WORLD RABBIT SCIENCE 1997, VOL.5 (3), 143-148

EFFECTS OF DIFFERENT DOSES OF PMSG ON OVARIAN RESPONSE AND *IN VITRO* EMBRYO DEVELOPMENT IN RABBITS

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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 TCM199 and embryos were morphologically evaluated. The zygotes were placed in TCM199 + 5% Foetal Calf Serum and cultured *in vitro* in a humidified incubator at 39°C with 5% CO_2 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 HFs (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%).

RESUME : Effets de différentes doses de PMSG sur la réponse ovarienne et le développement* in vitro *des embryons chez la lapine.
Le but de cette étude est de comparer l'effet de plusieurs doses de PMSG injectées à des lapines de race Néo-zélandais Blanc, sur la réponse ovarienne, le taux d'embryons récupérés et leur croissance in vitro. *Trente neuf lapines primipares, non allaitantes, ont reçu le 26ème jour post partum une injection intramusculaire du traitement suivant : Lot 1 (n=13) 0,1 ml d'eau distillée ; Lot 2 (n=13) : 20 UI de PMSG ; Lot 3 (n=13) : 100 UI de PMSG. Soixante douze heures après, les lapines ont reçu 0,8 µg de GnRH et ont été artificiellement inséminées. Les lapines ont été sacrifiées 48 heures après l'insémination ; le tractus génital prélevé a été conservé dans la solution tampon phosphato-saline de Dulbecco. Les ovaires ont été pesés et les follicules normaux non ovulés > 1mm (AF), les follicules hémorragiques (HF) et les corps jaunes ont été comptés. Les oviductes ont été lavés avec du TCM199 et on a évalué la morphologie des embryons. Les zygotes ont été placés dans TCM199 + 5 % de sérum de veau et cultivés* in vitro *dans un incubateur humide à 39°C dans une atmosphères contenant 5 % de CO_2. Après 24, 48 et 72 heures, on a estimé le développement embryonnaire. Les traitements n'ont pas affecté le poids des ovaires et le nombre total de AF+HF+CL n'est pas différent selon les lots (Lot 1 : 56,7 ± 6,2 ; Lot 2 : 57,0 ± 7,1 ; Lot 3 : 56,6 ± 6,7). Le PMSG accroît significativement le nombre de follicules hémorragiques (P<0,01) qui ont presque doublé dans le lot 2 (9,3 ± 3,5) et quadruplé dans le lot 3 (17,8 ± 3,3) comparé au lot 1 (4,3 ± 3,1). Les traitements n'ont pas influencé le taux de récupération des embryons. Dans les lots ayant reçu de la PMSG, le taux de développement embryonnaire* in vitro *a été significativement bas (Lot 2 : 74,7 % ; Lot 3 : 72,4 %* vs *Lot 1 : 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; CASTELLINI *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 (Ciclogonina, 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% CO_2 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 (BOITI *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 1 : Ovarian response in the control and PMSG treated groups (means ± SEM)

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

(*) = 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).

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

Groups	Total embryos		Embryo quality grade*			
	n		n	A-B	n	C-D
Control n=13	138	10.62 ± 1.25	118	9.12 ± 2.12	20	1.50 ± 0.79
PMSG 20 IU n=11	102	9.29 ± 1.45	95	8.64 ± 1.43	7	0.66 ± 0.91
PMSG 100 IU n=13	185	14.25 ± 1.75	164	12.66 ± 1.73	21	1.58 ± 0.64

(*) 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).

Table 3. Number of grade A and B embryos reaching blastocyst stage after 48 hours of *in vitro* culture

Groups	*In vitro* embryo development		
	Embryos A-B n	Blastocysts n	(%)
Control n=13	118	110	93.2^{A}
PMSG 20 IU n=11	95	71	74.7^{B}
PMSG 100 IU n=13	116	84	72.4^{B}

^{A,B}: Values in the same column with different superscripts differ (P<0.001).

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 (BOITI *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 HFs 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

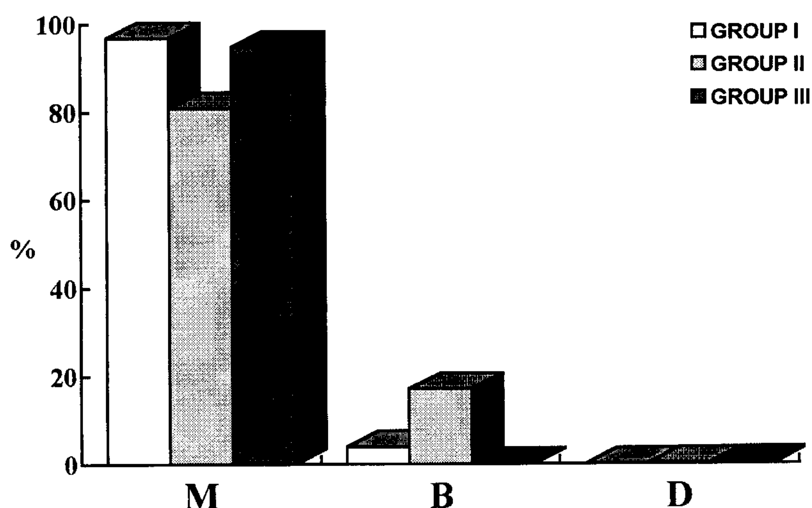

Figure 1. Embryo development after 24 hours of *in vitro* culture (M=morula, B=blastocysts, D=degenerates)

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

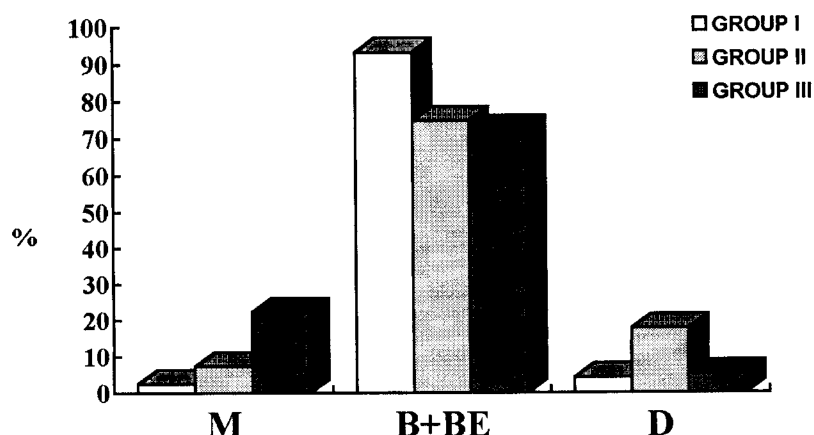

Figure 2 : Embryo development after 48 hours of *in vitro* culture (M=morula, B=blastocysts, EB=expanding blastocysts, D=degenerates)

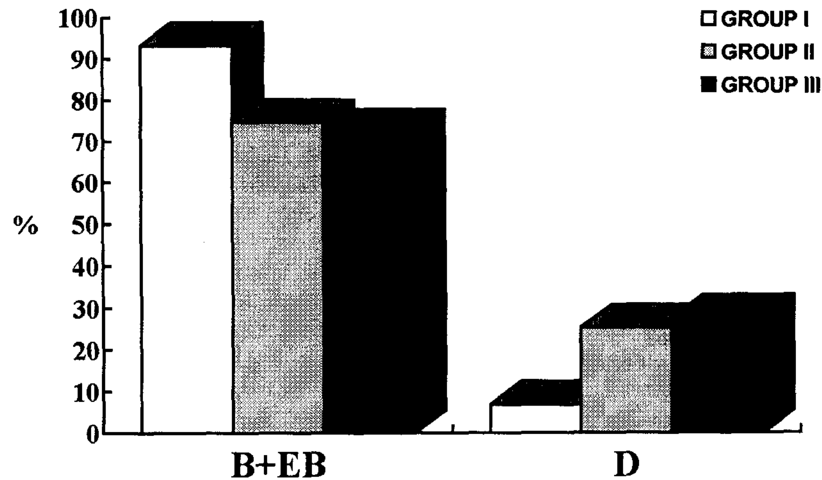

Figure 3. Embryo development after 72 hours of *in vitro* culture (B=blastocysts, EB=expanding blastocysts, D=degenerates)

alters follicular growth characterised by failure to ovulate (KENNELLY and FOOTE, 1965), ovulation of premature oocytes with deviant nuclear changes (HYTTEL *et al.*, 1991), interference with the ovum transport mechanisms (GREENWALD, 1961; TSUTSUMI *et al.*, 1980; BOURDAGE and HALBERT, 1988), and delayed zygote development (CARNEY and FOOTE, 1990). Superovulatory treatments may also affect oocyte maturation, fertilisation and embryo viability due to their effects on the reproductive tract as observed in cattle (HAWK, 1988) and in rabbit (TAKEDA *et al.*, 1978). However, the low levels of circulating progesterone observed in our study, the absence of embryos in the uterus 48 hours after AI, and the good quality of embryon on the day of collection induce us to exclude the disturbance of embryo transport as the cause of reduced *in vitro* embryo viability.

In summary, this study showed that low PMSG dosage (20 IU) did not increase ovulatory response and ovulation rate nor appear to 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 non lactating does (BOURDILLON *et al.,* 1992; THEAU-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.

Acknowledgements : This research was supported by a grant from MURST 40%.

Received :June 18th, 1997.
Accepted : October 3rd, 1997

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