Influence of day of oestrus on egg viability and comparative efficiency of *in vitro* fertilization in domestic cats in natural or gonadotrophin-induced oestrus

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Thirty-six domestic cats received 100 iu hCG (i.m.) on day 1, 2 or 3 of a natural, behavioural oestrus. Twenty-two anoestrous cats were injected with 150 iu pregnant mares' serum gonadotrophin (PMSG; i.m.) followed 84 h later by 100 iu hCG. Twenty-four to 26 h after hCG, all cats were examined laparoscopically to determine the number of ovarian follicles and to recover follicular eggs. Mature eggs were cultured with conspecific spermatozoa and examined 30 h later for cleavage. Within the natural oestrus group, cats on day 1 produced fewer ($P < 0.05$) follicles and total eggs than females on day 2 or 3, and 88.9% of the day 1 eggs were degenerate or immature and unsuitable for *in vitro* fertilization (IVF). Although only 54.5% of the cats in the PMSG/hCG group exhibited overt oestrus, mean ($\pm$ SEM) numbers of follicles (9.7 ± 0.8) and oocytes recovered (8.7 ± 0.8) were at least twofold greater ($P < 0.001$) than those measured in the natural oestrus group (3.7 ± 0.6 ; 3.4 ± 0.6 , respectively) or subgroups on day 2 (3.7 ± 0.4 ; 3.3 ± 0.4) and day 3 (5.7 ± 0.8 ; 5.3 ± 0.8). Overall, the proportion of eggs cleaving *in vitro* was similar ($P > 0.05$) between the natural oestrus group (48.3%) and the PMSG/hCG group (50.9%), but the latter group produced more than twice the number of embryos per donor. Embryo quality was unaffected ($P > 0.05$) by day of hormone treatment, and more than 80% of all two-cell embryos were rated good-to-excellent quality. In summary, there is a temporal relationship between day of sexual receptivity and follicular egg viability in the domestic cat: eggs on the first day of oestrus are not optimally responsive to an LH-like stimulus. There is also no evidence that PMSG/hCG treatment compromises egg quality or subsequent fertilizability *in vitro*. On the contrary, use of these gonadotrophins markedly improves overall IVF efficiency by increasing the total number of high quality embryos produced.

Introduction

One important element influencing fertilization and subsequent embryo development is the use and timing of exogenous gonadotrophins. For example, fertilization rate and resulting embryo quality are compromised in mated cats treated with commercial FSH (FSH-P; Burns-Biotec, Lincoln, NE) to induce oestrus (Goodrowe *et al.*, 1988a). For this reason, we have relied upon an alternative gonadotrophin, pregnant mares' serum gonadotrophin (PMSG), in developing a felid *in vitro* fertilization (IVF) system. When administered before hCG, PMSG stimulates the production of multiple ovarian follicles

containing eggs that readily cleave *in vitro* after insemination (Goodrowe *et al.*, 1988b; Johnston *et al.*, 1991a, b). However, egg viability appears closely related to the interval between administration of the two hormones; if the interval is too short or too long, egg quality and cleavage rate *in vitro* are markedly compromised (Goodrowe *et al.*, 1988b; Donoghue *et al.*, 1992).

The sensitivity of the domestic cat egg to the interval between the FSH-like and LH-like stimulus has been an incentive for considering other possible effects of exogenous gonadotrophins on IVF. It is well known that gonadotrophin therapies are associated with reduced fertilization rates *in vivo* and *in vitro* and poor embryonic development in mice (Maudlin and Fraser, 1977; Sato and Marrs, 1986), rats (Evans and Armstrong, 1984), rabbits (Fujimoto *et al.*, 1974) and sheep (Moore *et al.*, 1985). Problems frequently associated with exogenous gonadotrophin use in humans (ovarian hyperstimulation, cystic follicles, a perturbed endocrine milieu) have caused some to question whether IVF might be enhanced by using

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eggs from natural (nonhormone-stimulated) cycles (Messinis and Templeton, 1988; Osborn and Moor, 1988).

The domestic cat is an induced ovulator that exhibits distinct, overt signs of sexual receptivity for 5–7 consecutive days at 14–21 day intervals (see reviews, Goodrowe *et al.*, 1989; Wildt, 1991). The female can mate on any day of oestrus, and repeated copulations on any of the first 3 days of oestrus elicit pituitary release of LH that causes ovulation and the formation of three to seven corpora lutea (Wildt *et al.*, 1980, 1981; Schmidt *et al.*, 1983). No data are available on the temporal aspects of intra-follicular egg viability during the course of oestrus in the cat.

This investigation had two inter-related objectives. First, the impact of day of oestrus on number of eggs, maturation and ability to fertilize in culture was evaluated to determine whether follicular eggs were functionally comparable during different days of early oestrus. Second, the data from naturally oestrous cats were compared with those collected from PMSG/hCG-treated females to determine which type of egg was the most efficient for IVF.

Materials and Methods

Animals

Adult domestic cats were housed alone (males) or in pairs (females) and given commercial dry cat food (Purina Cat Chow, St Louis, MO) and water *ad libitum*. The colony room contained no windows, but 12 h of fluorescent lighting were provided daily.

Oestrus detection, induction of ovarian activity, laparoscopy and egg recovery

Fifty-eight female cats were monitored individually and twice a day for overt signs of oestrus (i.e. increased vocalization, rubbing, lordosis and treading of the hind legs) (Michael, 1961; Wildt *et al.*, 1981). The first 36 cats exhibiting overt sexual receptivity (the naturally oestrous group) received a single i.m. injection of 100 iu hCG (Sigma Chemical Co., St Louis, MO) on day 1 ($n = 12$), 2 ($n = 12$) or 3 ($n = 12$) of behavioural oestrus (10:00 h). This specific hCG dose induces ovulation in a high proportion of naturally developing cat follicles (Wildt and Seager, 1978). The remaining 22 females were designated as the PMSG/hCG group. During periods of no oestrous behaviour, these cats were subjected to laparoscopy to confirm that the ovaries were inactive. Laparoscopy was performed as previously described (Wildt *et al.*, 1977) under a surgical plane of anaesthesia induced and maintained with ketamine hydrochloride (Vetalar: Park Davis, Morris Plains, NJ; 20.0 mg kg⁻¹ body weight, i.m.) and acepromazine maleate (Ayerst Labs, Rouses Pt, NY; 2.0 mg kg⁻¹, i.m.). During laparoscopy, an ancillary, graduated Verres needle was used to manipulate the reproductive organs intra-abdominally to view all aspects of each ovary and to estimate follicle size. If no follicles ≥ 2 mm in diameter or corpora lutea were evident, each of these cats was injected i.m. with 150 iu PMSG (Sigma Chemical Co., St Louis, MO) followed 84 h later by 100 iu hCG (Sigma Chemical Co.). This PMSG dose was based

on previous studies performed in our laboratory (Goodrowe *et al.*, 1988b; Johnston *et al.*, 1991a, b) and by others (Niwa *et al.*, 1985). These cats were monitored twice a day for 5 consecutive days after PMSG administration for overt sexual receptivity to determine whether gonadotrophin treatment induced oestrous behaviour.

Twenty-four to 26 h after hCG, all females were subjected to laparoscopic egg collection (Goodrowe *et al.*, 1988b) and the follicular contents aspirated into collection tubes containing modified Krebs–Ringer bicarbonate medium (mKRB) (Niwa *et al.*, 1985; Tovoda and Chang, 1986) and 40 iu heparin ml⁻¹ (37°C). Contents of the tubes for each individual cat were emptied into a 60 mm $\times$ 15 mm plastic Petri dish and rinsed with 5 ml of equilibrated medium. Recovered eggs were transferred to fresh mKRB and placed in a 5% CO₂ in air, humidified incubator (37°C). After all collections were completed on a given day, each egg and its surrounding cumulus cell mass was assessed for maturational status on the basis of the following morphological criteria: (i) mature, if the corona radiata and cumulus oophorus cells were loosened and expanded; (ii) immature, if the egg had a tightly compacted corona radiata; or (iii) degenerate, if the egg appeared abnormal, pale or lacked an apparent corona radiata (Goodrowe *et al.*, 1988b; Johnston *et al.*, 1989, 1991a, b; Wildt, 1991). Only eggs designated as mature were inseminated *in vitro*. Within 2 h of collection, mature eggs were washed three times in mKRB under lightweight paraffin oil, placed in fresh mKRB (without heparin) and returned to the incubator.

Collection and processing of spermatozoa

Two male cats of proven fertility served as sperm donors and were used in rotation within groups and among subgroups to avoid the possibility of a male-specific effect (Fukui *et al.*, 1988). Electroejaculates (Wildt *et al.*, 1983) were subjected to swim-up processing (Goodrowe *et al.*, 1988b). In brief, this involved transferring semen into a 1.5 ml conical tube (Sarstedt Inc., Princeton, NJ, USA), diluting with an equal volume of mKRB and centrifuging for 8 min at 300 g. The supernatant was aspirated and discarded, and 150 μ l of mKRB was layered slowly onto the resulting pellet and the spermatozoa allowed a 1 h swim-up (22°C). The layered aliquot was aspirated and assessed objectively for sperm concentration and subjectively for percentage motility and forward progressive motility (rating of 0 = no forward movement of spermatozoa to a rating of 5 = rapid, linear, forward movement; Wildt *et al.*, 1983). Only swim-up aliquots containing spermatozoa with at least a 75% motility and 3.0 progressive motility rating were used for IVF. Spermatozoa were counted using a haemocytometer (Wildt *et al.*, 1983) and diluted in mKRB to a final insemination concentration of 2×10^5 motile sperm cells ml⁻¹.

In vitro fertilization

Up to ten mature eggs were placed in 100 μ l of sperm suspension (a total of 2×10^4 motile spermatozoa) under oil and co-cultured at 37°C in 5% CO₂, 95% air atmosphere (Goodrowe *et al.*, 1988b). After 12–18 h of culture, eggs were removed from fertilization dishes and washed in a 0.2% hyaluronidase

Table 1. Number of ovarian follicles and recovered eggs in cats given hCG on the first, second or third day of natural oestrus

Day of oestrus*	Number of follicles† (≥ 2 mm) per female	Total number of follicles	Number of eggs† collected per female	Total number of eggs
1	1.7 ± 0.6 ^a	20	1.5 ± 0.6 ^a	18
2	3.7 ± 0.4 ^b	44	3.3 ± 0.4 ^b	40
3	5.7 ± 0.8 ^c	68	5.3 ± 0.8 ^c	63

*When hCG was administered, $n = 12$ cats for each group.

†Mean ± SEM per female 24–26 h after hCG.

^{a,b,c}Values within columns with different superscripts are significantly different ($P < 0.05$).

Table 2. Mean number (± SEM) of eggs classified as mature, immature or degenerate in cats given hCG on the first, second or third day of natural oestrus or treated with PMSG and hCG

Treatment group	Mean number of eggs classified as		
	Mature (%)	Immature (%)	Degenerate (%)
Day 1 natural oestrus ($n = 12$)	0.2 ± 0.2 ^a (11.1)	0.2 ± 0.2 ^a (5.6)	1.3 ± 0.6 ^a (83.3)
Day 2 natural oestrus ($n = 12$)	1.6 ± 0.5 ^{ab} (47.5)	0.6 ± 0.4 ^a (17.5)	1.2 ± 0.4 ^a (35.0)
Day 3 natural oestrus ($n = 12$)	3.4 ± 0.7 ^b (65.1)	0.8 ± 0.4 ^a (15.9)	1.0 ± 0.3 ^a (19.0)
PMSG/hCG ($n = 22$)	7.3 ± 1.0 ^c (84.3)	0.4 ± 0.3 ^a (5.0)	1.0 ± 0.6 ^a (10.7)

^{a,b,c}Values within columns with different superscripts are significantly different ($P < 0.05$).

solution (Type 1-S from bovine tests, Sigma Chemical Co.) to remove cumulus cells and loosely attached spermatozoa. Eggs were returned to culture in 100 µl drops of fresh medium, overlaid with oil and cultured for an additional 12–18 h before assessing fertilization. Fertilized eggs were those that cleaved to the two-cell stage of development within 30 h of insemination (Goodrowe *et al.*, 1988b; Johnston *et al.*, 1991a, b). Embryos cleaving to the two-cell stage or greater were assigned a quality grade according to previously published criteria (Goodrowe *et al.*, 1988a; Johnston *et al.*, 1991a, b). In brief, embryos of good or excellent quality were those that were perfectly symmetrical (or only slightly asymmetrical), spherical in shape and uniformly dark. Fair or poor quality embryos were those that were partially or severely degenerate, pale in colour or contained lysed blastomeres. All embryos were stained using a DNA-specific Hoechst stain (Goodrowe *et al.*, 1988b) to confirm fertilization, and nuclei were counted.

Statistical analysis

Values are presented as means ± SEM. Differences in number of follicles or eggs collected between treatment groups were measured by analysis of variance using the Statistical Analysis System (SAS) general linear models program for factorial analysis (SAS, 1991). Treatment means were partitioned by least square means analysis. Chi-square (χ^2) analysis was used to compare the effect of natural oestrus versus PMSG/hCG treatment on the proportion of eggs fertilizing *in vitro*.

Results

Cats injected with hCG on day 1 of natural oestrus produced fewer ($P < 0.05$) ovarian follicles and total eggs than females given hCG on days 2 and 3 (Table 1). Because > 80% of the eggs collected from day 1 cats were classified as degenerate, too few eggs were available to test fertilizability *in vitro* (Table 2). Cats given hCG on day 3 produced more follicles and total eggs than did day 2 females ($P < 0.05$; Table 1), but the mean numbers of eggs categorized as mature, immature and degenerate were similar ($P > 0.05$) between these two subgroups (Table 2).

Of the 22 cats treated with PMSG, 12 (54.5%) exhibited behavioural oestrus. However, PMSG-treated females produced more than twice the number of ovarian follicles and recovered eggs ($P < 0.05$) than the combined group of naturally oestrous cats (Table 3). More than 80% of the eggs from PMSG/hCG-treated females were rated as mature compared with 65.1% for day 3 naturally oestrous females (Table 2). Overall, cats injected with both gonadotrophins consistently produced more ($P < 0.05$) total follicles ($n = 214$; mean 9.7 ± 0.8 per female), total eggs ($n = 191$; 8.7 ± 0.8 per female) and mature eggs ($n = 161$; 7.3 ± 1.0 per female) than any of the naturally oestrous subgroups (Tables 1 and 2). There appeared to be no subjective differences in ovarian follicular morphology (size, colour or vascularity) between the naturally oestrous and PMSG/hCG groups.

The proportion of fertilized two-cell embryos was similar ($P > 0.05$) between the naturally oestrous group (29 of 60

Table 3. Ovarian and egg characteristics and fertilization rates of natural oestrus versus PMSG and hCG stimulated cats

Day of oestrus	Mean* number of follicles (≥ 2 mm) per female	Mean* number of eggs collected per female	Number of IVF embryos per female†
Natural oestrus ($n = 36$)	3.7 ± 0.6^a	3.4 ± 0.6^a	1.8 ± 0.4^a
PMSG/hCG ($n = 22$)	9.7 ± 0.8^b	8.7 ± 0.8^b	4.3 ± 0.8^b

*Mean $\pm$ SEM 24–26 h after hCG.

†Mean $\pm$ SEM, includes only females from which eggs were inseminated (16 natural oestrus and 19 PMSG-hCG treated queens).

^{a,b}Values within columns with different superscripts are significantly different ($P < 0.05$).

mature eggs inseminated or 48.3%) and the gonadotrophin-treated group (82 of 161 mature eggs or 50.9%). However, the latter group produced more than twice the number of total embryos per egg donor than the former (Table 3). Regardless, 85.4% of the resulting embryos in the naturally oestrous group were rated as good-to-excellent quality which was similar ($P > 0.05$) to 80.0% of the embryos achieving the same ratings in the PMSG/hCG group.

Discussion

This is the first study that has examined the relationship of day of overt oestrus, *in vivo* follicular response to an LH-like stimulus and subsequent egg quality in cats. Overall, there was a strong association between the first day of sexual receptivity and an inability of the follicular egg to respond to hCG. There was no disadvantage to using PMSG as a priming hormone for provoking folliculogenesis for subsequent IVF. On the contrary, using this particular exogenous gonadotrophin combined with hCG increased the overall number of mature eggs recovered without compromising subsequent fertilization *in vitro* or embryo quality.

Results from within the naturally oestrous group revealed that behavioural signs of oestrus were a poor index of the readiness of the follicular egg to respond to hCG. This issue has not been addressed previously, but cats are known to occasionally experience 'silent' oestrus (Graafian follicle development in the absence of overt sexual receptivity) (Wildt *et al.*, 1978). In the study reported here, the total number of ovarian follicles and egg quality were enhanced if hCG administration was delayed until day 2 or 3 of oestrus. The cat will mate many times on any day of oestrus (Schmidt *et al.*, 1983; Goodrowe *et al.*, 1989), and these copulations are thought to (i) stimulate ovulation via the release of pituitary LH stores and (ii) ensure adequate numbers of spermatozoa at the site of fertilization (Wildt, 1991). On the basis of the few and poor quality of the day 1 eggs recovered in this study, it appears that the primary role of matings during early oestrus is to enhance pituitary gonadotrophin release to accentuate further follicular growth and egg maturation. This also probably explains why domestic cats (and other felid species) copulate so frequently during oestrus. Wildt *et al.* (1980) first demonstrated that cat ovarian follicles usually fail to rupture after only single matings, but sequential copulations

prolong LH release which increases the proportion of cats ovulating. Parallel studies have also demonstrated that few spermatozoa are needed to achieve conception. Although a single domestic cat ejaculate normally contains $57\text{--}61 \times 10^6$ motile spermatozoa, as few as 6×10^6 spermatozoa inseminated *in utero* can result in conception (Howard *et al.*, 1992). Frequent copulations during early oestrus therefore probably serve more to finalize folliculogenic events than to (i) cause immediate follicular rupture or (ii) ensure the presence of massive concentrations of deposited spermatozoa.

The finding that follicular development and egg integrity were compromised on the first day of natural oestrus was relevant to earlier observations concerning the sensitivity of the intrafollicular cat egg to exogenous PMSG/hCG. Extending the administration interval between these two gonadotrophins from 72 to 80 h decreases the proportion of degenerate eggs and increases the number of eggs fertilizing *in vitro* (Goodrowe *et al.*, 1988b). If the interval between PMSG and hCG is extended beyond 80 h (up to 12 additional h), eggs continue to be of high quality, but viability declines (Donoghue *et al.*, 1992). In the present study, if the interval between onset of oestrus and hCG administration was delayed for 24 (day 2) or 48 (day 3) h, then increasing numbers of follicles and mature, viable eggs were recovered. Taken together, these results demonstrate that timing of the LH-like stimulus in relation to naturally induced or gonadotrophin-induced folliculogenesis is important in predicting suitability of eggs for IVF. A poorly-timed hCG stimulus, even in naturally oestrous females, may interfere with eventual integrity of the egg and its viability. It is possible that the significant impact of the LH-like stimulus on egg function is closely related to the fact that the domestic cat is an induced ovulator. It is likely that this mating strategy serves a greater purpose in the domestic cat than simply causing follicular rupture. Copulations during early oestrus probably enhance follicular activity and subsequent egg integrity and fertilizability.

Results from the PMSG/hCG-treated group also confirmed that behavioural oestrus was uncoupled from either follicular or egg activity. More than 40% of these cats failed to exhibit overt sexual receptivity, yet mature eggs were recovered from 19 of 21 (90.5%) females. This incidence of oestrus was consistent with an earlier study using the same PMSG dose (Goodrowe *et al.*, 1988b). Although there were no apparent differences in gross follicular appearance, there was marked variation in the quality and maturation status of eggs from follicles on different

days of natural oestrus. It therefore appears that there is little value in relying upon follicular appearance as a predictor of egg viability in cats.

Unlike sexual behaviour or follicular morphometry, a subjective estimate of maturation (on the basis of cumulus cell mass expansion) was an accurate index of potential egg fertilizing capacity. Eggs meeting maturation criteria and derived from either naturally oestrous or PMSG/hCG-primed cats could fertilize *in vitro* at comparable rates. Because polar body extrusion is so difficult to identify in the uniformly dark, lipid-filled cat egg (Goodrowe *et al.*, 1988b), the degree of loosening and expansion of the corona radiata and associated cumulus mass continues to be the most reliable index of maturation (Goodrowe *et al.*, 1988b; Johnston *et al.*, 1991a, b). In this context, the cat egg is similar to that of the mouse (Gilula *et al.*, 1978), human (Brackett, 1985) and pig (Motlik *et al.*, 1986) in which these same criteria are convenient indices of egg maturity and fertilization potential.

As a gonadotrophin, PMSG is a potent stimulator of ovarian follicular development in cats. When given at a high dose or in sequential injections, PMSG causes ovarian hyperstimulation and the production of cystic follicles and abnormal endocrine profiles (Colby, 1970; Wildt *et al.*, 1978; Cline *et al.*, 1980). These adverse side effects are lessened using single doses ≤ 150 iu (Niwa *et al.*, 1985; Goodrowe *et al.*, 1988b, 1989). In addition to excessive folliculogenesis, PMSG has been associated with questionable egg quality and relatively poor IVF rates in an array of laboratory and livestock species (Fujimoto *et al.*, 1974; Maudlin and Fraser, 1977; Evans and Armstrong, 1984; Moore *et al.*, 1985; Sato and Marrs, 1986). In contrast, we detected no adverse consequences of using PMSG, at the described dose, to accelerate follicular growth in cats. On the contrary, compared with results from naturally oestrous cats, the total number of eggs recovered was increased more than twice, and more PMSG-stimulated eggs met maturation criteria, thus increasing the total number of embryos produced per egg donor. The mechanism by which PMSG may contribute to egg maturation is unknown. However, a recent study compared follicular granulosa cell proliferation in spontaneously cyclic women with women treated with human menopausal gonadotrophin (hMG) (Gougeon and Testart, 1990). Granulosa cell histology revealed a higher mitotic index in the hMG group suggesting that this gonadotrophin may be promoting granulosa cell growth. It is possible that there is a similar PMSG-mediated mechanism in cats to enhance egg development.

If the practical purpose of IVF is to generate large numbers of viable embryos, then these results demonstrate conclusively that this objective can be achieved more efficiently in domestic cats by using PMSG rather than by relying upon naturally produced follicles. The total number of recoverable eggs is increased, and PMSG combined with hCG appears to promote intrafollicular egg maturation, thereby allowing ready sperm-egg interaction *in vitro* and the formation of cleaved embryos.

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