Pregnancy resulting from cattle oocytes matured and fertilized *in vitro*

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Summary. Follicular oocytes ($n = 81$) collected from cattle at a local slaughterhouse were matured and fertilized *in vitro*. Of 27 ova 19 (70%) were penetrated by spermatozoa and 40/54 (74%) inseminated ova transferred surgically to the oviducts of a synchronized heifer were recovered by non-surgical flushing of the uterine horns 6 days later. Of the 40 ova 15 (38%) were at the morula, early blastocyst or diminutive morula stages. Culture *in vitro* sustained further development of all embryos and 9 were expanding or expanded blastocysts. One pregnancy resulted from non-surgical transfer of 2 blastocysts. The results demonstrate that immature oocytes from cattle can be matured and fertilized *in vitro*, subsequently develop to the blastocyst stage, and develop into a normal pregnancy after non-surgical transfer.

Introduction

All calves born after in-vitro fertilization have originated from in-vivo matured oocytes transferred surgically to the oviduct of synchronized recipients (Brackett *et al.*, 1982) or following incubation in rabbit or sheep oviducts (Sirard & Lambert, 1986). In-vitro fertilization of in-vitro matured oocytes has only resulted in the formation of early embryonic stages (Ball *et al.*, 1983). However, Critser *et al.* (1986) and K. H. Lu (personal communication) have obtained pregnancies resulting from in-vitro matured oocytes fertilized *in vitro*, cultured in ligated sheep oviducts and subsequently transferred to the final recipient heifer. Like Brackett *et al.* (1982) we have established pregnancies by transferring in-vivo matured and in-vitro fertilized oocytes to the oviducts of recipient heifers (Greve *et al.*, 1987). Here we present a study in cattle in which morulae/blastocysts and a pregnancy were obtained from slaughterhouse oocytes that were matured and fertilized *in vitro*, cultured 6 days in heifer oviducts and upon non-surgical recovery transferred non-surgically to a final recipient heifer.

Materials and Methods

Oocytes were collected from 10 heifers/cows at an unknown stage of the reproductive cycle at a local slaughterhouse by aspiration from small antral follicles (1–6 mm in diameter) within 30 min after slaughter. The aspirates were placed in tubes containing 4–5 ml Hepes-buffered Ham's F-10 medium (Flow Laboratories, Irvine, U.K.), supplemented with 20% heat-inactivated cow's serum (collected on the day of standing oestrus), 0.4 mM-glutamine, 50 i.u. penicillin/ml and 50 µg streptomycin/ml. Ovaries were collected and kept in a vacuum flask together with the tubes at 32–38°C during collection and transportation to the laboratory.

From the sediments at the bottom of the tubes 81 oocytes with compact cumulus layers (Fig. 1) were recovered and washed three times in the oocyte maturation medium, which was identical to the medium mentioned above except that bicarbonate buffer replaced Hepes. Granulosa cells were obtained by dissection of small follicles and subsequent perfusion of the follicular cavity with medium. There was no attempt to recover the oocytes of these follicles. The recovered granulosa cells were washed and added to the oocyte maturation medium to give an approximate concentration of 3×10^6 /ml. Oocytes were cultured for 27 h in 4-well multidishes (Nunc, Roskilde, Denmark) with 10–15 oocytes/1.0 ml medium/well in an incubator with 5% CO₂/air at 38°C with 100% humidity in order to achieve complete maturation.

Fig. 1. Oocytes before maturation. $\times 70$.

Fig. 2. A penetrated ovum, showing a male pronucleus on the right with sperm tail (arrow) and a female pronucleus on the left. $\times 300$.

Fig. 3. Two early blastocysts cultured for 4 h, which were at the morula stage at recovery. $\times 180$.

Fig. 4. One blastocyst and one expanded blastocyst cultured for 20 h, which were at the morula stage at recovery. $\times 180$.

Semen was obtained and spermatozoa processed by the method previously described by Parrish *et al.* (1986) and Greve *et al.* (1987). Briefly, frozen semen samples from two bulls of known fertility were thawed and washed in modified Tyrode's solution. The motile sperm fraction was obtained by a swim-up separation procedure, and then treated with $\sim 10 \mu\text{g}$ heparin/ml (Sigma, St Louis, MO, U.S.A.) for 15 min.

Oocytes matured *in vitro* were treated with 0.1% hyaluronidase (Sigma) to remove part of the cumulus cells. Five oocytes were placed in each 50 μl Tyrode's medium droplet covered with paraffin oil. Spermatozoa were added to reach a concentration of $\sim 0.5 \times 10^6/\text{ml}$. The sperm-oocyte co-culture conditions were identical to those for oocyte maturation except for the temperature, which was at 39°C .

After 22 h of co-culture, 54 ova were washed to remove most of the cumulus cells and the attached spermatozoa before transfer to the intermediate recipient. The remaining 27 ova were fixed 24 h after insemination in methanol: acetic acid (3:1, v/v) for 48 h and then stained with 1% aceto-orcein for microscopic evaluation of sperm penetration.

The intermediate recipient heifer was synchronized by an injection of 0.675 mg cloprostenol (2.5 ml Estrumat Vet.: H. Lundbeck & Co. A/S, Copenhagen, Denmark) 89 h before receiving ova by transfer to the oviducts through a midline incision under general anaesthesia as previously described by Greve *et al.* (1987) and Hyttel *et al.* (1988).

The uterus of the animal was flushed non-surgically 6 days after transfer. The recovered ova were further cultured in Ham's F-10 medium supplemented with 20% fetal calf serum, 0.4 mM-glutamine and penicillin and streptomycin.

Embryonic development was observed after 4 and 20 h *in vitro*. Since no appropriate recipient was available, 4 blastocysts were transferred to two intermediate recipients which had had the uterus flushed 4 h before. Pregnancy examination was carried out by rectal palpation after 6 weeks.

Results

Of the 27 fixed ova, 19 (70%) were penetrated by spermatozoa (Fig. 2), and no polyspermy was recorded. Of the 54 ova transferred to the oviducts of recipient heifers, 40 (74%) were recovered by non-surgical means: 15 of these (38%) were embryos at different stages, all of which developed after 4 and 20 h culture (Figs 3 & 4). The results from non-surgical recovery and in-vitro culture are presented in Table 1.

Each of the final recipients received 2 blastocysts by non-surgical transfer, and one was pregnant at 6 weeks, and is still pregnant at 4 months of gestation.

Table 1. Embryonic developmental stages from in-vitro matured and fertilized cattle ova incubated *in vivo* and then re-cultured *in vitro*

	No. at different developmental stages:		
	At recovery	After culture for 4 h	After culture for 20 h
Expanded blastocyst		1 }*	1
Blastocyst	1	5 }	2
Early blastocyst	1	3	—
Morula	4	1	1
Early morula	4	—	—
Very small morula	5	5	5†
1–12-cell egg	25	25	25

*Of these, 4 were transferred to recipients, and 2 were lost during the handling process.

†All formed a small blastocoele.

Discussion

The rates of penetration and polyspermy were similar to those in our earlier studies (Xu & Greve, 1988) and those reported from other groups (Parrish *et al.*, 1986). This indicates that a reliable in-vitro fertilization system has been established. One of the reasons for the high success rate may be the short time interval from slaughter until oocyte aspiration (< 30 min). It is well established that this early period is critical for subsequent germinal vesicle breakdown *in vitro* (Motlik *et al.*, 1978). The good facilities for oocyte aspiration at the slaughterhouse reduces the time between when the animals are killed and the oocytes are released from the ovaries. This might give better conditions for oocytes to mature *in vitro*.

Another contributing factor to the high rate of early embryonic development is the addition of serum from an oestrous cow to the oocyte maturation medium (Sanbuissho & Threlfall, 1985). When such serum is added to the medium the most striking morphological change is a complete expansion of the cumulus cells, including the corona radiata. This feature is one of the indications of oocyte maturation *in vitro* (Motlik *et al.*, 1986) and *in vivo* (Greve *et al.*, 1984). Addition of granulosa cells might also enhance the subsequent developmental competence (Motlik & Fulka, 1981; Staigmiller & Moor, 1984).

In the present experiment we used the heifer as an intermediate recipient, and a surgical

approach similar to that used by Trounson *et al.* (1977) and Shea *et al.* (1983). Oviducts from other species have been used as incubators (rabbit: Sirard & Lambert, 1986; sheep: Critser *et al.*, 1986), as it is believed that a 'block' of early cleavage, probably at the 8–16-cell stage in cattle, will occur during in-vitro culture (Eyestone & First, 1986) unless a cell-co-culture system is used.

Although the surgical procedure is more tedious for the cow than for rabbits and sheep, this approach has some advantages: e.g. (1) the oviduct condition is likely to be more favourable to the fertilized ova than that of other species, (2) after in-vivo incubation a non-surgical approach for recovery as well as for transfer can be used. With adequate training and reasonably good surgical facilities, surgical transfer of embryos to the intermediate recipient can easily be completed within 30–50 min.

Even though the birth of a live calf would be the ultimate proof of the efficiency of this technique, blastocyst formation is considered as a valid indication that normal oocyte maturation and fertilization have been accomplished (Moor & Trounson, 1977). The positive pregnancy diagnosis further supports this concept.

This work was supported by grants from the Danish Agricultural and Veterinary Research Council and Leo Research Foundation.

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Received 30 March 1987