ABSTRACT: The aim of the study was to compare ovarian response, embryo recovery rate and in vitro embryo growth in New Zealand White rabbits injected with different doses of PMSG. On day-26 post partum 39 primiparous non-lactating does were given i.m. the following treatments: Group I (n=13) 0.1 ml distilled water; Group II (n=13) 20 IU PMSG; Group III (n=13) 100 IU PMSG. Seventy-two hours later the animals received 0.8 μg GnRH and were artificially inseminated. Forty-eight hours after insemination, the does were sacrificed and the genital tracts were removed and kept in Dulbecco's Phosphate Buffer Saline. Ovaries were weighed and normal anovulated follicles >1mm (AF), haemorrhagic follicles (HF) and fresh corpora lutea (CL) were recorded. The oviducts were flushed with TC199 and embryos were morphologically evaluated. The zygotes were placed in TC199 + 5% Foetal Calf Serum and cultured in vitro in a humidified incubator at 39°C with 5% CO₂ in air. Embryo development was appraised after 24, 48 and 72 hours. Ovarian weights were not affected by treatments and there was no difference in the total number of AF+HF+CL among groups (Group I = 56.7 ± 6.2, Group II = 57.0 ± 7.1 and Group III = 56.6 ± 6.7). PMSG significantly increased the number of HF (P<0.01) as they were nearly double in Group II (9.3 ± 3.5) and four times as many in Group III (17.8 ± 3.3) compared with the control (4.3 ± 3.1). Embryo recovery rate was not influenced by treatment. In vitro embryo development rate was significantly lower (P<0.001) in the PMSG groups (Group II = 74.7% and Group III = 72.4% vs Group I = 93.2%).

INTRODUCTION

Good management of reproduction plays a key role in the profitability of intensive rabbit breeding where artificial insemination (AI) is widely practised. However, it is well known that female rabbits do not have a regular oestrous cycle and therefore it is not surprising that different methods are being employed to induce controlled sexual receptivity: management of light cycle, reduced or controlled access to nest box of lactating does, exogenous hormonal treatments. Among hormones the most commonly used is still PMSG (Pregnant Mare Serum Gonadotrophin) which is administered 2 to 3 days before insemination usually at dosages of 20 to 40 IU/animal (BOURDILLON et al., 1992; PAREZ, 1992; CECCHINI et al., 1992). Its overall effect on reproductive performance is still debated (see MAERTENS et al., 1995 for review). In fact, according to many authors (MAURER et al., 1968; CASTELLI et al., 1991; BONANNO et al., 1993), PMSG has been responsible for reduction in fertility and prolificacy which is particularly severe when high dosages are used or repeated treatments at frequent intervals are employed. This negative effect has been ascribed to an immunisation process (CANALI et al., 1991; BOITI et al., 1995), or impairment of ovarian function (KENNELLY and FOOTE, 1965), embryo transport (GREENWALD, 1961), or embryo development (CARNEY and FOOTE, 1990).

A single injection of PMSG at dosages from 40 to 150 IU has been frequently used for the induction of superovulation (ILLERA et al., 1990; SCHMIDT et al., 1992) for collection of embryos for in vitro studies.

The aim of this study was to assess ovarian response, embryo recovery rate and in vitro embryo development in rabbits treated with two different dosages of PMSG, a low one which is used for oestrous synchronisation under field conditions and a high one used for superovulation in research.

MATERIALS AND METHODS

To avoid interference between lactation and ovarian and pituitary functions (THEAU-CLEMENT and ROUSTAN, 1992), the present study was carried out on 39 primiparous non-lactating (day-26 post partum) New Zealand White does, weighing 3.8 ± 0.4 kg, 170 day old. The animals were housed in individual cages in a commercial rabbitry with controlled temperature (22-24°C), relative humidity (75%) and lighting cycles (14L:10D). They were fed ad libitum.
with a commercial pelleted diet, supplemented with mineral salts–vitamins, and had free access to water.

The animals were randomly assigned to three experimental groups receiving intramuscularly the following treatments: Group I (n=13) 0.1 ml of distilled water; Group II (n=13) 20 IU PMSG (Ciclogenina, SOLVAY VETERINARIA, Italy) and Group III (n=13) 100 IU PMSG. Seventy–two hours later, all animals were injected intramuscularly with 0.8 μg of GnRH (Receptal, HOECHST–ROUSSEL, Germany) to induce ovulation and were inseminated with 0.5 ml of pooled fresh semen (10–15x10^6 progressively motile spermatozoa/ml), in Tris buffer–Glucose extender supplemented with 20% egg-yolk, collected from two bucks of proven fertility.

Forty–eight hours post AI the does were sacrificed and the genital tracts, immediately removed, were placed in 50 ml conical tubes containing Dulbecco’s Phosphate Buffered Saline (GIBCO, Grand Island, NY, USA) at 25°C, supplemented with 2% antibiotics (200 IU of penicillin/ml – 200 μg of streptomycin/ml, SIGMA, St Louis, MO, USA) and transported to the laboratory within 2 hours.

Ovaries were dissected from the surrounding tissue and weighed; the number of normal anovulated follicles with external diameter greater than 1 mm (AF), fresh corpora lutea (CL), and anovulated haemorrhagic follicles (HF) was recorded.

Each isolated oviduct was flushed at room temperature with 1.0 ml of Tissue Culture Medium 199 (TCM199, GIBCO, Grand Island, NY, USA) supplemented with 5% inactivated Foetal Calf Serum (FCS, GIBCO, Grand Island, NY, USA) and 2% penicillin–streptomycin. Recovered embryos were morphologically evaluated for stage of development and assigned to a quality grade score (A=excellent, B=good, C= fair, D= degenerate or unfertilised oocytes) as outlined by LINDNER and WRIGHT (1983), using a Nikon TMS inverted microscope (10x). Embryo recovery rate (number of recovered embryos/number of CL) was calculated. Embryos of quality grades A and B were placed in multiwell plate in 0.5 ml TCM199 supplemented with 5% FCS, covered with 0.5 ml of sterile mineral oil (SIGMA, St Louis, MO, USA) and cultured in vitro in a humidified incubator at 39°C with 5% CO₂ in air. Embryo development rate was assessed after 24, 48 and 72 hours of in vitro culture.

In order to assess the ovarian function, progesterone was evaluated in blood samples withdrawn from the marginal ear vein and collected into heparinized tubes just prior to gonadotrophin administration, artificial insemination, and sacrifice. Plasma was stored at −20°C and then assayed by a specific and standardised RIA procedure (BOITT et al., 1974). The sensitivity of the assay for a sample volume of 200 μl was 0.08 ng/ml. The intra and interassay coefficient of variations were 5.3% (n=8) and 10.2% (n=4), respectively.

Data were analysed using General Linear Models, Frequency and Correlation procedures of the Statistical Analysis System Institute (SAS, 1991).

RESULTS

Plasma progesterone

The mean plasma progesterone concentrations were low in both control (0.94 ± 0.34 ng/ml) and PMSG treated groups (Group II = 0.90 ± 0.12 ng/ml and Group III = 0.91 ± 0.21 ng/ml) and was still at basal level (0.88 ± 0.32 vs 1.02 ± 0.15 and 0.94 ± 0.23 ng/ml, respectively) on the day of AI. Progesterone was higher two days later at embryo collection in both control (1.53 ± 0.47 ng/ml) and treated does (1.52 ± 0.32 and 1.48 ± 0.45 ng/ml, respectively).

Ovarian response

All does ovulated in response to GnRH based on the presence of fresh corpora lutea. Two does were eliminated from Group II because of purulent infection of the genital tract.

Ovarian weights (OW) were not significantly affected by treatment and were not correlated with the total number of the ovarian structures as previously defined (Table 1). However, in the PMSG treated groups the mean value of the OW was about 10–15% heavier than in the control animals.

When compared to the control group, the administration of 100 IU of PMSG nearly halved the number of anovulated normal follicles (P<0.01) while it did not increase the number of fresh corpora lutea.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ovarian Weight* (mg)</th>
<th>Anovulated Follicles (&gt;1 mm Ø)</th>
<th>Fresh Corpora Lutea</th>
<th>Haemorrhagic Follicles (&gt;1 mm Ø)</th>
<th>Total AF+CL+HF (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control n=13</td>
<td>796 ± 112</td>
<td>41.0 ± 5.9Aa</td>
<td>11.3 ± 1.1ab</td>
<td>4.3 ± 3.1B</td>
<td>56.7 ± 6.2</td>
</tr>
<tr>
<td>PMSG 20 IU n=11</td>
<td>914 ± 127</td>
<td>38.0 ± 6.9AAb</td>
<td>9.6 ± 1.2b</td>
<td>9.3 ± 3.5B</td>
<td>57.0 ± 7.1</td>
</tr>
<tr>
<td>PMSG 100 IU n=13</td>
<td>886 ± 82</td>
<td>23.1 ± 4.2Bb</td>
<td>15.7 ± 1.6a</td>
<td>17.8 ± 3.3A</td>
<td>56.6 ± 6.7</td>
</tr>
</tbody>
</table>

(*) = Total for both ovaries.
A,B : Values in the same column with different superscripts differ (P<0.05).
A,B : Values in the same column with different superscripts differ (P<0.01).
**Effect of PMSG on Rabbit Ovary and Embryo Growth**

Table 2: Total number of recovered embryos and their quality grades (A to D) evaluated at collection in control and PMSG groups (means±SEM)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total embryos n=13</th>
<th>n</th>
<th>Embryo quality grade* A-B n=11</th>
<th>n</th>
<th>Embryo quality grade* A-B n=11</th>
<th>n</th>
<th>C-D n=20</th>
<th>1.50±0.79</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>138</td>
<td>118</td>
<td>9.12±2.12</td>
<td>20</td>
<td>1.50±0.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMSG 20 IU</td>
<td>102</td>
<td>95</td>
<td>8.64±1.43</td>
<td>7</td>
<td>0.66±0.91</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMSG 100 IU</td>
<td>185</td>
<td>164</td>
<td>12.66±1.73</td>
<td>21</td>
<td>1.58±0.64</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(*) A = excellent; B = good; C = fair; D = degenerate and unfertilised oocytes

The number of haemorrhagic follicles in the low dosage PMSG treated group was nearly double and almost four times as much (P<0.01) in the high dosage PMSG as compared with controls (Table 1). Nevertheless, the total number of ovarian structures (AF+HF+CL) was not affected by gonadotrophin administration.

**Embryo recovery rate and in vitro development**

Data on embryos and their quality grades are summarised in Table 2. At collection, no significant differences were observed among the groups regarding the total number of recovered embryos and the number of A–B and C–D quality grade embryos.

The number of recovered embryos and unfertilised oocytes was highly correlated to the number of corpora lutea (r = 0.97; P<0.001) in all groups. Embryo recovery rate was not significantly affected by treatment (Group I = 0.92 ± 0.05; Group II = 0.89 ± 0.06; Group III = 0.86 ± 0.05).

After 24 hours of in vitro culture, the development was not significantly different among groups (Figure 1). However, the number of embryos (early morula–morula) of quality grades A and B reaching the blastocyst stages after 48 hours of in vitro culture (Table 3), was significantly lower (P<0.001) in the treated groups (74.7% and 72.4% in Groups II and III respectively, vs 93.2% of Control), whereas the number of embryos which became degenerate during in vitro culture (Figure 2) was significantly higher (P<0.001) in the low dosage PMSG treated group (n=17) than in the control (n=5). An high proportion (22.4%) of embryos recovered from the 100 IU PMSG treated animals remained at the morula stage and did not develop into blastocysts after 48 hours of in vitro culture (Figure 2).

After 72 hours in vitro culture we did not observe other embryos reaching the blastocyst stage and those embryos at morula stage showed signs of degeneration (Figure 3).

**DISCUSSION**

Blood basal progesterone levels were low throughout the experiment in treated and control groups and comparable with those observed in unstimulated does before ovulation (WATerson and MILLS, 1976), but lower than those found by TSUTSUMI et al. (1980) in 200 IU PMSG treated rabbits. In no case did we find progesterone concentrations higher than 2 ng/ml which have been reported to interfere with follicular growth (SETTY and MILLS, 1987; MILLS and STOPPER, 1989), reduce embryo recovery rate and increase the percentage of degenerated embryos (BOtti et al., 1996).

We did not observe the significant increase in ovarian weight which has been reported by KENNELLY and FOOTE (1965) and YOUNGLAI (1984) using 100–150 IU of PMSG or 0.5 mg of FSH.

The mean number of anovulated haemorrhagic follicles found in the ovary of does treated with PMSG increased especially when high dosage was employed. In a previous study (VERINI SUPPLIZI et al., 1994), we reported that the number of HF in 100 IU PMSG treated does was significantly reduced by the administration of monoclonal anti-PMSG. These findings suggest that the effect of PMSG is due to an overstimulation of ovarian follicles owing to its long half-life.

However, we can not exclude that 0.8 μg GnRH following 100 IU PMSG can be insufficient to trigger the ovulation of all stimulated follicles.

The mean number of recovered embryos in Groups I and II indicate the absence of a superovulatory effect.
induced by the injection of 20 IU of PMSG. By contrast, the high PMSG dose showed a superovulatory effect, as is already well known. We did not observe the engorgement of the reproductive tract nor find any difficulty in flushing oviducts as previously described by SCHMIDT et al. (1992) in does primed with a high dose of PMSG.

No difference in the gross appearance of recovered embryos between control and treated groups was observed on the day of collection. Nevertheless, the development rate of in vitro cultured embryos after 48 hours was significantly lower in PMSG groups than in the control (P<0.001) and an additional 24 hours of in vitro culture (Figure 3) did not increase the number of embryos reaching blastocyst stages, in contrast with the results obtained by ILLERA et al. (1990) using 120 IU of PMSG, and CARNEY and FOOTE (1990), using FSH superovulatory treatments. These discrepancies could be due to the different hormonal treatments used (dosages, association with monoclonal anti-PMSG), experimental protocol employed (time of embryo collection), and other conditions of in vitro culture. Our data (Figure 3) suggest that embryos recovered from PMSG treated does were less viable rather than delayed in developmental capacity compared with the control ones. FUJIMOTO et al. (1974) suggested that the low embryo development rate in 100 IU PMSG treated does could be due to the high incidence of chromosome abnormalities and the low mitotic index.

Moreover, exogenous gonadotrophins at superovulatory dosages often induce ovarian overstimulation resulting in abnormal follicular steroid production which causes severe environmental changes in the reproductive tract (FOOTE and ELLIGTON, 1988). Furthermore, this hormonal treatment

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Figure 1: Embryo development after 24 hours of in vitro culture (M=morula, B=blastocysts, D=degenerates)

Figure 2: Embryo development after 48 hours of in vitro culture (M=morula, B=blastocysts, EB=expanding blastocysts, D=degenerates)

IN SUMMARY, THIS STUDY SHOWED THAT LOW PMSG DOSAGE (20 IU) DID NOT INCREASE OVULATORY RESPONSE AND OVULATION RATE. AFFECT EMBRYO RECOVERY RATE AND EMBRYO QUALITY AT COLLECTION. OUR DATA ARE IN AGREEMENT WITH FIELD RESULTS CONCERNING LITTER SIZE AND FERTILITY, WHERE PMSG TREATMENTS (25 OR 35 IU) RESULTED EFFICIENT ONLY ON LACTATING FEMALES, WHEREAS IT WAS USELESS WHEN EMPLOYED ON NONLACTATING DOES (BOURLIDON ET AL., 1992; THEAUX-CLEMENT AND LEBAS, 1996).

THE DECREASED EMBRYO VIABILITY AND EMBRYO DEVELOPMENT IN PMSG-TREATED GROUPS AFTER 48 AND 72 HOURS OF IN VITRO CULTURE MAY BE DUE TO A NEGATIVE INFLUENCE ON OOCYTE MATURATION AND EARLY EMBRYO DEVELOPMENT INDUCED BY GONADOTROPHIN.

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