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Variation with Age in the Numbers of Ovulated Ova and Follicles of Wistar-Imamichi Adult Rats Superovulated with eCG–hCG

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Abstract: *There are large variations with age in the number of ovulated ova found in superovulated female Wistar-Imamichi rats. In this study we investigated the numbers of ovulated ova and follicles with the aim of developing a superovulation technique that minimises variations. We also examined the number of non-atretic follicles in untreated rats aged 7–14 weeks, for each week of age. The numbers of 250–549 μm non-atretic follicles in untreated rats and the numbers of ovulated ova in superovulated rats both reached a peak at 12 weeks of age. The coefficients of variation for both follicle numbers and ova numbers changed with each week of age, reaching a maximum at 9 weeks of age and a minimum at 12 weeks of age. In order to achieve stable numbers of ova from superovulated rats, satisfactory results will be achieved using 12-week-old rats, minimising individual variations, with high numbers of ova.*

Key words: *follicle, ovulation, rat, superovulation, variation*

Introduction

Superovulation is an essential basic technique of reproductive biotechnology. It is used for the collection of large numbers of ova and embryos; however, individual and seasonal variations in the numbers of ovulated ova obtained are a limiting factor in reproductive research [9–11]. In addition, there are age-related changes in the number of small to medium-sized non-atretic follicles, but the number of large non-atretic follicles (i.e. Graafian follicles) is constant with age [5]. Superovulation treatment comprises equine chorionic gonadotropin (eCG) to induce small follicles to develop into large follicles, and human chorionic gonadotropin (hCG) to induce ovulation. Mukumoto *et al.* demonstrated that changing the method of administering eCG–hCG to rats improved the overall result of superovulation, but had no effect on the individual variation in the number of ova collected [8].

In the present study, we investigated whether there is a relationship between the number of ova released after superovulation treatment and the number of non-atretic follicles according to age, in order to elucidate the reason for the variation in the numbers of ovulated ova. Our goal was to develop a technique of stabilizing the results of superovulation treatment.

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Materials and Methods

Animals

Adult Wistar-Imamichi rats were obtained from the Institute for Animal Reproduction (Ibaraki, Japan) and maintained at 23 ± 2°C, with illumination from 07:00 to 21:00. The midpoint of the dark period (02:00) was defined as 'midnight (00:00) colony time' and all times are reported in relation to that time. Proestrus females were caged with males at a ratio of 1:1 and the pups were weaned at 30 days after birth, at which age 1–6 female rats were housed per cage (aluminum; 335 × 410 × 210 mm [W × D × H]) with free access to food (Labo MR Stock, Nosan Co., Ltd, Yokohama, Japan) and water to prevent any nutritional effects on follicular growth [2]. Female rats are generally considered to reach sexual maturity at 50–80 days of age (7–11 weeks). Vaginal smears were taken daily, before 09:00 colony time, for at least two consecutive normal 4-day estrous cycles.

This study was approved by the Yamaguchi University Ethics Committee for the Care and Use of Laboratory Animals for Biomedical Research.

Number of non-atretic follicles

At each age group in weeks between 7 and 14 week of age, untreated rats (n=10) were sacrificed at 09:00 colony time on the day of metestrus. The ovaries were removed, fixed in Lavdovsky's solution, dehydrated and embedded in paraffin wax for serial sectioning at 10 μm thickness and staining with hematoxylin-eosin. All sections were examined and the diameter of each follicle was measured with a micrometer. We counted only follicles measuring 250–549 μm because those that are greater than 550 μm are considered to have ovulated [7], whereas with superovulation, 250–549 μm non-atretic follicles will ovulate [4].

In follicles containing oocytes, the average of the major and minor axes was regarded as the follicle size. Follicles with advanced atresia were excluded, and non-atretic follicles were differentiated from atretic follicles according to the criteria of Braw and Tsafriri [1], as follows. Non-atresia: follicles containing oocytes in the resting stage of prophase (dictyate) with neither pyknotic nuclei in the granulosa cells nor cell debris in the antrum; Stage I atresia (early stage): follicles containing oocytes in the dictyate stage with pyknotic nuclei in the granulosa cells and cell debris in the follicular fluid; Stage II atresia (advanced stage): most oocytes showing meiosis-like changes, such as chromosomes in metaphase or a polar body, with pyknotic nuclei in the granulosa cells but no cell debris in the antrum; Stage III atresia (more advanced stage): fragmented or pseudocleaved oocytes from which and most granulosa cells have disappeared.

Number of ovulated ova after superovulation

Female rats aged 7–14 weeks that had undergone at least two normal 4-day estrous cycles by the start of the study were induced to superovulate using Kagabu's method [3], which comprises injection into the femoral muscle (at 09:00 colony time on the day of metestrus) of eCG (Serotropin, Teikokuzoki, Tokyo, Japan) at 40 IU/rat, followed by an intraperitoneal injection (at 12:00 colony time on the day of proestrus) of sodium pentobarbital (Nembutal, Abbott, North Chicago, IL, USA) at 4 mg/kg body weight, and 23 h later an intramuscular injection of hCG (Gonatropin, Teikokuzoki, Tokyo, Japan) at 40 IU/rat. At 20 h after the injection of hCG, the rats were sacrificed, and the ova were collected by dissecting out the ovaries and oviducts and flushing the oviducts with saline containing 0.05% hyaluronidase.

The ovulated ova were counted and checked for morphological normality under a phase-contrast microscope.

Statistical analysis

Differences between the treated and control groups were analyzed by chi-square test. Values of $P<0.01$ and $P<0.05$ were chosen as indication of statistical significance.

Results

In the superovulated rats there were nearly 20 ovulated ova per rat between the ages of 7–9 weeks, and the number of ovulated ova increased to 75.5 and 101.1 ova per rat at 10 and 12 weeks of age respectively (Fig. 1). Of the collected ova, 97.1% were morphologically normal. The coefficient of variation in the number of ova collected was greater than 60% at both 8 and 9 weeks of age, and over 30% in rats aged 10 and 11 weeks (Fig. 2).

In the untreated rats the number of non-atretic follicles ranging from 250 to 549 μm in mean diameter

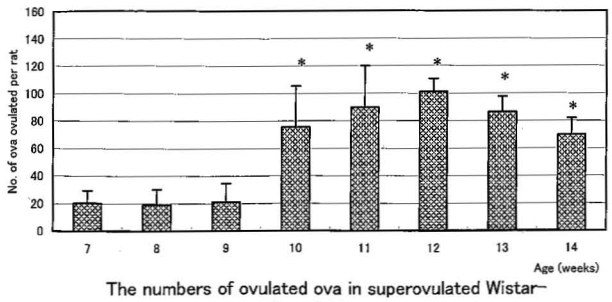

Fig. 1. The numbers of ovulated ova collected from superovulated Wistar-Imamichi rats (n=10 for each week of age). Data are means ± SD. *Significant difference with 7, 8, and 9 weeks of age ($P<0.05$).

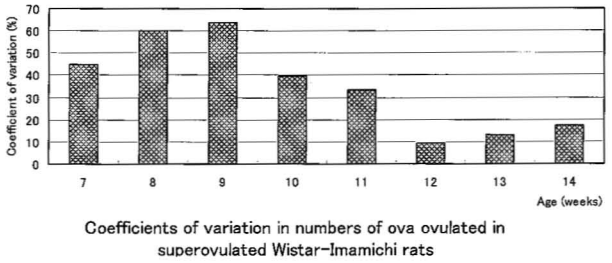

Fig. 2. Coefficients of variation in the numbers of ovulated ova collected from superovulated Wistar-Imamichi rats (n=10 for each week of age). Collated from non-atretic follicles of mean diameter 250–549 μm.

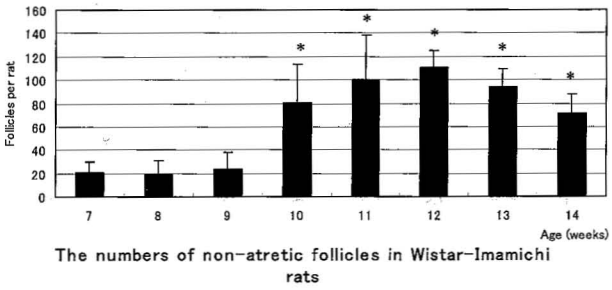

Fig. 3. The numbers of non-atretic follicles in Wistar-Imamichi rats (n=10 for each week of age). Data are means ± SD. Collated from non-atretic follicles of mean diameter 250–549 μm. *Significant difference with 7, 8, and 9 weeks of age ($P<0.05$).

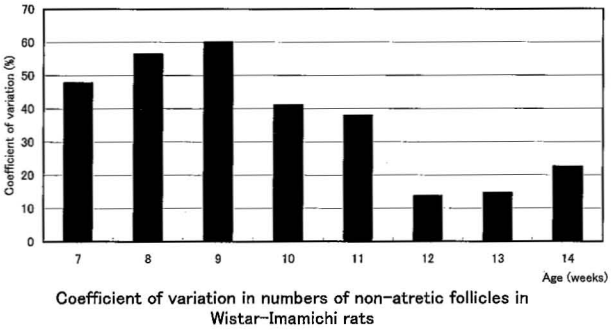

Fig. 4. Coefficients of variation in the numbers of non-atretic follicles in Wistar-Imamichi rats (n=10 for each week of age). Data are means ± SD. Collated from non-atretic follicles of mean diameter 250–549 μm.

was nearly 20 per rat at 7–9 weeks of age, increasing to 80.7 and 110.2 at 10 and 12 weeks of age respectively (Fig. 3). The coefficient of variation of the number of non-atretic follicles was greater than 40% between 7 and 10 weeks of age, and was 13.7% at 12 weeks of age, and 14.7% at 13 weeks of age.

The coefficients of variation for both the number of non-atretic follicles and the number of ovulated ova varied markedly with age. For the number of non-atretic follicles, it ranged between 39.7 and 60.0% for the ages 7–11 weeks; the highest coefficient of variation was 60.0% at 9 weeks and there was a low coefficient of variation of 13.7% at 12 weeks of age.

Discussion

The demand for ova is high in the field of reproductive engineering and although large numbers can be obtained through the use of superovulation treatments, there are also large individual variations in the number of ova collected. A number of possible causes exist for this variation between animals, and we investigated the relationship between the number of follicles and the number of ovulated ova, ignoring the seasonal influence on the results of superovulation, which we considered would be unlikely in laboratory animals housed in artificial conditions. We assumed that the number of ovulated ova would be more strongly influenced by the number of large follicles, so we studied the changes with age (in weeks) in the number of non-atretic follicles of a size thought to have developed in response to eCG. Our results are similar to those from an earlier study by Kagabu [5], showing that the number of non-atretic follicles of 250–549 μm diameter, the size thought to be involved in ovulation, varies dramatically with age.

In a recent study, eCG/hCG treatment induced superovulation at any stage of the estrous cycle in Wistar-Imamichi rats aged 12 weeks and the coefficient of variation for the number of ova was reported as 14.5–47.9% [6]. In contrast, in the present study the coefficient of variation for the number of ovulated ova ranged from greater than 30% between 7 and 11 weeks of age to greater than 60% at 9 weeks of age. The lowest coefficient of variation was 9.3% at 12 weeks of age. Similar patterns for the coefficient of variation for the number of non-atretic follicles and for the number of ovulated ova strongly suggest a correlation between the two.

Within the age range for sexual maturity of female rats (7–11 weeks) individual variations in the number of ovulated ova and non-atretic follicles were maximal at 9 weeks of age and the mean coefficient of variation for this period was also high, 63.5%.

Our results indicate a strong correlation between the number of non-atretic follicles and for the number of ovulated ova, which tended to be highest at 12 weeks of age. Therefore, to minimize individual variation and obtain consistently high numbers of ova, we recommend using 12 week old rats for superovulation treatment.

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