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Embryo cloning in cattle: the use of *in vitro* matured oocytes

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In vitro matured (IVM) bovine oocytes were examined to determine their potential viability in embryo cloning. Activation competence, as monitored by pronuclear formation, increased with oocyte age. Oocytes readily formed a pronucleus when challenged with an electrical pulse 30 h after the onset of maturation. Developmental competence of IVM oocytes tended to increase with oocyte age ($P = 0.079$). Selection of IVM oocytes on the basis of the presence of a polar body 24 h after the onset of maturation and the size of the follicle from which the oocyte was derived improved development of nuclear transfer embryos (polar body positive 25% versus polar body negative 10%, $P < 0.05$; large follicle oocytes 31% versus small follicle oocytes 14%, $P < 0.05$). When selected, IVM oocytes were compared with *in vivo* matured oocytes recovered from superovulated cows and heifers; no difference was detected for the frequency of embryos produced, pregnancies confirmed between days 50 and 60 of gestation, or the number of calves born. We conclude that selected IVM oocytes are equivalent to *in vivo* matured oocytes when used for bovine embryo cloning.

Introduction

The recent development of embryo cloning procedures in cattle has brought about widespread interest in its commercialization. The commercial application of bovine embryo cloning is in part dependent on the cost effective acquisition of mature oocytes. These oocytes serve as the reprogramming environment which allows a single embryonic cell to become a viable embryo and thus an embryo clone. Previous reports used oocytes at meiosis stage II matured *in vivo* after surgical collection from superovulated cows and heifers (Prather *et al.*, 1987; Bondioli *et al.*, 1990; Westhusin *et al.*, 1991). Prather *et al.* (1987) reported the use of *in vitro* matured oocytes for embryo cloning but obtained a lower frequency of development than with *in vivo* matured oocytes.

The following experiments describe procedures for the maturation and selection of abattoir recovered primary oocytes that yield a population of oocytes at meiosis stage II that are developmentally equivalent to *in vivo* matured oocytes when used for embryo cloning in cattle.

Materials and Methods

Maturation of oocytes

Oocytes were aspirated from 1 to 10 mm follicles from abattoir recovered bovine ovaries. Only oocytes with a compact oocyte–cumulus complex were selected. Cumulus–oocyte complexes were washed three times in Tyrodes-Hepes medium (Bavister *et al.*, 1983) supplemented with 3 mg BSA ml^{-1}, 1% penicillin: streptomycin liquid (100 U ml^{-1} and 100 mg ml^{-1}, respectively, Gibco Laboratories, Grand Island, NY) and the pH was adjusted to 7.4 before *in vitro* maturation. The duration of culture was 22–24 h at 39°C in a water saturated atmosphere of 5% CO_2 and air. Oocyte maturation medium consisted of TCM-199 supplemented with 10% heat-treated fetal calf serum (HTFCS), 45 μg recombinant bFSH ml^{-1} (Chappel *et al.*, 1988), 1 μg oestradiol ml^{-1}, 0.2 mmol pyruvate l^{-1} and 1% penicillin: streptomycin liquid. In Expts 1, 2 and 3 (see below) the addition of 19 μg recombinant bLH ml^{-1} (Integrated Genetics Inc., Framingham, MA) was included. We did not determine whether the addition of bLH had an effect on the parameters measured. *In vivo* matured (IVM) oocytes were recovered from superovulated dairy and cross-bred cows approximately 40 h after the expected onset of oestrus (Westhusin *et al.*, 1991).

Polar body selection and extended culture of IVM oocytes

At 22–24 h after the onset of culture, oocytes are at the metaphase stage II of meiosis (Sirard *et al.*, 1988). At that time oocytes were stripped of cumulus cells by vortexing for 2 min and 15 s and observed under a stereomicroscope (× 50 to 100) to determine the presence or absence of a polar body. Oocytes were then cultured for an additional 3 or 19 h in co-culture with oviductal, epithelial cells. Bovine oviductal epithelial cells were collected from oviductal flushings 36 to 46 h after the expected onset of oestrus. Culture conditions consisted of a co-culture of 20–30 oocytes with oviductal epithelial cells (3 μl packed volume) in 20 μl of TCM-199 supplemented with 10% HTFCS and antibiotic under oil (Dow Corning 200 Fluid, 50 CS viscosity, Specialized Products, Houston, TX). Incubation conditions were as described for oocyte maturation. Oviductal

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epithelial cell cultures were established 22–24 h before the addition of oocytes. When oocyte culture was extended to 19 h, an additional 20 μl of culture medium was added to co-cultures 3–5 h after oocytes were added.

Embryo cloning

Nuclear transfer was performed as described by Willadsen (1986) and modified as described by Westhusin *et al.* (1991). Only enucleated oocyte halves as determined by Hoechst staining were used as nuclear transfer recipients (Westhusin *et al.*, 1990). Donor nuclei were derived from fresh and frozen 5 and 6 day bovine embryos obtained by non-surgical collection from superovulated cows (Bondioli *et al.*, 1990; Westhusin *et al.*, 1991, 1992). Nuclei derived from such embryos have an equal probability of supporting development when used in nuclear transfer (Westhusin *et al.*, 1991). Electrofusion was used to transfer the blastomere nuclei into oocyte halves (Westhusin *et al.*, 1992). All manipulations were conducted at room temperature (23–26°C). Embryo clones were cultured to the morula and blastocyst stage in ligated ovine oviducts according to the procedures of Willadsen (1982). Nuclear transfer embryos at the morula or blastocyst stage of development were transferred to synchronous (±36 h) bovine recipients by non-surgical techniques.

In trials where oocyte activation was the desired endpoint, activation was induced by electrical stimulation performed exactly as conditions used for embryo cloning. In those trials, oocyte activation was defined as formation of a single pronucleus (Ware *et al.*, 1989).

Statistical analysis

Data for Expts 2, 3 and 5 were analysed by χ^2 test. Data presented from Expt 4 were analysed by the General Linear Models procedures (SAS Institute Inc., Cary, NC).

Experimental designs

Experiment 1 investigated the effect of oocyte age on activation competency. IVM oocyte activation was induced by electrical stimulation as described by Westhusin *et al.* (1991) at 26, 28, 30 and 32 h after onset of maturation. The experiment consisted of two replicates and each time point consisted of 30–40 oocytes. Oocytes were returned to culture for 12–16 h and subsequently mounted on glass slides and stained with aceto-orcein for determination of activation according to the methods of Ware *et al.* (1989).

Experiment 2 examined the effect of oocyte age on nuclear transfer embryo development. IVM oocytes from a given day were selected for polar bodies and divided into two treatments. In treatment 1, IVM oocytes were cultured for 3–5 h after the end of maturation, and in treatment 2 they were cultured for 19 h. Oocytes fused to blastomeres at 32 h after onset of maturation (treatment 1) were manipulated between 26 and 30 h after onset of maturation and then returned to culture for 2 h before fusion. Oocytes fused to blastomeres at 46 h after onset of maturation (treatment 2) were manipulated between 42 and 46 h after onset of maturation.

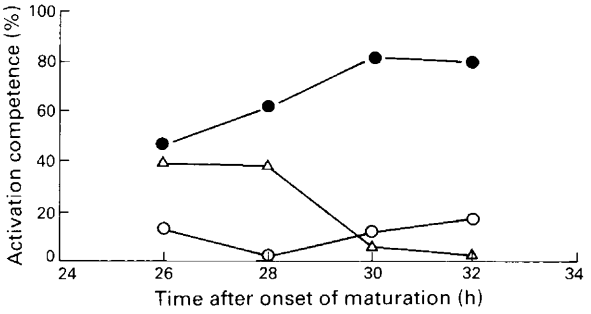

Fig. 1. Activation competence of *in vitro* matured bovine oocytes. IVM oocytes were induced to activate as described by Westhusin *et al.* (1991). The number of oocytes examined were 38, 39, 32 and 34 for 26, 28, 30 and 32 h after onset of maturation, respectively. The percent (%) pronuclear (●), metaphase (△) and lysed (○) oocytes are presented.

In Expt 3, the effect of the presence of a polar body on the development of nuclear transfer embryos was assessed. Oocytes were matured for 24 h as described. Oocytes were divided into two groups on the basis of the presence or absence of a polar body. Oocytes were then cultured for 19 h after maturation as described, and used for embryo cloning. The experiment was replicated nine times.

In Expt 4, the effect of follicle size on development of IVM oocytes used for embryo cloning was examined. Oocytes were aspirated from ovaries obtained from the abattoir and isolated from either 1–5 or 6–8 mm follicles. Oocytes were subsequently matured (22–24 h), selected for the presence of a polar body, cultured for an additional 19 h and used for embryo cloning. The experiment was repeated 20 times. Blastomeres from one or two, day 5 or 6 embryos were randomly distributed across follicle size treatments.

Experiment 5 investigated the use of *in vivo* matured versus IVM oocytes for bovine embryo cloning. Oocytes aspirated from follicles 3 mm or greater were matured as described and selected for the presence of a polar body and cultured for 19 h before embryo cloning. *In vivo* matured oocytes were collected from superovulated heifers as described by Westhusin *et al.* (1991). Blastomeres from a fresh or frozen–thawed donor embryo on any given day were randomly distributed between oocyte treatments. Embryo cloning was carried out as described above. Viability was assessed morphologically by the frequency of nuclear transfer embryos progressing to the morula or blastocyst stage of development after five to six days of culture in the sheep oviduct (Westhusin *et al.*, 1992). Pregnancies were diagnosed by rectal palpation between days 50 and 60 of gestation.

Results

The effect of oocyte age on embryo cloning

The ability of IVM oocytes to be activated increased with oocyte age (Fig. 1). Oocytes readily activated (81%, 26 of 32) following electroporation at 30 h after onset of maturation. This observation is in agreement with earlier investigations by Ware *et al.* (1989) in which different IVM and activation conditions

Table 1. Effect of follicle size on development of IVM bovine oocytes used for embryo cloning

Follicle size (mm)	Morulae and blastocysts/embryos recovered (mean ± SEM)
1–5	17/121 (14 ± 4)[a]
6–8	39/125 (31 ± 4)[b]

[a,b]Frequencies with different superscripts are significantly different; $P < 0.05$ (General Linear Models procedures, SAS).

were used. In another study, it was found that 41 to 43 h after onset of maturation, oocytes readily activate when subjected to an electrical pulse (Powell and Barnes, 1992). It appears that oocyte ageing beyond the time required for nuclear maturation (Sirard *et al.*, 1989) is a general requirement for activation competence of IVM oocytes.

Culture of IVM oocytes beyond the time required for activation competence did not significantly improve ($P < 0.05$) the developmental potential of IVM oocytes used in embryo cloning. However, there was a trend for oocytes cultured for 19 h after polar body selection to give better results ($P = 0.079$). The frequency of morula and blastocyst stage development was 12% (18 of 153) for IVM oocytes cultured for 19 h after maturation versus 7% (17 of 253) for those cultured for 3–5 h after maturation. The frequency of development was low and considered to be due to low donor embryo quality. Other experiments in our laboratory have indicated donor embryo quality to be a source of variation. All subsequent experiments used oocytes with an overall age of 41–43 h after maturation at the onset of manipulation.

Selection criteria for IVM oocytes used for embryo cloning

The presence of a clearly visible polar body enhanced the selection of developmentally competent IVM oocytes used for embryo cloning. Sixty-five per cent of the oocytes (156 of 241) examined had a membrane bound, clearly visible polar body at 24 h after maturation. When IVM oocytes with a polar body were compared with those without a polar body for development following nuclear transfer, development to the morula and blastocyst stage was increased with polar body-containing oocytes, 25% (28 of 111) versus 10% (7 of 70). It was concluded that IVM oocytes with a clearly visible polar body at 24 h after maturation resulted in increased developmental potential when used for embryo cloning.

The size of follicle from which an IVM oocyte was derived influenced the development of embryo clones. Neither fusion rate (1–5 mm 50% versus 6–8 mm 61%) nor frequency of oocyte lysing (1–5 mm 27% versus 6–8 mm 18%) were affected by the follicle size category of the recipient oocyte. Oocytes derived from 6–8 mm follicles resulted in a higher frequency of development to morulae/blastocysts when compared with oocytes derived from 1 to 5 mm follicles (Table 1).

In vivo *matured versus IVM oocytes for embryo cloning*

Selected IVM oocytes were developmentally equivalent to oocytes matured *in vivo* when used for embryo cloning (Table 2). No differences were observed between IVM or *in vivo* matured oocytes for fusion rate, production of viable nuclear transfer embryos, pregnancy rates upon transfer, or number of calves born.

Discussion

The results presented have demonstrated the efficacy of *in vitro* matured bovine oocytes for embryo cloning. Duration of maturation and culture, oocyte selection based on follicle size and presence of a polar body have an influence on developmental ability of IVM oocytes. When IVM oocytes were selected by these parameters, development of nuclear transfer embryos was equivalent to the development of nuclear transfer embryos derived from *in vivo* matured oocytes collected from superovulated cows and heifers.

The trend for older oocytes to yield improved results following embryo cloning has been observed by others. Willadsen (1989) noted that IVM oocytes matured for 44–46 h as well as *in vivo* matured oocytes that are "... a good deal older" than freshly ovulated oocytes yielded the highest frequency of viable embryo clones. Similarly, Sims *et al.* (1991) reported that oocyte and blastomere fusion delayed for 18 h after manipulation enhanced development of embryo clones. The data presented here support these observations.

The developmental potential of IVM oocytes was greater when a clearly visible polar body was present at 24 h after maturation. IVM oocytes generally progress to metaphase II 100% of the time when examined by aceto-orcein stain at 24 h after maturation (data not shown). These mounting and staining procedures make it difficult to determine whether the putative polar body lies within or outside the oocyte plasma membrane. Frequently, a polar body plasma membrane cannot be detected. Preliminary observations suggested that not all IVM oocytes contained a clearly visible polar body when examined by stereomicroscopy. This result suggests that IVM oocytes that have a clearly visible polar body upon completion of maturation represent a more developmentally normal population of oocytes.

Selection of oocytes at 24 h after maturation was based on previously published reports describing the time required for nuclear maturation (Hyttel *et al.*, 1986; King *et al.*, 1986; Sirard *et al.*, 1989). It has been observed that the rate of meiosis varies considerably in IVM oocytes (Sirard *et al.*, 1989); thus, a time-point corresponding to the rate of meiosis found in *in vivo* matured oocytes was used (Hyttel *et al.*, 1986; King *et al.*, 1986). It is possible that the presence of a polar body at 24 h represents a normal rate of meiosis. Hyttel *et al.* (1989) indicated that the first polar body is often degenerate 7 h after the LH peak. This suggests that the stability of a polar body changes over time. The experiment described eliminated oocytes in which the polar body membrane was lysed or in a state of degeneration. Polar body selection at 24 h after maturation may have effectively removed oocytes that either progressed through meiosis too rapidly or those that failed to mature to meiosis stage II.

The size of follicle from which an oocyte is derived before *in vitro* maturation influences its subsequent developmental potential when used for embryo cloning. Since the report by Motlik and Fulka (1986) describing the RNA synthetic activity

Table 2. Development of embryo clones with *in vitro* matured or *in vivo* matured bovine oocytes

Treatment	Successful fusions/attempted fusions	Viable embryos/embryos recovered	Pregnancies/embryos transferred	Calves born/embryos transferred
In vitro matured	840/961 (87)	161/906 (18)	33/125 (26)	27/125 (22)
In vivo matured	884/1074 (82)	161/1008 (16)	33/135 (24)	24/135 (18)

Values in parentheses are percentages.
$P > 0.05$ for all comparisons (χ^2).

of oocytes derived from different follicle size categories, it has been postulated that the follicle size from which an oocyte is derived may ultimately affect its developmental competence. Preliminary studies from this laboratory demonstrated that oocytes derived from 1–5 and 6–8 mm follicles result in 21% (37 of 180) and 42% (10 of 24) development to the morula and blastocyst stage, respectively, after *in vitro* fertilization and *in vitro* culture. Tan and Lu (1990) have reported similar results. These data suggest that critical events required for embryo development take place in the oocyte during the final stages of folliculogenesis. Although oocytes derived from large follicles are more developmentally competent, the maturation procedure imposed may be more suitable to these oocytes. Future maturation protocols that inhibit the resumption of meiosis for 2–3 days as well as alter gonadotrophin and steroid parameters may prove advantageous for recruiting oocytes derived from small follicles.

The use of *in vitro* matured oocytes for the cloning procedure is a prerequisite if embryo cloning of livestock is to be of commercial relevance. The above studies indicate that with these maturation conditions, selected IVM oocytes may be used for commercial embryo cloning of cattle.

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