.45%) presented significantly lower pregnancy rates compared with those superovulated at the estrous stage (66.67 ± 9.20%). After superovulation at the proestrous and estrous stages, mucin 1 (MUC1) and *let-7a*/*let-7b* microRNA (miRNA) expression levels were significantly attenuated and enhanced on embryonic day 3.5 (E3.5), respectively, whereas no significant differences in the expression level were found in mice superovulated at the other two stages. A higher number of developing and Graafian follicles was observed in the ovarian sections 48 h after the administration of pregnant mare serum gonadotropin (PMSG) at the proestrous and estrous stages. The sections from mice treated at the metestrous and diestrous stages, however, presented more corpora lutea. Therefore, mice superovulated at the proestrous and estrous stages exhibited the best pregnancy rates. Furthermore, the disordered expression of MUC1 and *let-7a*/*let-7b* miRNA in mice superovulated at the metestrous and diestrous stages may impair reproduction performance.

1. Introduction

The administration of gonadotropins, *i.e.*, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), or pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) is commonly used to induce superovulation to increase the number of oocytes obtained in animals. Mice can be superovulated regardless of their natural estrous cycle stage [1,2] because the dosage of exogenous gonadotropins is sufficiently high to override the effect of endogenous hormones [3]. However, the oocytes obtained from mice induced by exogenous gonadotropins at different estrous cycle stages may display dissimilar characteristics. It has been found that higher percentages of cumulus-free oocytes, oocytes without polar bodies, or oocytes with intracytoplasmic mitochondrial aggregates and chromosome scattering are present in oocytes obtained from mice that had been administered PMSG at the metestrous stage compared with those obtained from the mice treated at the estrous, diestrous, and proestrous stages [4]. A clinical study reported that oocytes obtained from women with endometriosis are of poor quality and show an abnormal mitochondrial structure and low mitochondria mass, which could be directly responsible for problems related to fertilization and embryonic development in patients with endometriosis [5]. In short, the synchronization of the innate estrous cycle and administration of gonadotropins can optimize the quality of oocytes, which is an important factor for a successful pregnancy [4,5]. Hence, the timing of gonadotropin administration is critical. The administration of exogenous gonadotropins in mice should be synchronized with the innate estrous cycle to obtain high-quality oocytes [4]. Additionally, the recovery rate of oocytes from superovulated mice is not very consistent. Occasionally, we have observed that very few or poor-quality oocytes are obtained.

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Furthermore, superovulated mice did not produce more offspring than non-superovulated mice (personal observation). Therefore, identifying the reason for these phenomena will provide valuable information.

In addition to influencing oocyte quality, superovulation may result in delayed or impaired implantation, pre- and post-implantation mortality, low fetal weight, and fetal growth retardation in mice [6,7]. It has been shown that PMSG and hCG administration may cause metabolic disturbance in the ovaries, which may be responsible for the increased prenatal mortality observed in superovulated mice [8]. Thus, although the exogenous administration of gonadotropins may increase the number of oocytes, which improves the overall chance for their further application, the negative effects of superovulation resulting in higher concentrations of steroids may disturb the milieu of the reproductive system, including the ovaries, oviducts, and uterus, and affect the embryos and the endometrium at the time of implantation [8].

Mucin 1 (MUC1), the first transmembrane glycoprotein in the mucin family to be identified, is a potential marker for endometrial receptivity. MUC1 is abundantly expressed on the apical surface of the luminal and glandular epithelia of the uterus during the non-receptive phase, which leads to the formation of the glycocalyx at the mucosa layer of the uterus [9–12], and it is believed to act as an anti-adhesion molecule, or a barrier to prevent the embryo from attaching to the endometrium [13,14]. In mice, MUC1 is lost on embryonic day 3.5 (E3.5) before blastocyst attachment, suggesting that the loss of MUC1 is a prerequisite for a receptive uterus [15]. In addition, the expression of MUC1 is regulated by estrogen and progesterone [16].

The *let-7* family of miRNAs regulates implantation during early pregnancy. *Let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7g*, and *let-7i* are upregulated at implantation sites in the mouse uterus, and *let-7a* is expressed in the glandular and luminal epithelia on E3.5 and shows a strong signal in the decidua on E5.5. In addition, *let-7b* expression in the mouse uterus is also upregulated from E0.5 (pre-receptive phase) to E3.5 (receptive phase) [17,18]. These studies indicate that the *let-7* family plays a critical role during embryo implantation.

Based on our observations and other documented reports regarding the inconsistent recovery rate of oocytes from superovulated mice, the aim of this study was to investigate the effect of superovulation timing on the production of oocytes and the associated responses of the ovary and uterus. In the present study, female CD1 mice were superovulated at each stage of the estrous cycle, and we assessed the effect of superovulation on pregnancy performance, fetal development, endometrial MUC1 expression, and *let-7a*/*let-7b* miRNA expression.

2. Materials and methods

2.1. Animals

All of the animals and the procedures for animal handling and treatment were approved by the Institutional Animal Use and Care Committee (IACUC) at the National Chung Hsing University (no. 99-83). Sexually mature CD1 mice at 8 weeks of age were purchased from BioLASCO Taiwan Co., Ltd., and maintained at 25 °C and 60% relative humidity for 1 week prior to the treatments. All of the mice were given the same reverse-osmosis-purified water and standard feed (MFG, Oriental Yeast Japan Co., Ltd.).

2.2. Determination of the estrus stages and superovulation

To collect cells from the vagina, a pipette tip with 20–50 μL of phosphate buffered saline (PBS) was gently inserted into the vagina of a restrained mouse, and cells were collected and suspended by gentle pipetting. The cells were transferred to pre-heated dry glass slides and smeared. The smeared slides were dried by heating and then stained with Wright-Giemsa stain (Sigma-Aldrich, St. Louis, MO, USA) for 30 s. The slides were rinsed with tap water and dried again before examination under a microscope. The stage of the estrus cycle was determined based on the presence or absence of leukocytes, cornified epithelial cells, and nucleated epithelial cells. After determining the stage, the mice were treated with 10 IU PMSG (Sergona X09; China Chemical & Pharmaceutical Co., Ltd., Taipei, Taiwan) and 10 IU hCG (Gona-500X08; China Chemical & Pharmaceutical Co., Ltd., Taipei, Taiwan) by intraperitoneal injection at 48-h intervals. After injection of hCG, all mice copulated with male mice. Mice with a vaginal plug in the following morning were designated as embryonic day 0.5 (E0.5). Only superovulated mice with a vaginal plug were used in the present study. The number of mice with a vaginal plug and mice were pregnant at each stage of the estrous cycle was recorded. The litter size of each pregnant mouse was also recorded, and the fetal weight gained at 2, 4, and 6 weeks was monitored.

2.3. Histological study of the ovaries

The stage of the estrous cycle in each mouse was determined by vaginal smear, as mentioned above. Mice at each stage of the estrous cycle (proestrus, estrus, metestrus, and diestrus) were treated with PMSG via intraperitoneal injection, and ovaries were collected for histological study 48 h after injection. The ovaries were fixed in 4% (v/v) formaldehyde and embedded in paraffin after serial dehydration in alcohol. The tissues were cut into 4-μm-thick slices using a microtome (Leica HM315R microtome, Leica Microsystems Inc., Bannockburn, IL, USA), which were stained with hematoxylin and eosin using standard procedures. The number of follicles and corpora lutea in three sectioned slides at the different estrous stages was counted under an upright microscope according to the previous study [19].

2.4. Immunohistochemistry of the uterine horns

Uterus samples obtained from female mice on E0.5 and E3.5 were fixed in 4% (v/v) formaldehyde and embedded in paraffin after serial dehydration in alcohol. The tissues were cut into 4-μm-thick slices using a microtome. For immunohistochemical staining, the slides were deparaffinized and immersed in boiling sodium citrate buffer (pH 6.0, 0.01 M) containing 0.05% (v/v) Tween-20 for 10 min to retrieve the antigen. After cooling at room temperature for 20 min, the slides were washed three times with PBS containing 0.1% (v/v) Tween-20 (PBS/Tween-20), and the endogenous peroxidase was removed with 3% (v/v) H_2O_2 for 10 min. After washing three times with PBS/Tween-20, the slides were blocked with SuperBlock Blocking Buffers (Thermo Scientific, Rockford, IL, USA) for 30 min at room temperature and then incubated with rabbit anti-MUC1 polyclonal antibody (GTX15481, GeneTex International Corporation, Hsinchu, Taiwan) diluted in blocking buffer (1:50 dilution) for 1 h at room temperature. After washing three times with PBS/Tween-20, the slides were incubated with goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) (KPL, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) diluted in blocking solution (1:200 dilution) for 30 min. Finally, after washing three times with deionized and distilled water, the slides were stained with 3,3′-diaminobenzidine (DAB, Invitrogen, Life Technologies, Grand Island, NY, USA) and counterstained with hematoxylin. Images were quantified using Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA).