Estrous Stage- and Animal Age-Independent Superovulation in the BrlHan:WIST@Jcl(GALAS) Rat

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Abstract: In most strains of rats, the effects of treatment for the induction of superovulation show major strain differences and are strongly influenced by the stage of the estrous cycle. This study demonstrated, however, that superovulation was easily induced in Wistar strain Brl Han:WIST@Jcl(GALAS) rats by PMSG and hCG administration. To confirm the effects of such treatment, we studied age differences in egg collection efficiency. After superovulation was induced by intraperitoneal administration of 150 IU/kg PMSG and 75 IU/kg hCG given 48 h apart, the mean numbers of oocytes obtained from rats at 4, 8, 12, 20 and 28 weeks of age were 38.9, 33.5, 46.1, 26.9 and 21.3, respectively. No differences caused by the estrous stage at the PMSG administration were observed. In an embryo transfer experiment, fertilized eggs obtained from superovulated rats at each week of age showed equivalent viability until full-term to those from untreated rats. These results suggest that estrous stage-independent superovulation is effective not only in the pre-pubertal stage but also in adult rats.

Key words: estrous cycle, hCG, PMSG, superovulation

Introduction

In mice, superovulation is conventionally induced by combined administration of pregnant mare serum gonadotrophin (PMSG) and human chorionic hormone (hCG), and is used to obtain a large number of eggs at one time. Such eggs can be used not only as experimental materials for development of reproductive technology (e.g. IVF, IVC and cryopreservation), but also as a source for production of transgenic or knock-in/-out mice. In rats, on the other hand, the effects of treatment for superovulation show major strain differences [3, 12]. This could be one of the reasons for the delay in practical application of reproductive biotechnology techniques in rats. Since rats are the most widely used experimental animals next to mice and numerous experimental data using rats have been accumulated in research fields such as medicine, pharmacology, physiology, nutrition, psychology and genetics, there is an urgent demand for the application of reproductive technology in rats.

In most strains of rats, the effect of treatment for superovulation is strongly influenced by the stage of the estrous cycle [5]. There are two main protocols to induce superovulation. One is to emphasize efficacy of endogenous gonadotrophins, in which rats at the diestrus stage are selected to initiate exogenous hormonal
treatment (PMSG administration), or in which gonadotrophins are gradually stimulated by constant infusion of purified FSH with a short half-life via a subcutaneously or intraperitoneally implanted osmotic mini-pump [1, 5, 11]. However, the superovulatory effect is influenced by rat strain in the above-mentioned cases. The other protocol involves the use of immature rats at 4 to 6 weeks of age [1, 4, 8, 11, 13, 14], which are not regulated by endogenous hormones since rats begin to exhibit stable estrous cycles with a defined 4-day period at around 8 weeks of age. However, the number of collected fertilized eggs is low because immature female rats generally won’t mate with adult males [2]. Accordingly, it is difficult to say that the superovulation procedure in rats has been established at present.

Wistar-Imamichi rats are easily superovulated independently of the estrous cycle [7], although it is not clear whether superovulation can be induced in other Wistar strain rats by PMSG and hCG administration. Therefore, to confirm the effects of such treatment on Wistar strain Brl Han:WIST@Jcl(GALAS) rats, closed colony rats often used for safety tests in toxicology and pharmacology, we studied age differences in egg collection efficiency and dependence on the estrous cycle.

**Materials and Methods**

**Animals**

BrlHan:WIST@Jcl(GALAS) (Han:Wistar) female rats (CLEA Japan Inc., Tokyo, Japan) at 4, 8, 12, 20, and 28 weeks old and males at more than 12 weeks of age were used. They were maintained in a room at a controlled temperature of 22 ± 2°C, humidity of 55 ± 5% with lights on at 6:00 and off at 20:00, and were given commercial diet (CA-1, CLEA Japan Inc., Tokyo, Japan) and tap water ad libitum.

**Superovulation**

Female rats were randomly selected for the superovulation procedure. Superovulation was induced by intraperitoneal administration of 150 IU/kg PMSG (Serotropin, Teikoku-zoki Ltd., Japan) and 75 IU/kg hCG (Gonatropin, Teikoku-zoki Ltd., Japan) given 48 h apart (at 12:00 to 14:00) to the females as reported previously [9]. To collect fertilized eggs at the pronucleus stage, some of the rats were mated with males of the same strain after hCG administration.

**Collection of eggs**

At 24 to 26 h after hCG administration, ovulated oocytes or pronuclear-stage zygotes were collected from oviductal ampullae of the female rats by puncturing with a 27G needle. They were placed in PBI medium [15] containing 300 units/ml hyaluronidase and cumulus cells were removed by pipetting. The oocytes/eggs obtained were transferred to PBI medium and their number and morphology were recorded. As a control, oocytes/eggs were collected from intact adult female rats (mated naturally) at 10 to 12 weeks of age.

**Confirmation of estrous cycle and copulation**

The estrous cycle of the rats at 8 and 12 weeks of age was confirmed on the day of PMSG administration and the day after hCG administration (day of ovulation). The estrous cycle was assessed in the morning (9:00–12:00 a.m.) by observing vaginal smears under an optical microscope. Copulation was confirmed by observing sperm present in the vaginal smears.

**Cryopreservation and Embryo transfer**

The viability of the fertilized eggs was assessed by embryo transfer. For experimental convenience, all eggs were cryopreserved immediately after collection. Cryopreservation was performed by a vitrification method as reported previously [10], but the procedure was partially modified. Eggs together with 5 µl of 1 M DMSO solution were transferred to cryotubes and cooled on crushed ice for 1 min. Then, 95 µl of DAP213 solution was added to the tubes. After further cooling on crushed ice for 1 min, the tubes were plunged into liquid nitrogen and stored before embryo transfer. Within 1 h after warming, the eggs were transferred to the oviducts of SD female rats on day 1.0 of pseudopregnancy, and fetal development was examined at days 18 to 20 of gestation.

**Statistical assessment**

Differences were analyzed by t-test, Cochran-Cox test or Chi-square test in comparisons between the treated and control groups. A value of P<0.05 was chosen as indication of statistical significance.

**Results**

The number of ovulated oocytes showed age differ-
The percentage of ovulating rats at 4 to 28 weeks of age ranged from 72.7 to 100%, with the lowest rate in the youngest group. The mean number of morphologically normal oocytes obtained from superovulated rats ranged from 21.3 to 46.1, with a tendency toward lower values in the two aged groups. All of these values from the superovulated rats were significantly higher than that from untreated controls (12.4 oocytes).

The estrous cycle of the rats at 8 and 12 weeks of age was confirmed on the day of PMSG administration and the day after hCG administration, and correlation with the number of ovulated oocytes was investigated (Table 2). Regardless of the stage of estrous cycle at the PMSG administration, all rats were in estrus 3 days after initiation of the hormone treatment. The numbers of ovulated oocytes obtained from 8-week-old rats ranged from 25.2 to 41.2, while those from 12-week-old rats ranged from 41.0 to 56.4. No correlations between estrous stage at the PMSG priming and superovulatory response were found although the value was highest in the rats in estrus.

The efficiency of collecting fertilized eggs from superovulated rats at various ages is shown (Table 3). The copulation rate of the rats ranged from 77.3 to 100%, with the lowest rate in the youngest group. The mean numbers of fertilized eggs ranged from 20.0 to 34.5, showing that significant superovulatory effects were found in the rats at 4 to 28 weeks of age when compared with untreated rats (11.5 zygotes). The percentages of fertilized eggs among total harvested eggs (=fertilization rate) in superovulated rats ranged from 70.8 to 80.3%, all of which were significantly lower than that of untreated rats (93.5%).

Fertilized eggs at the pronuclear stage were used in an embryo transfer experiment after cryopreservation for experimental convenience (Table 4). The morphological survival of post-thawed zygotes was high in all the superovulated groups (93.8 to 98.4%), and develop-
mental competence of the zygotes in the recipient females (implantation rates 70.8–82.0%, fetus rates 56.9–70.5%) was quite comparable with those of the control group (68.7 and 59.7%, respectively), except for a significant difference between 8-week-old and control groups in the implantation rate (no biological significance found).

**Discussion**

The present study clearly showed that, in the same way as the mouse, Han:Wistar rats can be successfully superovulated by a simple, single intraperitoneal PMSG/hCG administration procedure, and the effect of superovulation is inducible in rats at 4 to 28 weeks of age. Furthermore, in the rats at 8 and 12 weeks of age, the effect was not dependent on the estrous cycle unlike most other rat strains. These results confirmed that Han:Wistar is an unique rat strain in which superovulation can be effectively induced not only in the pre-pubertal stage but also in adulthood.

Although the doses of PMSG and hCG were based on IU/kg body weight in order to ensure an accurate dosage of hormones for each rat, the efficiency of superovulation showed some differences in rats at each week of age. The highest number of oocytes was obtained from rats at 12 weeks of age, and the proportion of non-ovulating rats was high at 4 weeks of age as compared with the others, although the number of oocytes collected was relatively high. This result is probably caused by age difference of sensitivities to exogenous hormones. In this study, the doses of injected PMSG and hCG were uniformly 150 and 75 IU/kg body weight, respectively, while it was previously reported that, using SD strain rats in the pre-pubertal stage, the effect of superovulation was the highest when both PMSG and hCG were injected at the dose of 300 IU/kg body weight [6]. According to that report, the superovulatory response should be improved by adjustment of the dose of injected gonadotrophins. In the present study, the number of ovulated oocytes decreased after 20 weeks of age, suggesting that sensitivity to ex-

### Table 3. Efficiency of collecting fertilized eggs from superovulated Han:Wistar rats

<table>
<thead>
<tr>
<th>Weeks of age</th>
<th>No. of rats</th>
<th>Mean no. ± S.D. of oocytes morphologically normal</th>
<th>Mean no. ± S.D. of eggs fertilized</th>
<th>Percentage ± S.D. of eggs fertilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 2</td>
<td>21</td>
<td>12.3 ± 2.2</td>
<td>11.5 ± 3.4</td>
<td>93.5 ± 24.8</td>
</tr>
<tr>
<td>4</td>
<td>22</td>
<td>48.7 ± 34.1*</td>
<td>34.5 ± 28.4*</td>
<td>70.8 ± 37.9*</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>33.5 ± 25.1*</td>
<td>25.4 ± 19.2*</td>
<td>75.8 ± 35.9*</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>46.8 ± 22.0*</td>
<td>33.4 ± 22.0*</td>
<td>71.2 ± 30.1*</td>
</tr>
<tr>
<td>20</td>
<td>16</td>
<td>29.9 ± 12.1*</td>
<td>23.4 ± 13.5*</td>
<td>78.3 ± 27.4*</td>
</tr>
<tr>
<td>28</td>
<td>16</td>
<td>24.9 ± 14.3*</td>
<td>20.0 ± 12.6*</td>
<td>80.3 ± 27.8*</td>
</tr>
</tbody>
</table>

1) Values calculated from the copulated rats. 2) Untreated rats at 10 to 12 weeks of age. * Significant difference from the control (P<0.05).

### Table 4. Viability of vitrified eggs obtained from superovulated Han:Wistar rats

<table>
<thead>
<tr>
<th>Weeks of age</th>
<th>No. of fertilized eggs (%)</th>
<th>Vitrified</th>
<th>Survived (=transferred)</th>
<th>Implanted</th>
<th>Developed to fetus 1, 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 3</td>
<td>75</td>
<td>67 (89.3)</td>
<td>46 (68.7)</td>
<td>40 (59.7)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>69</td>
<td>66 (95.7)</td>
<td>47 (71.2)</td>
<td>41 (62.1)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>65</td>
<td>61 (93.8)</td>
<td>50 (82.0)*</td>
<td>43 (70.5)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>61</td>
<td>60 (98.4)</td>
<td>44 (73.3)</td>
<td>38 (63.3)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>65 (94.2)</td>
<td>46 (70.8)</td>
<td>37 (56.9)</td>
<td></td>
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<tr>
<td>28</td>
<td>64</td>
<td>61 (95.3)</td>
<td>47 (77.0)</td>
<td>41 (67.2)</td>
<td></td>
</tr>
</tbody>
</table>

1) Percentages calculated from the transferred eggs. 2) Examined at days 18 to 21 of gestation. 3) Untreated rats at 10 to 12 weeks of age. * Significant difference from the control (P<0.05).
ogenous hormones tends to decrease, as rats grow older.

Fertilized eggs were effectively collected from superovulated rats at 4 to 28 weeks of age, while the copulation rate of immature rats at 4 weeks of age was significantly lower than that at more than 8 weeks of age, as previously reported [2]. One possible reason is that mating behavior is obstructed by differences of body size between female rats at 4 weeks of age and adult male rats. Besides, it is assumed that the disparity of the copulation rate is related to the sensitivity to exogenous hormone since the number of 4-week-old rats with induced ovulation was relatively low.

The proportion of fertilized eggs in superovulated rats was low overall compared with naturally mated rats (see Table 3), as reported previously [14]. Details of this phenomenon are unclear, but one possible reason is that the environment in the female reproductive organs is modified by administration of exogenous gonadotrophins. It appears that the difference of reproductive activity in each male rat was amplified as a result of this modification.

In rats, it is known that the effect of superovulation treatment is strongly influenced by the stage of the estrous cycle. Nevertheless, Wistar-Imamichi rats are also easily superovulated, independent of the estrous cycle [7]. Since both Wistar-Imamichi and Han:Wistar rats strains carry a similar genetic background to Wistar strain rats, it is assumed that some strains of Wistar rat have a high sensitivity to superovulation by administration of exogenous gonadotrophins. Although the details are unclear, it hints at how ovulation efficiency in rats with poor superovulatory response might be improved. Fertilized eggs obtained from superovulated rats at various ages showed a high developmental potential to term, as do those from non-treated rats. Thus, Han:Wistar rats are expected to be valuable tools for improvement of techniques in reproductive biotechnology and a useful source for production of genetically manipulated rats.

**Acknowledgments**

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