Factors Influencing the Number of Eggs Recovered from Rabbits Superovulated with FSH or PMSG: Analysis of Five Years of Data from 509 Rabbits

by ***Shuji Kitajima[1,*], Tatsuhiro Maeda[1], Enqi Liu[1,2], Kazutoshi Nishijima[1], Masatoshi Morimoto[1], Teruo Watanabe[1] & Jianglin Fan[3]***

[1]Analytical Research Center for Experimental Sciences, Saga University, Saga, Japan

[2]Laboratory Animal Center, Xi'an University School of Medicine, Shaanxi, China

[3]Department of Molecular Pathology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan

Summary

To determine the best conditions for superovulation in rabbits, we analyzed the influence of age, season and hormone treatment on the numbers of eggs collected over five years from 509 rabbits aged 4–10 months using follicular stimulating hormone (FSH) or pregnant mare serum gonadotropin (PMSG) hormone stimulation. The number of eggs recovered was significantly higher in younger rabbits in both treated groups ($P < 0.01–0.05$). The number of eggs collected from rabbits treated with FSH were significantly higher than from rabbits treated with PMSG at all ages ($P < 0.01$). Seasonal differences were not observed in either hormone treatment group as they were maintained under constant temperature, humidity and light cycle through the year. Thus, younger rabbits are more sensitive to hormonal superovulation treatment with both FSH and PMSG, and FSH offers a better regimen for egg collection.

Introduction

Rabbits have several advantages over other laboratory animals as they have a unique lipid metabolism similar to that of humans (*Fan & Watanabe, 2003*). Transgenic rabbits have been used as animal models of genetic and acquired diseases, especially in the fields of lipid metabolism, atherosclerosis, obesity and inflammation (*Fan et al., 2001; Kitajima et al., 2004; Wang et al., 2004*), and as bioreactors for producing recombinant proteins (*Fan & Watanabe, 2003; Foote & Carney, 2000; Hiripi et al., 2003*). To generate transgenic rabbits, superovulation is an important technique needed before one can move to gene injection by microinjection and subsequent embryo transfer. However, the efficiency of generating transgenic rabbits by microinjection is still low (*Fan & Watanabe, 2003; Murakami et al., 2002*) and we require many embryos. Therefore, efficient superovulation techniques will help to reduce the costs in producing transgenic rabbits. In rabbits, both follicular stimulating hormone (FSH) and pregnant mare serum gonadotropin (PMSG) have been used for superovulation (*Cheng et al., 1999; Fan et al., 1999; Hashimoto et al., 2004; Kauffman et al., 1998; Mehaisen et al., 2006; Naik et al., 2005; Treloar et al., 1997*). Efficient egg recovery from rabbits might be influenced by several factors such as the strain, age, season and hormone stimulation regimen. In our laboratory, we have superovulated more than 500 rabbits by FSH or PMSG for producing transgenic rabbits over the past five years. Here we have analyzed the influence of age, seasons and hormones on the efficacy of collection based on these data.

**Correspondence:* Dr S. Kitajima, DVM, PhD
Analytical Research Center for Experimental Sciences, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan
Tel +81-952-34-2431
Fax +81-952-34-2024
E-mail kitajims@cc.saga-u.ac.jp

Materials and Methods

Animals

Japanese White (JW) rabbits were used in this study, obtained from Japan SLC Inc., Shizuoka, Japan. They were housed individually in metal or glass fiber cages in a room maintained at constant temperature (24+/- 2 ˚C), humidity (55 +/- 15%) and light cycle (light on 08:00 to 20:00). They were fed commercial pellets (CRB-1, CLEA Japan Inc., Tokyo, Japan) at 120 g per day and given free access to water. The experimental protocols were approved by the Saga University Animal Care and Use Committee and performed according to the Saga University Guidelines for Animal Experimentation.

Superovulation

To induce superovulation, rabbits were treated with FSH (Kawasaki Pharmaceutical Co. Ltd., Kanagawa, Japan) or PMSG (Teikoku Hormone MFG Co. Ltd., Tokyo, Japan). In the FSH protocol, the rabbits were injected six times with 0.5 AU of FSH subcutaneously at intervals of 12 h (total 3.0 AU) (*Cheng et al., 1999; Hashimoto et al., 2004*). These rabbits were mated on the afternoon following the last injection. In the PMSG protocol, the rabbits were given a single intramuscular injection of 150 IU PMSG (*Fan et al., 1999*) and they were mated three days later. For mating, each female rabbit was mated with two bucks of confirmed fertility and was then immediately injected intravenously with 100 U of human chorionic gonadotropin (hCG) (Teikoku Hormone MFG Co. Ltd., Tokyo, Japan).

Egg recovery

For egg collection, rabbits were euthanized with an overdose of thiamylal sodium (Kyorin Pharmaceutical Co. Ltd., Tokyo, Japan) 17–20 h after mating. The oviducts were removed and each was flushed with 5 mL of M2 medium. The number of all eggs, which contains both of fertilized and unfertilized, recovered from each rabbit was counted. Then, the eggs to M199 medium containing 20% fetal bovine serum (FBS) and cultured in an incubator at 37.0 to 38.5 °C under 5% CO_2 in air until used for microinjection.

Statistical analysis

All data are expressed as the mean ± SEM. Analysis of variance (ANOVA) was performed for multiple comparisons of ages and seasons. The Tukey HSD post hoc test was applied when significant differences were observed between treatment groups by ANOVA. For comparing hormone treatments, Student's *t*-tests or Welch's *t*-tests were applied according to the *F* value. Significance was assumed at $P < 0.05$.

Results

Influence of age on the numbers of eggs recovered

To increase the reliability of the results, the analysis was restricted to animals aged 4–10 months, used only for egg collection: 232 were treated with FSH and 277 were treated with PMSG. First, we analyzed the influence of age and season on the numbers of eggs recovered by two-factor ANOVA in each of the FSH and PMSG treatment groups. Data were grouped by rabbit age: 4–5, 6–7 and 8-10 months. Seasons of treatment were divided into four groups: I from January to March, II from April to June, III from July to September and IV from October to December. The results are shown in Figure 1. There was a significant difference in age, but not in season, in both the FSH and PMSG groups. We did not find any interaction of age and season on the number of eggs recovered in either of the hormone treatment groups. In both groups, significantly more eggs were collected from the 4–5 month old rabbits than in the 6–7 and 8–10 month old groups ($P <$ 0.01–0.05).

Next, we analyzed the influence of age on the numbers of eggs recovered in both FSH and PMSG groups. The results are shown in Figure 2. The numbers of eggs recovered tended to decrease with age in both hormone treatment groups. For the 4-month-old rabbits, there were 51.4 ± 3.1 eggs recovered in the FSH group and 35.5 ± 3.0 eggs in the PMSG group. Among the FSH-treated age groups, there were significant differences between the 4 and 6 month ($P < 0.01$), 4 and 7 month ($P < 0.05$), 4 and 8 month ($P < 0.05$), 4 and 10 month ($P < 0.05$), and 5

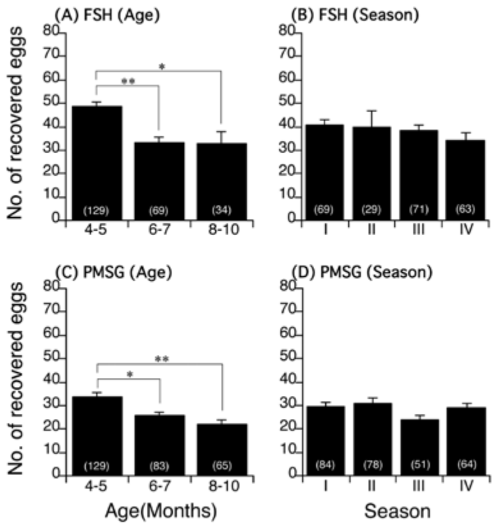

Figure 1. Effects of donor age and seasons on the number of eggs recovered in FSH- or PMSG-treated rabbits. *$P < 0.05$; **$P < 0.01$. The number of rabbits in each group are shown in brackets. The four seasons of treatment were: I, January to March; II, April to June; III, July to September and IV, October to December.

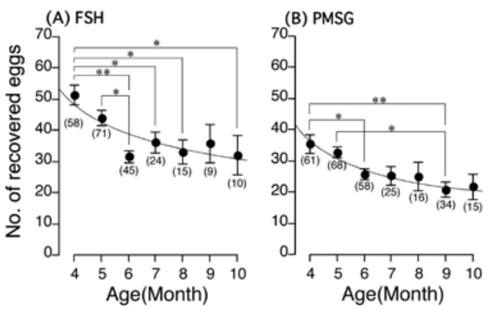

Figure 2. Effects of age on the number of eggs recovered in FSH or PMSG-treated rabbits.*$P < 0.05$; **$P < 0.01$. The number of rabbits in each group are shown in brackets.

and 6 month groups ($P < 0.05$). Among the PMSG-treated groups, there were significant differences between the 4 and 6 month ($P < 0.05$), 4 and 9 month ($P < 0.01$), and 5 and 9 month groups ($P < 0.05$).

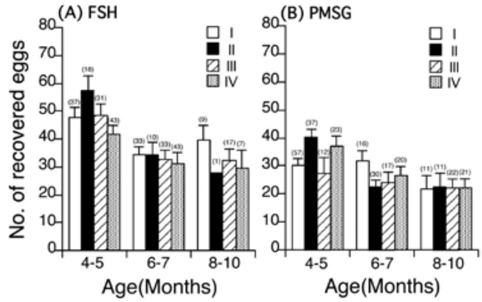

Figure 3. Effects of season on the number of eggs recovered in FSH or PMSG-treated rabbits. The number of rabbits in each group are shown in brackets. The four seasons of treatment were: I, January to March; II, April to June; III, July to September and IV, October to December.

Influence of season on the number of eggs recovered

Age obviously influenced the number of eggs recovered. Therefore, the analysis was repeated with the data divided into age groups of 4–5, 6–7 and 8–10 months to avoid any bias of the age distribution between the four seasons. However, we could not find any significant differences in the number of eggs recovered between seasons for any age group or hormone treatment by ANOVA (Fig. 3), except for the FSH treatment in the 8–10 month group. However, this did not contain enough data for analysis.

Influence of hormone treatment, FSH versus PMSG on the numbers of eggs recovered

Finally, we analyzed the influence of hormone treatment on the number of eggs recovered. We divided the rabbits into three groups aged 4–5, 6–7 and 8–10 months to avoid any bias of age distribution between the two hormone treated groups. The data are summarized in Table 1. There were significantly more eggs recovered from FSH-treated rabbits than from PMSG-treated rabbits at all ages ($P < 0.01$). The maximum number of eggs recovered was 133 from a 20-week-old rabbit treated with FSH and five rabbits each provided more than 100 eggs in

Table 1. Effects of FSH and PMSG superovulation on the numbers of eggs recovered from each age group

Age (months)	Hormone	*n*	No. of eggs recovered	*P*
4–5	FSH	129	47.3 ± 1.9	<0.01
	PMSG	129	33.9 ± 1.7	
6–7	FSH	69	33.2 ± 1.7	<0.01
	PMSG	83	25.6 ± 1.5	
8–10	FSH	34	33.5 ± 2.8	<0.01
	PMSG	65	22.0 ± 1.9	

Data are expressed as the mean ± SEM. Rabbits were divided into three age groups to avoid any bias of age distribution in the FSH and PMSG treatment groups. Donor age is an important factor affecting egg recovery following both FSH and PMSG hormone treatments (Figs 1, 2).

the FSH-treated group (age range 4–5 months). On the other hand, in the PMSG-treated group the maximum number of eggs recovered was 89 from an 18-week-old rabbit. We sometimes encountered non-responsive rabbits producing fewer than 10 eggs in spite of the superovulation treatment. There were significantly more non-responders in the PMSG-treated group (40/277, 14.4%) than in the FSH-treated group (4/232, 1.7%; $P < 0.01$).

Discussion

In this study, we analyzed several factors influencing the numbers of eggs recovered, such as age, season and hormone treatment, based on our five years of data. The most important factor influencing the number of eggs recovered was the age of the rabbits. No seasonal difference influencing recovery was observed in either of the FSH and PMSG-treated groups. Fischer & Meuser-Odenkirchen (*1988*) also reported little difference in rabbit fertility between seasons. We believe that this was because environmental factors such as temperature, humidity and the light-dark cycle were controlled throughout in our laboratory.

FSH treatment produced significantly more eggs than PMSG, similar to previous reports (*Mehaisen et al., 2006*). There were also significantly more non-responders in the PMSG-treated group than in the FSH-treated group (14.4% vs. 1.7%; $P < 0.01$). There are several protocols for PMSG superovulation in rabbits, using dosages ranging from approximately 25 IU to 200 IU per head (*Fan et al., 1999; Mehaisen et al., 2006; Peinado et al., 1995*). We do not know whether other protocols show similar frequencies of non-response to PMSG treatment, but it was 14% in our protocol using 150 IU/head of PMSG.

In terms of the quality of collected eggs, we counted the total numbers recovered in this study. These might have included unfertilized oocytes and abnormal and degenerated zygotes. We did not distinguish these in the records. However, the percentages of normally fertilized eggs suitable for microinjection were not significantly different: 64.4% in the FSH group and 68.4% in the PMSG group: (data not shown). This suggests that there little difference in the ratio of normally fertilized pronuclear stage embryos suitable for microinjection between FSH and PMSG treatment. However, PMSG treatment reportedly increased the incidence of chromosomal abnormalities (*Fujimoto et al., 1974*) and decreased the cell numbers constituting blastocysts (*Carney & Foote, 1990*). Therefore, for the generation of transgenic rabbits we should pay more attention not just to the total numbers of recovered eggs but also the results of embryo transfer after microinjection. From these observations, PMSG treatment seemed to have little advantage over FSH treatment except that administration simply involves a single intramuscular injection.

In terms of the relation between age and egg recovery in other laboratory animals, immature mice (*Sugiyama et al., 1992*) and rat (*Popova et al., 2002*) and prepubertal miniature pigs (*Shimatsu et al., 2000*) have also shown good results. In our analysis, the most eggs recovered were from rabbits aged 4 months old in both the FSH and PMSG treatments. We have not tried to collect eggs from JW rabbits younger than 16 weeks. However, Foote & Carney (*2000*) reported that maximum superovulation rates were not obtained until Dutch-belted female rabbits were 16 weeks old and New Zealand White rabbits were 20–24 weeks old. Moreover, in prepubertal rabbits the ovaries contain a few Graafian follicles that will ovulate when rabbits are 12 weeks old.

Both FSH and PMSG treatments increased the numbers of recovered eggs compared with natural ovulation. However, superovulation treatments can adversely affect the development of embryos *in vitro* and *in vivo*, and reduce the viability of cryopreserved embryos after de-vitrification (*Kauffman et al., 1998; Mehaisen et al., 2006*). Moreover, the developmental abilities of embryos derived from differently aged donors is not clear for rabbits. Therefore, we will need to examine the influence of the age of the donor on embryo development to establish the most efficient conditions for superovulation in rabbits.

In conclusion, our findings based on JW rabbits suggests that younger rabbits are the most sensitive to hormonal superovulation by both FSH and PMSG, and that FSH is more suitable for egg collection in conditions lacking seasonal influences.

Acknowledgments

The authors thank Y. Nakanishi for her technical assistance. This study was partly supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Numbers 16590281, 17500287 and 19500362).

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