In vitro production of cattle embryos: review and Belgian results

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ABSTRACT This paper reviews the overall process of *in vitro* production and cryopreservation of bovine embryos in Belgium. Three laboratories are involved in this field, one at the University of Liège, one at the University of Ghent and ours at the University of Louvain-La-Neuve. Each one uses this technology as a tool to reach different goals. This paper refers mainly to the work done in Louvain-La-Neuve. Oocytes are obtained by punction of 2-4 mm follicles on slaughtered cow ovaries. They are matured in hormone-supplemented TCM199 containing 10% heat-treated fetal calf serum. *In vitro* fertilization by Percoll-selected spermatozoa is followed by *in vitro* culture in oviduct-conditioned medium for 7-9 days. Six calves have been born from *in vitro* produced blastocysts. Recently, full development was obtained in conditioned medium without protein supplementation. This finding will allow further investigations on oviduct/embryo molecular communication and research of oviduct-secreted embryotrophic proteins which were impaired in previous culture systems using serum-supplemented media. *In vitro* produced blastocysts were frozen-thawed and non-surgically transferred: 7/19 recipients remained pregnant beyond 2 months. Embryo loss was high between day 21 and 35 (31%).

KEY WORDS: *IVM, IVF, IVC, bovine, embryo, blastocyst, breeding, oviduct, conditioned medium, in vitro fertilization*

Introduction

The first calf produced from *in vitro* fertilization of an oocyte matured *in vivo* was a male born in 1981 (Brackett *et al.*, 1982). Since then considerable effort has been devoted to developing and improving *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes as well as *in vitro* culture (IVC) of zygotes (see reviews by First and Parrish, 1987; Greve and Madison, 1991; Marquant-Le Guienne, 1991). The reason for the considerable interest in this technology is the ability to produce at low cost many synchronized embryos at a specific stage of development. Among the most significant potential applications of IVF in cattle are:

- gene injection for the production of transgenic animals. Gene injection is done at the pronuclear stage and as the integration rate is low, a large number of fertilized oocytes is necessary.
- cloning by nuclear transfer (Prather and First, 1990), which requires mature enucleated oocytes as recipients and 8- to 16-cell embryos as nuclei donors;
- production of transferable embryos of high genetic value from oocytes collected by ultrasound-guided puncture of small ovarian follicles in living animals. This new method for bovine embryo production could represent an alternative to current embryo transfer technology (Kruip *et al.*, 1991);
- low cost mass production of beef cattle embryos that will improve the commercial value of calves produced in dairy breeds (Gordon, 1990);
- overcoming disorders of the genital tract (Sirard, 1989);
- disease control as viral contaminations are easier to avoid *in vitro* than *in vivo* ;
- fertility prediction in artificial insemination bulls (Marquant-Le Guienne *et al.*, 1990b);
- fundamental research on the mechanisms of early embryonic development.

Large numbers of bovine oocytes and embryos are needed for all these purposes and in most of the European countries, including Belgium, research groups are involved in programs of *in vitro*

Abbreviations used in this paper: BSA, bovine serum albumin; CM, conditioned medium; COC, cumulus-oocyte complex; DMSO, dimethylsulfoxide; FCS, fetal calf serum; FSH, follicle stimulating hormone; ICM, inner cell mass; IVC, *in vitro* culture; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; LH, luteinizing hormone; MII, second meiotic metaphase; PB, polar body; PN, pronucleus; PROH, 1,2-propanediol; TALP, Tyrode medium supplemented with bovine serum albumin, lactate and pyruvate.

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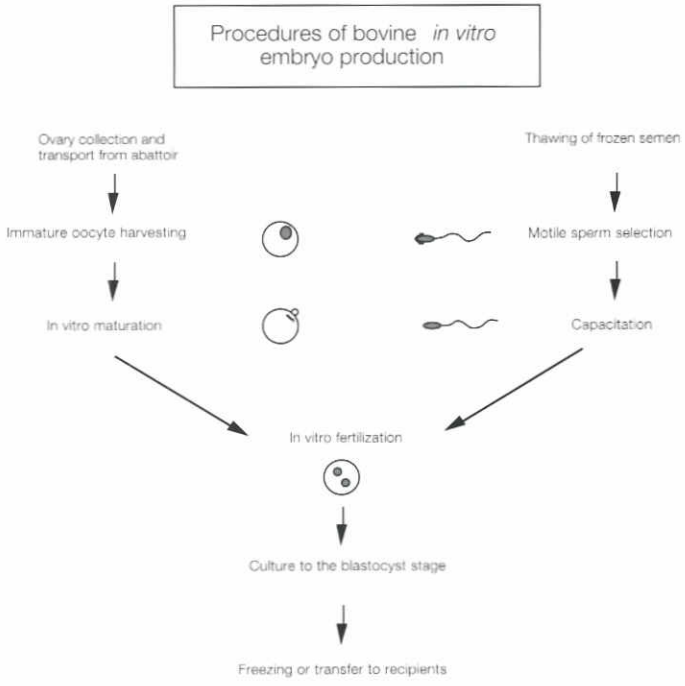

Fig. 1. Diagram of the different steps in bovine embryo *in vitro* production from slaughtered cow ovaries and from frozen semen. *The steps described in this review are in bold.*

production of bovine embryos. In Belgium three groups are concerned:

- one group at the University of Liège (F. Ectors);
- one group at the University of Gent (A. De Kruif) and
- our group at the University of Louvain-la-Neuve (F. Dessy and A. Massip).

In vitro production of embryos requires development of technology in five areas (see Fig. 1): oocyte harvesting, *in vitro* oocyte maturation, *in vitro* fertilization, *in vitro* embryo development and oocyte or embryo freezing. This paper will review the recent progress in these areas, mainly describe the work of our laboratory and refer to some interesting results obtained by the other Belgian groups.

Harvesting of immature oocytes

The first IVF attempts were performed on *in vivo* matured oocytes recovered from superovulated cows by laparotomy (Brackett *et al.*, 1982) or laparoscopy (Lambert *et al.*, 1983; Sirard and Lambert, 1985). However, some specific applications of IVF technique require the production of large numbers of embryos. This need led numerous authors to develop immature cattle oocyte collection methods and to study appropriate conditions for their *in vitro* nuclear maturation (reviewed by Sirard, 1989).

Cow ovaries are harvested in abattoir, soon after slaughter and taken to the laboratory within a few hours. Yang *et al.* (1990) examined the optimal time and temperature conditions of ovary transport. They reported that ovaries can be stored up to 8 h at 25°C without significant loss of developmental competence. However, a storage at 4°C or 37°C during 4 h results in decreased oocyte viability. In our laboratory, time of ovary storage does not exceed 4 h and the storage temperature is allowed to slowly decrease from 37°C to ambient temperature (about 20°C).

Follicle size does not seem to influence either the rate of nuclear maturation (Fukui and Sakuma, 1980) or the oocyte fertilization rate (Leibfried-Rutledge *et al.*, 1985). However, since oocytes from small follicles have not completed their growth, they have not been able to complete their stock of mRNAs before transcription arrest and fail to develop after fertilization (Crozet, 1989). A number of authors recommend the use of medium-sized follicles (Staigmiller, 1988; Tan and Lu, 1990) without morphological evidence of atresia (Grime and Ireland, 1986). The follicle diameters measured on the ovary surface reflect the diameter of dissected follicles (Spicer *et al.*, 1987). In our experiments, after repeated washing of ovaries in a large volume of saline, follicles with an apparent diameter ranging from 2 to 4 mm are aspirated through a 28G1/2 needle connected to a controlled depression of 4 cm Hg (Boccart *et al.*, 1991b). In these conditions, an average of 6 (ranging from 0 to 25) fair quality cumulus-oocyte complexes (COC's) are harvested from each ovary.

Maturation

Mammalian oocytes are arrested at the diplotene stage of the first meiotic division. In response to the preovulatory surge of gonadotrophins some of the oocytes undergo resumption of meiosis characterized by germinal vesicle breakdown, chromosome condensation, formation of the first meiotic spindle, expulsion of the first polar body and arrest in metaphase of the second meiotic division. These events are defined as oocyte maturation and lead to an ovulated fertilizable oocyte.

Ovaries collected from slaughter provide an abundant and economic source of oocytes but these oocytes have to be matured *in vitro*. More than fifty years ago, Pincus and Enzmann (1935) demonstrated that oocytes removed from their follicle are able to mature spontaneously, but the first successful system of ruminant oocyte maturation *in vitro* mimicked the *in vivo* conditions: explanted follicles with enclosed oocytes were incubated in the presence of gonadotrophins (Moor and Trounson, 1977). However, the technical problems associated with the culture of isolated follicles gave rise to improvements in the conditions of extrafollicular oocyte maturation. Co-culture of COC's with granulosa cells enhances the developmental capacity of the oocytes. This method, initially proposed for sheep (Staigmiller and Moor, 1984) has been extended to cattle (Critser *et al.*, 1986; Xu *et al.*, 1987; Fukui and Ono, 1989). The co-culture medium was originally supplemented with FSH, LH, and 17ß-estradiol. However, supplementation of the medium with serum from an estrus cow was as efficient as hormonal addition in cattle (Lu *et al.*, 1987; Le Guienne *et al.*, 1988).

In our laboratory, the harvested oocytes are examined under a stereomicroscope and only those surrounded by more than three compact layers of cumulus cells are selected. They are washed in Tyrode modified medium (low bicarbonate TALP, Parrish *et al.*, 1986) and cultured in groups of about 100 in 4-well tissue culture plates with 500 µl maturation medium. Maturation medium is TCM199 containing 10% heat-treated fetal calf serum, 1 mg/ml 17ß-estradiol, 5 µg/ml pLH and 0.5 µg/ml pFSH. They are incubated for 24 h at 39°C under a 5% CO_2 in air, humidified atmosphere. The maturation rate determined after fixation and staining with fluorochrome bisbenzimide (Hoechst 33 342) varies from 75% to 90%. The morphological criterion for maturation is the presence of metaphase II spindle and of the first polar body (Fig. 2A).

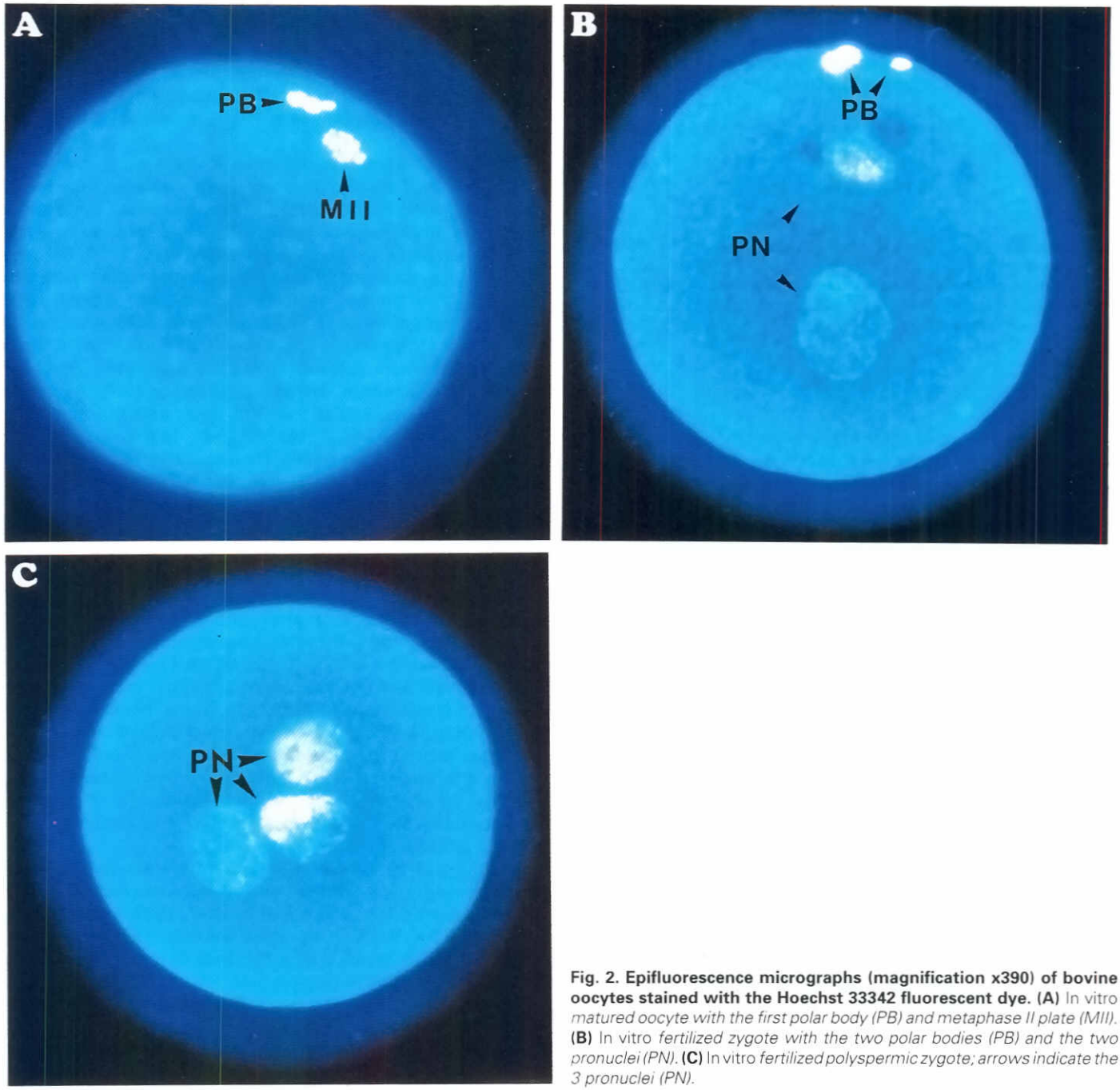

Fig. 2. Epifluorescence micrographs (magnification x390) of bovine oocytes stained with the Hoechst 33342 fluorescent dye. (A) In vitro *matured oocyte with the first polar body (PB) and metaphase II plate (MII).* **(B)** In vitro *fertilized zygote with the two polar bodies (PB) and the two pronuclei (PN).* **(C)** In vitro *fertilized polyspermic zygote; arrows indicate the 3 pronuclei (PN).*

Recently it has been shown by Ectors *et al.* (1992) that protein supplementation may be omitted during IVM without affecting subsequent fertilization and embryo development except that the rate of blastocyst hatching is lowered in these conditions.

Fertilization

Fertilization represents an interesting step in the *in vitro* cattle embryo production procedure. It can be a key point for development of new technologies such as production of embryos of known sex by sperm selection (Upreti *et al.*, 1988) or transgenic offspring obtained by sperm electroporation with foreign DNA (Gagné *et al.*, 1991). In addition, *in vitro* fertilization could represent a new alternative tool for bull fertility testing (Marquant-Le Guienne *et al.*, 1990a).

The overall success of the *in vitro* fertilization process requires good control of different steps: sperm selection, sperm capacitation and fertilization itself (Fig. 1). Semen is known to contain factors that can prevent capacitation and/or fertilization. Abnormal spermatozoa, dead cells, enzymes and bacteria are also present

TABLE 1

PERCENTAGES OF BLASTOCYST FORMATION IN THE DIFFERENT MEDIA IN THE PRESENCE OR ABSENCE OF CUMULUS CELLS (FROM MERMILLOD *et al.*, 1992b)

Condition	Cumulus removed		Cumulus not removed	
CM	16% [a]	n=113	21% [a]	n=436
NCM	0% [b]	n=68	9% [c]	n=91
NCM+C	11% [c]	n=70	n.d.	

Culture medium was TCM199 supplemented with 10% FCS. (CM) Oviduct conditioned medium. (NCM) Non-conditioned medium. (NCM+C) Non-conditioned medium supplemented with cumulus cells at the beginning of embryo culture. Numbers of oocyte and development rates are indicated. [abc] Means with different superscript are significantly different (Chi square, $p<0.05$).

that could be the cause of IVF failure or unreproducible results. In addition, the concentration of sperm suspensions has to be controlled to ensure the continuity of the IVF method. Numerous techniques have been proposed for removing undesirable semen components and concentrating the motile sperm fraction in a suspension of known concentration. The most conventional is the swim-up method (Parrish *et al.*, 1986; Parrish and Foote, 1987) but other methods have been described involving either centrifugation on BSA (Wall *et al.*, 1984) or Percoll (Bolton and Braude, 1984) density gradients or Sephadex column separation (Drobnis *et al.*, 1991). Centrifugation on Percoll density gradients has been reported as the most suitable method for human IVF, the pregnancy rate increased from 18% after swim-up to 31.5% after Percoll sperm separation (Guérin *et al.*, 1989). In our experiments with bovine, the use of Percoll gradients reduced the rate of polyspermy (Fig. 2B) which was the major cause of IVF failure (Mermillod *et al.*, 1990).

The next critical point encountered in IVF is sperm capacitation. Ejaculated spermatozoa are more suitable than epididyme collected sperm for bovine IVF and give rise to more normal eggs (Pavlok *et al.*, 1988) but they have to undergo a capacitation treatment prior to fertilization (reviewed by First and Parrish, 1987; Greve and Madison, 1991). The mechanisms of mammalian sperm capacitation, which normally takes place in the female genital tract, are not yet well understood but several *in vitro* capacitation methods have been proposed: washing with high ionic strength solution (Brackett *et al.*, 1982); treatment with bovine follicular fluid (Fukui *et al.*, 1983) or oviduct fluid (Parrish *et al.*, 1989); co-culture onto oviduct cell monolayers (Guyader and Chupin, 1991); incubation with the Ca^{2+} ionophore A23187 (Shorgan, 1984). However, the most common method used in bovine IVF involves heparin (Parrish *et al.*, 1986). Heparin, as well as other glycosaminoglycans present in the female genital tract (Lenz *et al.*, 1982), is able to capacitate bovine fresh sperm within 4 h (Parrish *et al.*, 1988) and frozen-thawed sperm within 15 min (Parrish *et al.*, 1986).

Some recent studies pointed out that the semen from different bulls strongly differ in their reactions to heparin treatment and the optimal heparin concentration ranges from 0.05 (Marquant-Le Guienne *et al.*, 1990b) to 100 µg/ml (Fukui *et al.*, 1990). As a consequence, each bull and perhaps each ejaculate has to be carefully tested for optimal heparin dosage and/or optimal sperm concentration before use in IVF (Leibfried-Rutledge *et al.*, 1989; Lancaster *et al.*, 1990; Mermillod *et al.*, 1990; Van Soom and De Kruif, personal communication). Furthermore, recent results indicate that bull also influences later embryonic development rate (Eyestone and First, 1989b; Marquant-Le Guienne *et al.*, 1990a; Van Soom and De Kruif, personal communication) and this effect seems not to be correlated to bull's *in vivo* fertility (Ohgoda *et al.*, 1988).

In general, the medium used for sperm/oocyte co-culture is TALP (Parrish *et al.*, 1986) supplemented with the appropriate heparin concentration, in 50 µl droplets under mineral oil and containing 10 oocytes and $1x10^6$ sperm/ml (Parrish *et al.*, 1986). We have used this method with some success (Mermillod *et al.*, 1990; Boccart *et al.*, 1991a,b; Mermillod *et al.*, 1991) but after some troubles due to toxicity of oil batches we decided to avoid the oil overlay in co-culture (unpublished data). Finally, we use Percoll sperm selection and heparin (10 µg/ml) sperm capacitation. Fertilization is done in 4-well tissue culture plates with about 100 oocytes per well and 10^6 sperm in 500 µl/well of heparin supplemented TALP. Oocytes and sperm are incubated for 18 h at 39°C under a 5% CO_2 in air, water saturated atmosphere. Fertilization quality is assessed using the same method as for maturation. The rate of zygotes presenting both male and female pronuclei (Fig. 2C) is about 80% with the Belgian Blue sire that we use in routine.

Embryo culture

As reported for many mammalian species, bovine embryos encounter a developmental block as they are removed from their natural environment. In the bovine, this developmental arrest takes place between the 8- and 16-cell stage (Thibault, 1966). The embryos remain alive but cleavage is arrested at the G2 stage of the cell cycle (Eyestone and First, 1988). Some methods have been described to overcome this developmental block. The first of these methods involved the transient incubation of cow embryos in ewe or rabbit oviducts (Eyestone *et al.*, 1987). This observation led to the first IVF attempts in Belgium, and the first IVF offspring was obtained after the transient transfer of IVF cow embryos in rabbit oviducts (Ectors *et al.*, 1989).

Later experiments have shown that embryo development may be performed in a fully *in vitro* system involving either oviduct epithelial cell co-cultures (Marquant-Le Guienne *et al.*, 1989) or use of oviduct cell culture supernatants (Eyestone and First, 1989a; Eyestone *et al.*, 1991). Such systems were successfully used in Belgium. The first calves obtained in Belgium from IVM-IVF embryos co-cultured on bovine epithelial cell monolayers (Boccart *et al.*, 1991a; Mermillod *et al.*, 1991) were born in April 1991 in Louvain-la-Neuve (Boccart *et al.*, 1991b). Some good results were also obtained by the other two groups, using similar methods (Fontes *et al.*, 1991; Van Soom and De Kruif, personal communication).

Further improvement of the technique led us to use oviduct-conditioned media for embryo culture (Mermillod *et al.*, 1992b). Oviduct cell cultures are initiated as described by Eyestone and First (1989a). Cow oviducts are obtained from abattoir without control of the estral cycle status of the donor. After dissection, they are briefly immersed into ethanol 70% and epithelium is gently scraped using a microscope slide. Mucosal tissue is then transferred to a conical tube containing 10 ml of culture medium (TCM199 supplemented with 10% of heat treated fetal calf serum). Tissue is then washed twice in the same medium and finally resuspended in 50 volumes of medium for seeding in 25 cm^2 culture flasks (6 ml per flask). After

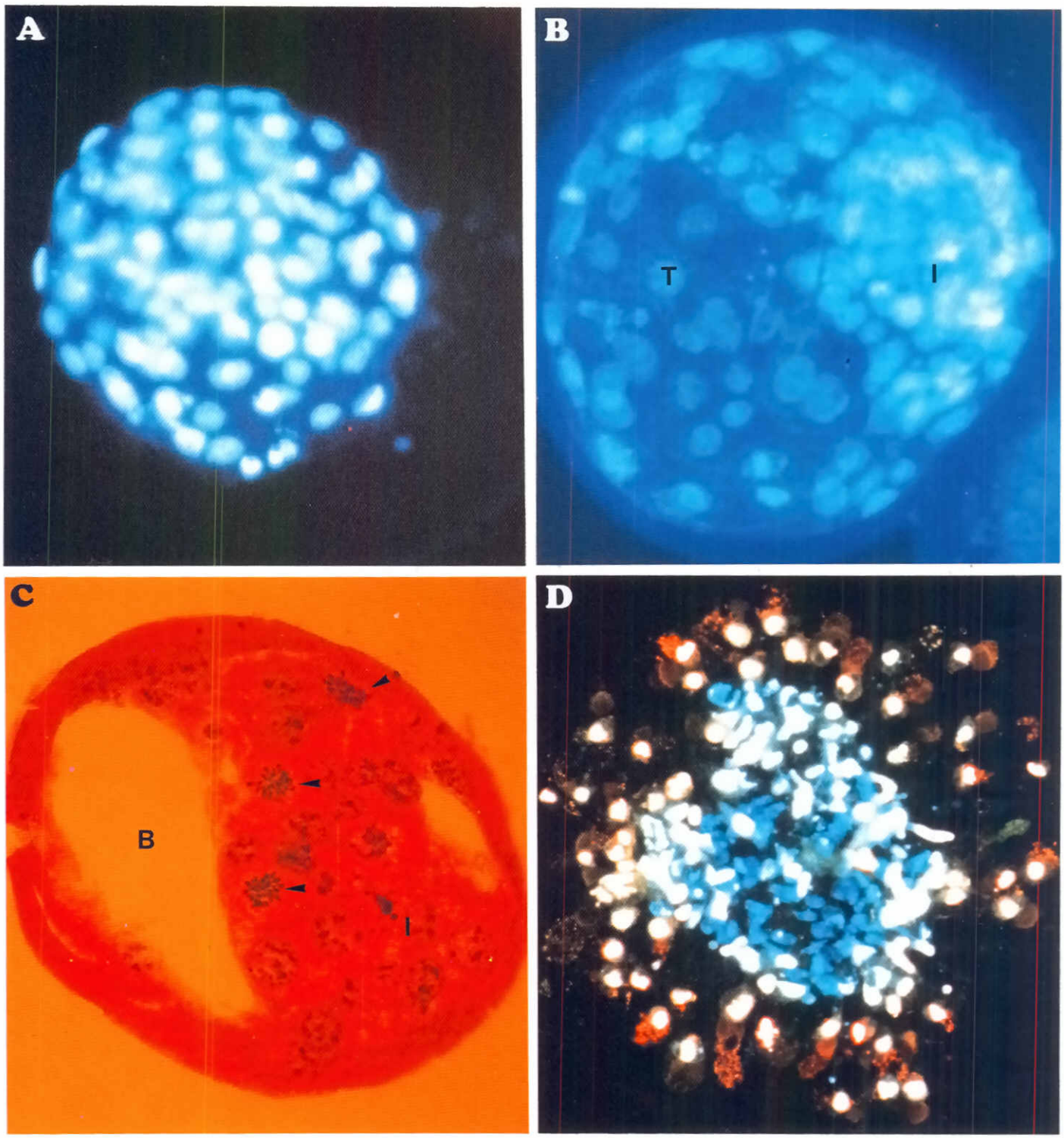

Fig. 3. Micrographs (magnification x190) of bovine *in vitro* produced embryos. (A) *Late morula stage embryo stained with the Hoechst 33342 fluorescent dye.* **(B)** *Expanded blastocyst stained with Hoechst 33342 showing inner cell mass (I) and trophectoderm (T) nuclei.* **(C)** *Histological section of a blastocyst stained with hematoxylin-eosin; inner cell mass (I) and blastocoele (B) are visible, arrows indicate mitotic cells.* **(D)** *Differential staining of an expanded blastocyst after immunosurgery; inner cell mass nuclei are stained in blue (Hoechst 33258) and trophectoderm nuclei in pink-white (propidium iodide).*

TABLE 2

NUMBERS OF EMBRYOS THAT CLEAVED (2 DAYS) AND REACHED THE EXPANDED BLASTOCYST STAGE IN SERUM-FREE MEDIUM (FROM MERMILLOD *et al.*, 1992b)

Culture medium	n	Cleaved (%)		Blastocysts(%)	
SCM	333	261	(78.4)	44	(13.2)
CM+S	97	78	(80.4)	15	(15.5)
CM	276	182	(65.9*)	32	(11.6)

Culture medium is TCM199. (SCM) Medium supplemented with 10% FCS and then conditioned on oviduct cells. (CM+S) Serum free conditioned medium supplemented with 10% FCS before embryo culture. (CM) Serum free conditioned medium. *Significantly different (Chi square, $p<0.01$).

4 days, medium is renewed and unattached cells are discarded. Confluence is reached 6 days after seeding. Then, conditioned medium is collected every two days, centrifuged at 500 x g for 10 min and stored refrigerated (4°C). After three harvestings (six days) on the same monolayer, conditioned medium is pooled and frozen (-20°C) in 1ml aliquots.

Fertilized ova are washed in culture medium. Cumulus cells are then removed by repeated pipetting and the embryos are cultured in droplets of conditioned medium (1 µl per embryo) under mineral oil. The rate of expanded blastocysts is determined on day 7 of culture; this rate ranges from 10 to 30% .

The oviduct effect in overcoming developmental block in numerous species led some authors to postulate a central oviduct function in the regulation of preimplantation embryo development (reviewed by Gandolfi *et al.*, 1989a). This hypothesis is still controversial since embryo growth improvement may also be achieved either by adaptation of basic culture media composition (Ellington *et al.*, 1990a; Pinyopummintr and Bavister, 1991), by adaptation of physical culture conditions (Fukui *et al.*, 1991) or by the use of co-culture with other cell types such as trophoblastic vesicles (Heyman *et al.*, 1987), granulosa or cumulus cells (Goto, *et al.*, 1989a; Mermillod *et al.*, 1992b) or fibroblasts (Gandolfi and Moor, 1987). Table 1 compares bovine embryo development rates obtained either in oviduct-conditioned medium or by cumulus cell co-culture and shows that oviduct-conditioned medium gives better results in our hands.

Nevertheless, culture results have to be analyzed not only in terms of zygote development rates but also in terms of blastocyst quality and viability (Gandolfi *et al.*, 1989a). Gandolfi and Moor (1987) have shown that sheep fibroblasts allow some embryos to develop beyond the 8-cell block but that the pregnancy rates obtained with such embryos were significantly lower than those from oviduct cell co-cultured embryos. Embryo quality could be investigated by total embryonic cell count using histological (Marquant-Le Guienne *et al.*, 1989) or fluorescent techniques (Ellington *et al.*, 1990b). More accurate results may be obtained by cell number allocation between inner cell mass (ICM) and trophectoderm (TE, Iwasaki *et al.*, 1990). An epifluorescence micrograph of a late morula stained with the Hoechst 33342 fluorescent dye is represented in Fig. 3A, Fig. 3B represents an expanded blastocyst stained with the same method and Fig. 3C a histological section in a day 7 blastocyst. Fig. 3D shows a day 7 bovine blastocyst submitted to immunosurgery (selective complement attack of TE cell membranes) prior to staining with Hoechst 33258, a blue fluorescent dye permeating all cells and propidium iodide, a red fluorescent dye only entering fragilized membranes. Marquant-Le Guienne *et al.* (1989) compared total cell numbers and ICM/TE cell repartition between *in vivo* and *in vitro* obtained bovine blastocysts. The conclusion of their study was that ICM seems smaller in embryos co-cultured with bovine oviduct epithelial cells than in *in vivo* embryos. Cytogenetics (Iwasaki and Nakahara 1990), metabolic activity measures (Thompson *et al.*, 1991), ultrastructural studies (Hyttel and Niemann, 1990; Van Blerkom *et al.*, 1990) and analysis of embryo secretions (Kaaekuahiwi and Menino, 1990) could also represent good tools in the approach of embryo quality.

A key point in the knowledge of embryonic requirements will certainly be a better understanding of oviduct-embryo molecular communication. Analyses of oviduct fluid proteic content have been done in a number of species, such as sheep (Gandolfi *et al.*, 1989b), pig (Buhi *et al.*, 1989), cattle (Malayer *et al.*, 1988; Boice *et al.*, 1990; Gerena and Killian, 1990; Wegner and Killian, 1991), mouse (Kapur and Johnson, 1985) and human (Verhage *et al.*, 1988).

Studies in cattle have shown an estrous cycle-related modulation of proteic content in oviduct fluid (Boice *et al.*, 1990) associated with changes in phospholipid and cholesterol levels (Killian *et al.*, 1989). Some experimental results strongly indicate that oviduct-secreted glycoproteins may mediate oviduct action on embryo development. First, a 97kD oviduct glycoprotein has been reported to be mainly expressed during the first five days of estrous cycle in cow, which is the period of embryo presence in oviduct (Boice *et al.*, 1990). Second, some mitogenic activity on NIH 3T3 fibroblasts of a 92kD sheep oviduct glycoprotein has been reported by Gandolfi et al. (1989b). The same authors also recently detected a similar mitogenic activity in bovine oviduct fluid (Gandolfi *et al.*, 1991). Third, some oviduct-secreted glycoproteins have been reported to bind to the zona pellucida and to embryos themselves in sheep (Gandolfi *et al.*, 1989b) and cattle (Wegner and Killian, 1991). Fourth, recent investigations of Pollard *et al.* (1991) have shown that Xenopus oocytes injected with bovine oviduct epithelial cell mRNAs are able to support bovine sperm capacitation and embryo development. Unfortunately, studies of oviduct protein intervention in embryo development were generally impaired by serum content of the media used in embryo culture.

However, recent results have shown that serum, like any other proteic supplement, can be omitted from the culture medium of bovine IVM-IVF embryos during either oviduct co-culture (Fontes *et*

TABLE 3

***IN VITRO* MATURATION OF IMMATURE BOVINE OOCYTES AFTER EXPOSURE TO INCREASING CONCENTRATIONS OF VARIOUS CRYOPROTECTANTS (FROM DEPIESSE *et al.*, 1991)**

Concentration	Glycerol		Propanediol		DMSO		Ethylene glycol	
10%	31/59	(53%)	25/47	(53%)	12/51	(24%)	25/48	(52%)
20%	28/67	(42%	24/57	(42%)	16/51	(31%)	17/36	(47%)
30%	13/51	(26%)	17/48	(35%)	8/54	(15%)	7/27	(26%)
40%	42/132	(32%)	3/40	(8%)	0/54	(0%)	8/53	(15%)
50%	6/69	(9%)	0/52	(0%)	0/49	(0%)	3/40	(8%)

The maturation rate without exposure to cryoprotectant was 92% (n=258).

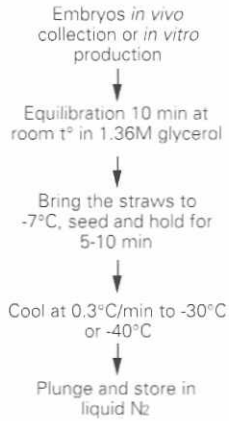

Fig. 4. Diagram of the different steps of a classical freezing procedure for bovine blastocysts.

al., 1992) or culture in oviduct-conditioned medium (Mermillod *et al.*, 1992a). Table 2 shows that similar development rates were obtained after culture in either serum-containing conditioned medium or serum-free conditioned medium. These data are of great interest for further investigations on development control by oviduct-secreted proteins, which is now one of the goals in our laboratory.

Oocyte and embryo cryopreservation

The entire process of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) is tedious and time consuming. So efforts are made to find freezing procedures adapted to each step from oocyte to blastocyst in order to create gamete and embryo banks and gain more flexibility in their use.

Freezing of oocytes

Freezing of oocytes offers many advantages. It will

- greatly facilitate IVF studies relating to the process of fertilization;
- provide a germ-plasm bank to evaluate the fertility of future sires by IVF;
- in combination with semen bank, allow greater flexibility in genetic management of a breed;
- economically provide fertilized eggs (via IVF) in the pronuclear stage for gene insertion;
- provide for the preservation of oocytes of individual cows beyond their normal limits of fertility and allow for IVF with the sperm of future sires.

So far, oocytes from farm animals have not been frozen successfully. Recently some blastocysts were obtained from mature oocytes frozen in 1M glycerol by the classic method used for embryos (Lim *et al.*, 1991). A point to be taken into account when considering the freezing of oocytes is the effect of organic solvents on the cytoskeleton before freezing. Both dimethylsulfoxide (DMSO) and 1,2-propanediol (PROH) for example modify the organization of tubulin in cattle oocytes and may induce a partial disassembly of the meiotic spindle at room temperature. This could explain reduced survival following transfer.

In our laboratory we have investigated the toxicity of four cryoprotectants on immature bovine oocytes by exposing them to increasing concentrations of each one (Depiesse *et al.*, 1991). Survival was evaluated by determining IVM rate after cryoprotectant treatment. The results are given in Table 3. They show that DMSO is toxic and that glycerol, propanediol and ethylene glycol could be interesting candidates although maturation rates do not exceed 50%.

Freezing of early stage embryos

Early embryonic stages such as zygotes, 2- and 4-cell stages or even up to the precompacted bovine morula have attracted significant interest in the context of embryo technologies such as cloning and gene transfer. Up to now, very few studies on freezing of early stage embryos from livestock have been reported. Preliminary data on precompacted bovine morulae indicate that these stages are extremely sensitive to cooling even at 4°C (Looney *et al.*, 1989). Experiments on freezing bovine 2- and 4-cell stages using combinations of PROH and sucrose revealed a survival rate of 40 to 60%; one pregnancy was established upon transfer (Vincent and Heyman, 1986). Frozen day-5 embryos have been used for nuclear transfer (Bondioli *et al.*, 1990).

The data discussed here refer to *in vivo* embryos. To our knowledge no information is available on freezing of early stages obtained *in vitro*.

Freezing morula and blastocyst stages

These stages are those collected non-surgically in embryo transfer practice. When frozen by controlled freezing and thawing procedures, survival rates may reach 90% to 100% based on morphological evaluation after thawing and pregnancy rates of 50

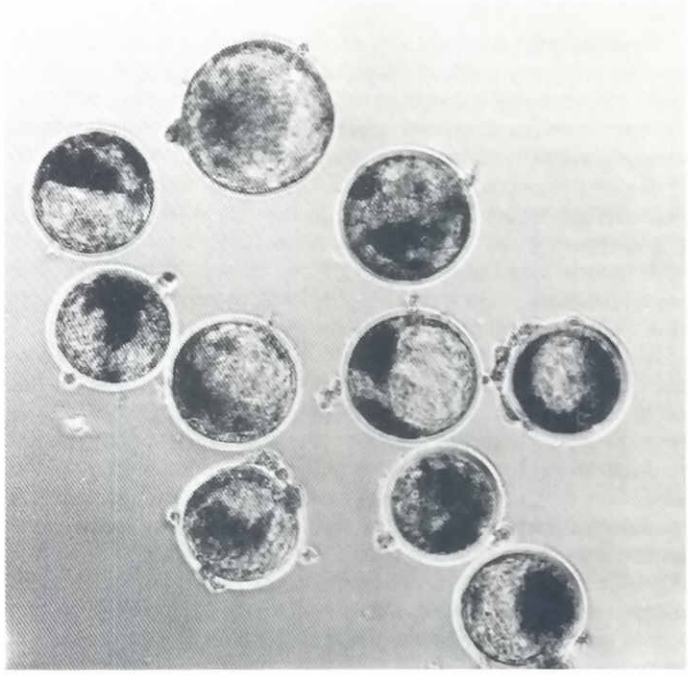

Fig. 5. Bovine *in vitro* produced expanded blastocysts (day 7 of culture) selected for freezing (magnification x95).

Fig. 6. Diagram of a 0.25 ml straw just before cooling.

to 60% following non-surgical transfer are obtained (Niemann, 1991). Late morulae to blastocysts derived from *in vitro* techniques have also been frozen successfully by the same or slightly modified method used for blastocysts collected *in vivo* (Lu *et al.*, 1988; Goto *et al.*, 1989b; Lu *et al.*, 1990; Reichenbach *et al.*, 1991; Suzuki *et al.*, 1991). The common steps of this freezing method are described in Fig. 4.

The transfer results are generally displayed in such a way that it is difficult to evaluate the exact survival rate. In some studies two or more embryos were transferred to the same recipient and/or pregnancy rate was established at 1 month. So Lu *et al.*, (1988) have frozen 148 late morulae to expanded blastocyst stages obtained by IVM, IVF and subsequent culture *in vivo* in the sheep oviduct for six days. One hundred and forty-one embryos were examined after thawing and culture for 4-6 h. The survival rate for embryos graded 1-2 was 49% (29/59), and was 11% (9/82) for embryos graded 3. A pregnancy rate of 67% (4/6) was established at one month when two frozen-thawed embryos were transferred per recipient. When a single embryo was transferred, a pregnancy rate of 42% (5/12) was found.

More recently, Reichenbach *et al.* (1991) have frozen 248 late morulae and early blastocysts graded morphologically excellent or good. After thawing and culture for 8-10 h, 65 (26%) were selected and transferred to -1 day asynchronous recipients. Pregnancy tests were performed by both plasma progesterone concentration on day 21 and rectal palpation on day 35. On day 21, 51% (33/65) had progesterone levels >1.4 ng/ml. On day 35, 43% (28/65) were diagnosed pregnant. With fresh embryos, the pregnancy rate was 60%. Embryo loss between days 21 and 35 was 15% (5/33). The overall pregnancy rate was 11% (28/248). In only one study (Goto *et al.*, 1989b) were births and normality of calves reported.

The quality of embryos produced *in vitro* has been stressed as an important factor determining their developmental capacity after transfer or their freezability. This quality is dependent on the culture systems used. In our laboratory we routinely use oviduct-conditioned medium throughout the culture period from the zygote to the blastocyst stage, *i.e.*, for seven days. Only expanded blastocysts are selected for freezing (Fig. 5). The freezing-thawing procedure is derived from that of Massip and Van der Zwalmen (1984) and slightly modified. The cryoprotectant medium is a solution of phosphate buffered saline (PBS) containing 1.36M glycerol and 0.25M sucrose; the dilution medium contains 0.25M sucrose in PBS. These two media are aspirated into transparent 0.25 ml straws (IMV France) in the proportion of 1 part of freezing medium and 10 parts of diluent (Fig. 6). The successive steps are those given in Fig. 4 except that slow cooling at a rate of 0.3°C/min is interrupted at -25°C before plunging in liquid nitrogen because sucrose incorporated in the freezing medium predehydrates the embryos during equilibration. Thawing is done in water at 20°C during 30 seconds. Then the cryoprotectant is diluted by the sucrose of the straw.

In a first series of experiments viability after thawing was assessed by *in vitro* culture on oviduct cell monolayers for 4 days. The criteria for survival were re-expansion after 24 h and hatching after 4 days. A total of 147 frozen-thawed blastocysts were checked in culture. After 24 h, 81% (120/147) had re-expanded, of which 50% (60/120) were hatched 3 days later.

Developmental capacity was evaluated by transferring embryos looking morphologically normal after thawing and dilution. Naturally cycled recipients were +/- 1 day asynchronous. The non-surgical transfer of 21 frozen-thawed blastocysts to 19 recipients resulted in 7 ongoing pregnancies beyond 2 months. All pregnancies derive from transfer of a single embryo. These results show that blastocysts totally produced *in vitro* can be frozen successfully but the pregnancy rate is still low — 37% per recipient and 33% per embryo transferred. Total embryonic loss was 46%, of which 31% occurred between days 21 and 35. The cumulative effects of culture and freezing could explain this high mortality rate.

Conclusion

Within the last decade important progress has been made in the control of *in vitro* maturation, *in vitro* fertilization and *in vitro* culture of cattle embryos. This control has to be enhanced but it is clear that we are now able to easily manipulate *in vitro* very early bovine embryos in high quantities. This means that in the near future we will have at our disposal the needed amounts of embryos to try and improve transgenesis and cloning in cattle. This could have very important economical consequences in the livestock production of the 21st century and could also lead to a better understanding of the mechanisms involved in early development.

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