Influence of temperature and gas atmosphere on in-vitro fertilization and embryo development in domestic cats

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Summary. The influence of culture temperature and gas atmosphere on in-vitro fertilization and embryo development was examined in the domestic cat. In Exp. 1, eggs were fertilized and cultured in 5% CO₂ in air at 37, 38 or 39°C. Experiment 2 evaluated the effects of 5% CO₂ in air; 5% CO₂, 5% O₂ and 90% N₂; and 10% CO₂ in air. Fertilization (cleavage) and development to the morula/blastocyst stage were not influenced ($P > 0.05$) by variations in temperature and gas composition. Despite changing these culture conditions, egg cleavage averaged ~75% and >80% of the 2-cell embryos proceeded to morulae *in vitro*. However, the partial in-vitro morula-to-blastocyst developmental block normally observed in this species was not removed.

Keywords: cat; fertilization; embryo; in-vitro culture; incubation; temperature; gas atmosphere

Introduction

There are 3 reasons for sustaining in-vitro-fertilized felid embryos in culture until later developmental stages. First, access to more-advanced embryos allows transfer of embryos directly to the uterus by less invasive approaches than surgical oviducal transfer. Secondly, successful embryo cryopreservation may require later-stage embryos because, in some species, embryos containing 8 or fewer blastomeres have reduced post-thaw viability (see review, Wilmot, 1986). Thirdly, understanding the fundamental nutritive and energy requirements for preimplantation cat embryos provides comparative information for other carnivore taxa, most of which have never been studied. In-vitro fertilization (IVF) and embryo transfer could play an important role in managing rare felid species (Wildt, 1990). Recently, advances in IVF and culture techniques in domestic cats (Goodrowe *et al.*, 1988; Johnston *et al.*, 1990) have been adapted for leopard cats (Goodrowe *et al.*, 1989), pumas (Miller *et al.*, 1990) and tigers (Donoghue *et al.*, 1990). Felid IVF embryos, surgically transferred to the oviducts, are viable as demonstrated by the production of live offspring in the Indian desert cat (Pope *et al.*, 1989) and tiger (Donoghue *et al.*, 1990). However, for IVF to be practical in such species, it will be necessary to culture embryos to morulae and blastocysts before transfer directly into the uterine lumen by an atraumatic technique such as laparoscopy (Schiewe *et al.*, 1984).

Although IVF and in-vitro development to morulae can be achieved routinely in domestic cats, most of the embryos exhibit a developmental block and are unable to advance to blastocysts (Johnston *et al.*, 1991). A similar observation has been made for cultured tiger embryos produced by IVF (Donoghue *et al.*, 1990). We recently reported that the type of culture medium and protein supplement used has a greater impact on embryo growth than on IVF in cats (Johnston *et al.*, 1991). Although supplementing the culture medium with serum partially circumvents the morula-

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to-blastocyst block, 70–80% of these embryos remain incapable of forming blastocysts. There are several reports that incubation temperature influences oocyte maturation, fertilization and cleavage rates (Lenz *et al.*, 1983; Eng *et al.*, 1986; Lavy *et al.*, 1988). Altering CO₂ content in the culture environment can also affect in-vitro embryo development (see review, Wright & Bondioli, 1981; Carney & Bavister, 1987).

This study examined the role of temperature and gas-phase composition on IVF and embryo development in domestic cats.

Materials and Methods

Animals. Twenty-nine adult, female cats and a single male sperm donor were housed as described previously (Goodrowe *et al.*, 1988; Johnston *et al.*, 1991), fed a commercial feline diet (Purina Cat Chow, Ralston Purina, St Louis, MO, USA) and provided with water *ad libitum*. All cats were exposed to a 12-h light:12-h dark cycle.

Induction of ovarian activity and egg recovery. Females were checked daily for oestrus (Wildt *et al.*, 1978) and those demonstrating no signs of sexual receptivity were given 150 iu pregnant mares' serum gonadotrophin (PMSG; i.m., Sigma Chemical Co., St Louis, MO, USA) to stimulate ovarian follicular development (Goodrowe *et al.*, 1988); 84 h later, each female was injected with 100 iu human chorionic gonadotrophin (hCG; i.m., Sigma Chemical Co.) and, 24–27 h after that, eggs were recovered laparoscopically from ovarian follicles (≥ 2 mm in diameter) (Goodrowe *et al.*, 1988). Follicular content was aspirated into collection tubes containing Ham's F10 (Johnston *et al.*, 1991; Irvine Scientific, Irvine, CA, USA) supplemented with 40 units of heparin/ml. Collection tubes were maintained at 35–37°C during and after aspiration. The content of each tube was emptied into a plastic culture dish and searched for mature (Goodrowe *et al.*, 1988) eggs, which were transferred to fresh Ham's F10. On any given day, eggs from all donors (usually 3–4 cats/day) were pooled and then assigned randomly for use in Exp. 1 (temperature effects) or Exp. 2 (gas effects).

Collection and processing spermatozoa. A single, proven breeder male was used as a sperm donor to control potential male effects on fertilization and embryo development (Fukui *et al.*, 1988). Semen was collected using a standardized electroejaculation technique (Wildt *et al.*, 1983) and a highly motile spermic fraction was recovered by the swim-up technique (Goodrowe *et al.*, 1988; Howard *et al.*, 1990). In brief, this involved transferring the electroejaculated semen into a 1.5-ml conical tube (Sarstedt Inc., Princeton, NJ, USA), diluting with an equal volume of Ham's F10 medium and then centrifuging for 8 min at 300 *g*. The supernatant was aspirated and discarded and 150 μ l of Ham's F10 medium was layered slowly onto the resulting pellet and the spermatozoa were allowed to swim-up for 1 h at room temperature. The layered aliquot was aspirated and assessed objectively for sperm concentration and subjectively for percentage motility and progression status (0 = no forward movement of spermatozoa to 5 = rapid, linear, forward movement) (Wildt *et al.*, 1983; Howard *et al.*, 1990). Only swim-up aliquots containing sperm with 75% motility and 3–0 progression status rating were used for IVF. Spermatozoa were diluted to a final concentration of 2×10^5 cells/ml using the appropriate temperature- and gas-equilibrated media according to specific Exp. 1 and 2 protocols.

In-vitro fertilization, embryo culture and assessment. Mature eggs were cocultured with processed spermatozoa (6–12 oocytes/insemination drop) at the appropriate temperature (Exp. 1) or gas atmosphere (Exp. 2). After 12–18 h of culture, eggs were removed from fertilization dishes and washed in a 0.2% hyaluronidase solution (Type 1-S from bovine testis, Sigma Chemical Co.) to remove cumulus cells and loosely attached spermatozoa. Eggs were placed in 100- μ l drops of fresh medium, overlaid with oil and cocultured under specific treatment conditions for an additional 12–18 h before assessing fertilization. To determine the rate of parthenogenesis, control eggs (2 or more representative mature eggs from each collection) were cultured under each treatment condition in medium containing no spermatozoa.

Fertilized eggs were those which cleaved to the 2-cell stage of development within 30 h of being placed in culture. Cleaved embryos, maintained in specific treatments, were monitored for quality and stage of development at intervals of 24 h for 7 days. Embryos considered viable were those which: (i) were symmetrical (or only slightly asymmetrical), (ii) contained spherical and uniformly dark blastomeres and (iii) demonstrated development over time (increased number of blastomeres). Embryos were considered to be degenerate if blastomeres were asymmetrical, pale or lysed and the embryo had arrested in growth while maintained *in vitro*. Embryos with ≥ 32 cells were considered as morulae, whereas embryos with a developing blastocoelic cavity were classified as early blastocysts. All embryos were stained using a DNA-specific Hoechst stain (Goodrowe *et al.*, 1988; Johnston *et al.*, 1989) and nuclei were counted.

Experiment 1. Ham's F10 medium, supplemented with foetal calf serum (FCS, 5%, Irvine Scientific) was equilibrated under 5% CO₂ in air at 37, 38 or 39°C. This was accomplished by simultaneously using 3 incubators maintained at one of these temperatures, which were chosen because 37°C is commonly used for mammalian embryo culture, whereas 38 and 39°C simulate the normal core body temperature variation for domestic cats. A single electroejaculate, processed with Ham's F10, was divided, diluted and maintained at each temperature. Each experiment was repeated 3 times and the medium was prepared fresh on 3 occasions over 2 months. Eggs were

divided randomly among the test temperatures (37°C, $n = 57$; 38°C, $n = 50$; 39°C, $n = 51$) and then cultured with spermatozoa previously maintained at each specific temperature.

Experiment 2. Three gas phases were evaluated: 5% CO₂ in air; 5% O₂, 5% CO₂ and 90% N₂; and 10% CO₂ in air. Based on the findings from Exp. 1, 38°C was chosen as the optimal culture temperature for Exp. 2. Ham's F10 was equilibrated for a minimum of 2 h before use in each of the gas phases. Spermatozoa, prepared as described above, were divided into aliquots and diluted in medium equilibrated with each gas phase. The number of experimental replicates and times that medium were prepared were similar to Exp. 1. The number of eggs distributed randomly among the treatments were: 5% CO₂, $n = 68$; 5% CO₂, 5% O₂ and 90% N₂, $n = 62$; and 10% CO₂, $n = 67$.

Statistical analysis. The proportion of eggs fertilized after culture with spermatozoa and the proportion of embryos developing to a specific stage are expressed as percentages. Differences in fertilization were evaluated by χ^2 analysis.

Results

None of the control eggs, cultured in the absence of spermatozoa, in Exp. 1 or 2 cleaved.

Experiment 1

There was no effect ($P > 0.05$) of the temperatures tested on IVF success or the proportion of 2-cell embryos developing to the morula or blastocyst stages (Table 1). All temperatures allowed at least 72% of all eggs to form 2-cell embryos after 24 h *in vitro* and >85% of these developed into morulae by 96 h of culture. Regardless of temperature, <30% of all cleaved embryos reached the blastocyst stage by 120 h in culture.

Table 1. Effect of incubation temperature and gas phase on in-vitro fertilization and embryo development in domestic cats

Treatment	No. of oocytes	No. of 2-cell embryos (%) [*]	No. of 2-cell embryos developing to morulae (%) [†]	No. of 2-cell embryos developing to blastocysts (%) [‡]
Temperature (°C)				
37	57	46 (80.7)	40 (86.9)	10 (21.7)
38	50	38 (76.0)	37 (97.4)	11 (28.9)
39	51	37 (72.5)	32 (86.5)	5 (13.5)
Gas phase				
5% CO ₂ in air	68	52 (76.5)	45 (86.5)	12 (23.1)
5% CO ₂ , 5% O ₂ , 90% N ₂	62	49 (79.0)	40 (81.6)	17 (34.6)
10% CO ₂ in air	67	49 (73.1)	41 (83.7)	11 (22.4)

*By 24 h in culture.

†By 96 h in culture.

‡By 120 h in culture.

Experiment 2

The number of eggs fertilized *in vitro* and the proportion of embryos developing into morulae or blastocysts were not influenced by the variations in gas phase (Table 1). At least 73% of all eggs cleaved within 24 h of insemination and >80% of these developed into morulae by 96 h in culture (Table 1). As in Exp. 1, ≤35% of the cleaved embryos developed into blastocysts by 120 h in culture.

Discussion

The efficiency of the IVF system developed for domestic cats was unaffected by the changes in culture temperature or gaseous components. These findings, together with our recent report evaluating the effects of medium and protein supplement (Johnston *et al.*, 1990), show that successful IVF and early embryo cleavage in domestic cats is remarkably resilient to substantial alterations in *in-vitro* culture conditions. The precise biological characteristics of spermatozoa–oocyte interaction in cats are unknown, but the ability of cat spermatozoa to capacitate, bind and penetrate follicular oocytes routinely *in vitro* will permit further examination of this phenomenon (see review, Wildt, *in press*). Within the limits already tested, it is now apparent that most conventional types of medium, protein supplement, temperature and gaseous environment will support high rates of oocyte cleavage after IVF.

Imposing various experimental variables on the system appears to influence the development of 2-cell cat embryos to later preimplantation stages more than fertilization itself. Although IVF cat embryos develop efficiently to the morula stage *in vitro*, the present study confirmed our earlier observation (Johnston *et al.*, 1991) of a partial morula-to-blastocyst developmental block in this species. This arrest in embryonic development may be related to chromosome anomalies, perhaps associated with exogenous gonadotrophin treatment (Sengoku & Dukelow, 1988). We chose to assess possible environmental causes. For example, we demonstrated recently that more cat embryos cultured in Ham's F10 reach the morula stage *in vitro* than in a modified Tyrode (TALP) solution (Johnston *et al.*, 1991). More embryos cultured in Ham's F10 advance to blastocysts when foetal calf serum or oestrous cat serum was the protein supplement rather than bovine serum albumin or a nonprotein control (polyvinylalcohol).

Statistical analyses revealed no marked effects of temperature or gas phase on embryo development or on the proportion of embryos advancing from morulae to blastocysts. In this context, fertilization and embryo development appear somewhat different in cats from other species. For example, greater IVF rates have been achieved in cow eggs cultured at 39 rather than 37°C (Lenz *et al.*, 1983), but, at a high temperature (41°C), mouse embryo development *in vitro* is compromised (Lavy *et al.*, 1988). Our results indicated that we have not yet defined the critical maximum adverse temperature for cat embryo viability *in vitro*.

For several mammalian species, successful embryo development *in vitro* is at least partially dependent on the gaseous environment. The CO₂ component is important for regulating intracellular pH and serving as a carbon source for development (Wales *et al.*, 1969; Graves & Biggers, 1970). Recently, Carney & Bavister (1987) demonstrated that increasing CO₂ from 5 to 10% improves *in-vitro* development of 8-cell hamster embryos to blastocysts. The stimulation by increased CO₂ is attributed, in part, to its ability to act as a weak acid, thereby maintaining intracellular pH. However, doubling the CO₂ content had no effect on the ability of cat embryos to become morulae, and, unlike in the hamster, CO₂ did not play a role in blastocyst formation. Lowering O₂ also did not influence embryo growth. Similar studies in other species have produced equivocal results. For example, the development of 1- and 2-cell mouse embryos (Whitten, 1971; Quinn & Harlow, 1978) or 2–8-cell cattle embryos (Wright *et al.*, 1976) *in vitro* is enhanced by lowering the O₂ atmosphere to 5%, but varying the oxygen tension in the culture of 1-cell sheep embryos is highly dependent on the incubation vessel (Tervit & Rowson, 1974; Wright *et al.*, 1976; Betterbed & Wright, 1985).

Overall, these results demonstrate that fertilization of domestic cat oocytes *in vitro* and subsequent growth to morulae are independent of incubation temperature or gas atmosphere. Many of the traditional culture variables (medium, protein source, incubation temperature and gas phase) can be changed (within conventional limits) without severely compromising fertilization or culture success rates. Despite alterations in culture environment, egg cleavage rate is usually ~75%; >80% of these 2-cell embryos proceed to morulae *in vitro*, but, even under optimum

conditions, >65% of all IVF embryos remain arrested as morulae in culture. We conclude that the partial developmental block to blastocyst formation in cats is a highly complex phenomenon regulated by factors other than type of medium, temperature or gaseous constituents in the culture environment.

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