

***IN VITRO* PRODUCTION OF RUMINANT EMBRYOS: RESULTS, LIMITS AND PERSPECTIVES**

P. Mermillod*, Y. Locatelli, R. Dalbiès-Tran, S. Uzbekova, G. Baril, F. Guignot, C. Perreau, N. Poulin, J. L. Touzé, S. Pennetier, B. Schmaltz, and Y. Cognié

INRA, UMR Physiologie de la Reproduction et des Comportements, INRA, CNRS, Université de Tours, Haras Nationaux, 37380 Nouzilly, France

* Corresponding author e-mail : Pascal.Mermillod@tours.inra.fr

ABSTRACT

After artificial insemination and multiple ovulation and embryo transfer (MOET), in vitro production of embryos (IVP) represents the third generation of techniques aimed at a better control of animal reproduction. This technique involves four major steps (Figure 1): oocyte collection, oocyte in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro development of the resulting embryos (IVD). These different steps are now well established in domestic ruminant species (cattle, sheep and goat) although the variability of the number and quality of the oocytes collected and the low viability of frozen – thawed in vitro produced embryos still limit the large-scale use of this promising technology. Beyond the potential use of IVP in breeding schemes, this technique is also required for the establishment of new biotechnologies such as cloning and animal transgenesis. Additionally, the knowledge of oocyte and embryo physiology acquired through IVP techniques may stimulate the further development of other techniques such as MOET.

KEY WORDS: Blastocyst, Development, Fertilization Freezing, Maturation, Oocyte, Transfer.

INTRODUCTION

The knowledge and use of reproductive technologies have been increasing worldwide all along the second part of the 19th century in most domestic mammalian species. First generation technologies (estrous synchronization, semen collection, semen freezing and artificial insemination) are now commonly used in domestic species breeding.

The second generation of reproductive techniques have then been introduced and their use have been promisingly increasing until early nineties. This generation included embryo based techniques, i.e. female superovulation, embryo recovery, freezing and transfer to recipients. At that time, as breeding industry was facing an increasing competition context worldwide, several health crisis, i.e. Bovine Spongiform Encephalopathy (BSE) and foot and mouth disease, negatively impacted the breeding economy and reduced the expansion of assisted reproductive techniques in European countries.

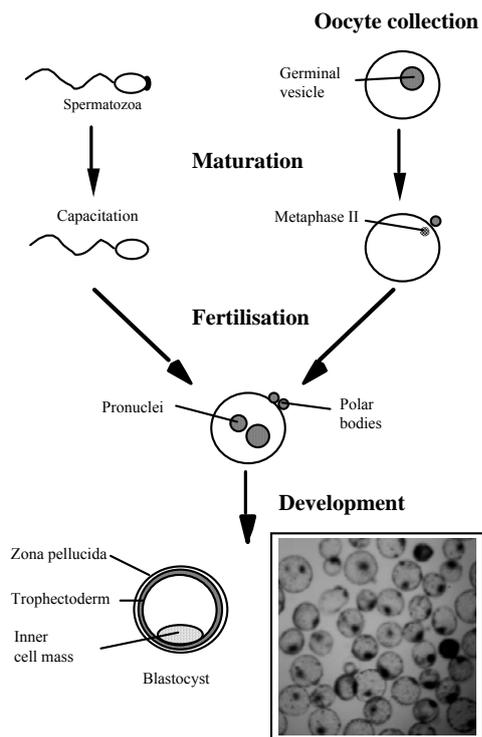


Figure 1. Major steps of in vitro embryo production (IVP)

In addition, following these health crisis, there was an increasing rejection by consumers and public opinion of the use of animal originated products (such as pituitary extracts) in animal breeding and husbandry. This rational concern has been triggered by a more philosophical claim for “green” products obtained in highly natural conditions (i.e. with lowest as possible human intervention). This increasing demand for high quality and natural products by consumer, has progressively decreased the need for advanced reproductive techniques aimed at improving herds productivity.

Basically, IVP includes three major steps (Figure 1) : in vitro maturation (IVM), in vitro fertilization (IVF and in vitro development (IVD) of the resulting embryos. However, primary oocytes collection should be added upstream of these major steps and embryo management (freezing, transfer) should be added downstream to give a complete overview of the whole process. Since the birth of the first IVP calf in 1982, thanks to intensive research programs worldwide, cattle IVP has done significant progress. However, some residual shortcomings are still limiting the larger commercial use of this promising technique.

The focus of this review paper will be to describe the different steps of IVP and giving the results currently obtained. We will also describe the main limitations impairing a wider use of this promising technology and provide some research directions that may allow to overcome these limitations in the future. Bovine species will be presented as a general models but interesting particularities of other ruminant species will be also described.

OOCYTE COLLECTION

For the set up of the techniques as well as for the large scale production of average genetic merit embryos, large quantities of material can be obtained at low cost by collecting ovaries at slaughterhouses. These ovaries are shipped to the laboratory (dry or in warmed saline) where the content of the follicles is aspirated. The needle used as well as the aspiration vacuum are important factors in determining the number and quality of the oocytes collected. These parameters have to be established for each species. In the cow, good results can be obtained with 18 gauge needle connected to a 3 cm Hg vacuum. Alternatively, the ovaries may be sliced with a razor blade and washed with PBS to

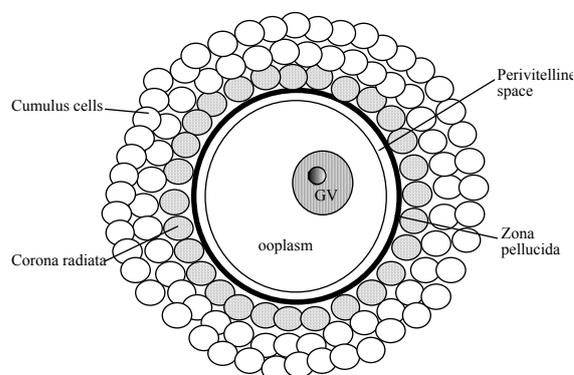


Figure 2. Schematic representation of a cumulus oocyte complex. Oocyte diameter is about 120 mm.

collect the oocytes. An average of 5 to 10 oocytes can be collected from each bovine ovary (Mermillod *et al.* 1992) and one to two from sheep or goat ovaries during the breeding season (Cognie 1999). After aspiration or slicing, the collected fluid is screened under a stereomicroscope to select the oocytes. The oocytes are surrounded by several layers of somatic cells (cumulus oophorus, Figure 2). These cells establish contacts together as well as with the oocyte to allow cell communication through gap junctions, which is necessary for efficient maturation of the oocyte (Van Soom *et al.* 2002). Only cumulus oocyte complexes (COC) with complete and compact cumulus investment should be selected (Leibfried and First 1979; Stangl *et al.* 1999). Cumulus expansion observed at oocyte collection may reflect follicular atresia or premature meiotic resumption in the oocyte. Cresyl brilliant blue vital staining could be used for better selection of COC according to the glucose-6-phosphate dehydrogenase activity in cumulus cells, that reflects their vitality and functionality (Pujol *et al.* 2004). The morphology of the ovaries themselves may also help to predict the developmental potential of enclosed oocytes (Gandolfi *et al.* 1997).

To use this technique in a breeding scheme, the COC could be obtained from females of high genetic merit. This could be done after accidental culling of the female or at the end of her life to ensure a last reproduction (in the same way as described for slaughterhouse oocytes) or from living female, through the Ovum Pick Up (OPU) technique.

In the bovine, OPU is mainly done by transvaginal aspiration of follicle content under ultrasonographic control. This technique has been developed in humans and has been then adapted in cattle (Pieterse *et al.* 1988). Aspiration parameters are as important in OPU procedure than they are during aspiration of oocytes from slaughterhouse ovaries. Increasing aspiration flow increases oocyte recovery rate but decreases oocyte quality (Bols *et al.* 1997). The ratio of oocyte collected from aspirated follicles is usually around 60-70 %. OPU could be performed as frequently as twice a week, with or without hormonal stimulation of follicular growth, over several months without affecting further reproductive life of the donor (Bols, 2005; van Wagendonk-de Leeuw, 2006). It has been shown that two OPU sessions per week allow the collection of oocytes of higher quality, translating into higher rate of development to the blastocyst stage as compared with one session per week (Chaubal *et al.* 2006). This may be due to the appearance between weekly sessions of a dominant follicle which negatively influences the quality of oocytes in subordinate follicles. Indeed, the removal of the dominant follicles by OPU between two weekly sessions increased the blastocyst yield. An average of ten oocytes per cow and 8 per heifer may be collected at each OPU session in a twice a week protocol without stimulation, resulting in an average of 2.5 transferable embryos per cow per session and 1.5 per heifer (Galli *et al.* 2003). This protocol presents the advantage to avoid the repeated use of exogenous gonadotropins which could induce several reproductive pathologies and may lead to subfertility. In goat, it has been shown that LOPU could be repeated up to five times at various intervals without decrease in efficiency (Pierson *et al.* 2005). However, we have shown that repetition of LOPU on a weekly basis in goat under pFSH stimulation progressively decreases oocyte recovery (Baril *et al.*, unpublished data). This decrease may be due to the repeated starvation of the donors before LOPU.

Alternatively, OPU could be done on a weekly basis after FSH priming with similar yield of total good quality oocytes. After one shot FSH stimulation (80 mg i.m. and 120 mg s.c. of pFSH), it is possible to perform two successive OPU sessions at 30 h and 126 h after FSH injection with good embryo production rate (Chaubal *et al.* 2006). The combined intra muscular and subcutaneous administration of FSH allows a rapid stimulation (i.m.) as well as

a sustained release of the hormone (s.c.) that avoid repeated handling of animals and ensure continuous follicular growth stimulation in a twice a week stimulation protocol. In the case of stimulated protocols with multiple injections (300 mg pFSH i.m., 6 injections 12 h apart), it has been shown that a coasting period of 48 h allows to increase the rate of oocytes reaching the blastocyst stage, specially when LH (Lutropin, Vetrepharm, Canada, i.v.) was administered 6 h before OPU (Blondin *et al.* 2002). We did not find similar effects of this coasting period in goat LOPU (Baril *et al.*, unpublished results). In young heifers (2-3 months of age), good IVP results (up to 3 transferable embryos per calf) could be obtained after eCG stimulation followed by oocyte collection under laparotomy (Galli *et al.* 2004).

Table 1. Embryo production after OPU in a twice a week unstimulated protocol (adapted from Galli *et al.* 2003).

	OPUs	Oocyte (per OPU)	Cleavage	Embryos (per OPU)
Cows	2,641	26,532 (10.0)	70.9 %	7,403 (2.8)
Heifers	2,143	16,786 (7.8)	67.0 %	3,284 (1.5)
Total	4,784	43,318		10,687

In small ruminants, OPU may be done by laparotomy or, less invasively, by laparoscopy guided aspiration of ovarian follicles (Baldassarre *et al.* 2002; Cognie *et al.* 2004). In laparoscopic ovum pick-up (LOPU), aspiration conditions (size of the needle, bevel shape, aspiration flow, tubing internal diameter) should also be controlled and adapted to the species, as in OPU, to improve recovery rate and oocyte quality (Rodriguez *et al.* 2006). Good results can be obtained by LOPU in goat and sheep with or without ovarian stimulation by FSH or eCG (Baldassarre *et al.* 2003; Morton *et al.* 2005).

In wild ruminant species, oocyte recovery technique should be adapted to the size of the donor. Transvaginal OPU could be used in large deer species such as wapiti and red deer (Berg and Asher, 2003), whereas LOPU is recommended for smaller animals like sika deer. In sika deer, it has been shown that LOPU after oFSH stimulation of ovarian activity allows the recovery of good quality oocytes and in vitro production of viable embryos all year long, even during the non breeding season (Locatelli *et al.* 2006).

IN VITRO MATURATION

Nuclear maturation

In the ovary, all primary oocytes are blocked at the prophase stage of the meiotic cycle. The block of meiotic progression during follicular growth is controlled by unknown factors produced by the surrounding somatic cells (Mermillod and Marchal, 1999). At this stage, the round shaped nucleus, called the germinal vesicle (GV), contains diffuse chromatin. During early folliculogenesis, the oocyte grows, undergoes strong morphological modifications and stores molecules (proteins, RNAs) that will be determinant for the success of fertilization and early embryo development. The zona pellucida, a glycoprotein envelope surrounding the oocyte membrane, is synthesized by the oocyte during this period of growth. The zona

pellucida has important function in regulating fertilization and protection of the embryo until hatching. The oocytes resume meiosis and progress to the metaphase II stage only if they encounter a gonadotropin surge in the preovulatory follicle. They stop their progression at this MII stage until fertilization in the oviduct, after ovulation.

Oocytes that are collected from growing follicles for IVP are blocked at the prophase stage of meiosis. As soon as they are removed from the follicular inhibitory environment, meiotic resumption occurs spontaneously and progress to metaphase II (Sirard, 2001). This spontaneous meiotic resumption of the oocyte outside of the follicle is the basis of in vitro maturation.

Cytoplasmic maturation

Cytoplasmic maturation covers all morphologic and molecular events accompanying nuclear maturation after LH surge in preovulatory follicles and preparing oocyte cytoplasm to successful fertilization and embryo development. Cytoplasmic maturation includes well known morphological modifications, such as the migration of cortical granules in the cortical region of the ooplasm (Cran, 1989). These granules are stored during oocyte growth and release their enzymatic content in the perivitelline space after fertilization. These enzymes modify the structure of the zona pellucida, preventing the penetration of additional spermatozoa. Meiotic competence is acquired during early folliculogenesis, soon after the apparition of the antral cavity in the follicle (Mermillod *et al.* 1999). However, after this acquisition, the oocyte requires a further differentiation period during late follicular growth to reach the full competence for cytoplasmic maturation (Sirard *et al.* 2006). This late differentiation occurs under the meiosis inhibiting signal sent by somatic follicular cells that maintain the meiotically competent oocyte at the prophase stage.

During IVP, more than 90% of the oocytes collected from follicles larger than 3 mm in cattle are able to complete nuclear maturation. However, few of them are competent for cytoplasmic maturation. Consequently, only 30 to 40% of the oocytes reach the blastocyst stage after IVF and IVD (Figure 3). This contrast highlights the functional importance of cytoplasmic competence (Sirard *et al.* 2006). When oocytes harvested from larger follicles or in vivo matured oocytes are processed under the same IVF – IVD techniques, the success rate is increased (Hue *et al.* 1997; Lequarre *et al.* 2005; Marchal *et al.* 2002). Ovarian stimulation by gonadotropins increases the number of follicles growing to large size classes by protecting them against involution through the atresia process. Consequently, ovarian stimulation increases the number of competent oocytes collected through the OPU process.

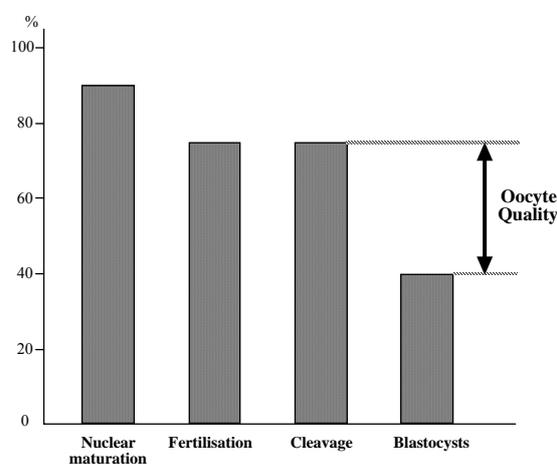


Figure 3. Success rates of the successive IVP steps.

Increasing developmental competence during late follicular growth is suspected to be related to storage in oocyte of transcripts that will be necessary for the success of early embryo development. Indeed, in ruminant species, embryo genome activity only starts after the third embryo cell cycle (8-16 Cell stage, (Memili *et al.* 1998). Transcription activity has

been studied in growing oocytes by following ribosomal RNA synthesis (Hyttel *et al.* 2001). These studies allowed to establish that the oocyte is transcriptionally quiescent in primordial follicles. RNA synthesis appears in secondary follicles, peaks in tertiary follicles, at later stages of oocyte growth, and decreases thereafter to return to undetectable levels. Meiotic resumption in late preovulatory follicle is preceded by a short burst of RNA synthesis (Memili *et al.* 1998) which seems to be functionally important for the success of embryo development, as evidenced by α -amanitin (inhibitor of RNA polymerase II) inhibition of transcription at this stage. Several maternal effect genes, such as Mater (Maternal Antigen That Embryo Require) or zygote arrest 1 (Zar1) have been identified in mouse oocytes (Tong *et al.* 2000; Wu *et al.* 2003). The storage of these maternal transcripts in oocytes is required to allow embryos to develop beyond the 2-Cell stage. Orthologues of these genes have been recently identified in oocytes from domestic species (Dalbies-Tran *et al.* 2005; Pennetier *et al.* 2006; Pennetier *et al.* 2004) and their expression profile has been characterized (Pennetier *et al.* 2006). In addition, cDNA arrays are used for a more comprehensive analysis of oocyte's expression patterns. These studies rely on the use of both heterologous arrays (Dalbies-Tran and Mermillod, 2003) or specific arrays builded from SSH selected oocyte specific cDNA banks (Pennetier *et al.* 2005; Sirard *et al.* 2005; Vallee *et al.* 2006). These studies have allowed the identification of several hundreds of genes expressed in the bovine oocyte, some of them being preferentially expressed in oocyte (and sometimes testis) and not detected in other tissues. However, up to now, none of these genes has been identified as a key element of oocyte developmental competence acquisition during late folliculogenesis.

In vitro maturation

In vitro maturation is probably the most critical part of the whole process of in vitro embryo production. Ruminant oocytes are usually matured at 39°C under a 5% CO₂ in a humidified atmosphere. The optimal maturation time (more than 90% of the oocytes at the metaphase II stage) is 22-24h. Due to the high lipid content of oocytes cytoplasm, it is not possible to follow the progression of their nuclear status during culture. Additionally, the presence of the cumulus cells is required during IVM for efficient cytoplasmic maturation and these cells mask the oocyte. Consequently, the only visible sign of oocyte maturation during IVM is the expansion of the cumulus cells. These cells produce hyaluronic acid which is secreted and polymerized in the extracellular matrix, leading to the increase of intercellular space.

Several culture media have been proposed for IVM (MEM, Waymouth, Ham-F12). However, the most widely used seems to be the TCM199 medium, bicarbonate buffered and containing minerals, carbon and energy sources (glucose, glutamine) as well as vitamins and amino acids. The medium is generally supplemented with high molecular weight molecules that exert a surfactant effect (BSA) and provide hormones and growth factors (fetal calf serum, serum of female in estrus, follicular fluid). These complex additives from animal sources may raise sanitary questions due to possible presence of pathogen agents, and decrease the reproducibility of experiments. They may be successfully replaced by high molecular weight polymers (Ali and Sirard, 2002) for the surfactant effect (such as polyvinyl alcohol) and by cocktails of purified or recombinant hormones and growth factors (Lonergan *et al.* 1996).

Hormones are usually added to the maturation medium (FSH, LH, estradiol), in addition to biological fluids. Their exact function is not clearly established, although the effect of FSH on cumulus expansion and metabolism has been clearly established (Sutton-McDowall *et al.* 2004). Estradiol effect on oocyte is exerted through intracellular receptors and seem to be

detrimental to meiotic progression, inducing chromosomal abnormalities (Beker-van Woudenberg *et al.* 2004). Growth hormone (GH) stimulates some aspects of cytoplasmic maturation (cortical granules migration) as well as nuclear maturation, resulting in a higher blastocyst yield (Bevers and Izadyar, 2002). GH action seems to be mediated by cumulus cells (Izadyar *et al.* 1997). Epidermal growth factor (EGF) has been shown to stimulate nuclear and cytoplasmic maturation in a wide variety of species including mouse, pig, cattle, deer goat and sheep (Abeydeera *et al.* 2000; Cognie *et al.* 2004; Comizzoli *et al.* 2001b; De La Fuente *et al.* 1999; Guler *et al.* 2000; Lonergan *et al.* 1996). High blastocysts yields could be obtained after maturation of cattle oocytes in TCM199 only supplemented by EGF. EGF action is exerted, at least partly, directly on the oocyte and recent studies showed that EGF family of growth factors (epinephrin, amphiregullin, betacellulin) could be produced by granulosa cells under gonadotropins stimulation and could thus mediate LH signaling inside the follicle, transmitting the ovulatory signal to the oocyte (Park *et al.* 2004). Insulin like growth factor-I (IGF-I) has also a positive effect on cytoplasmic maturation of cattle oocytes (Bevers and Izadyar 2002). Indeed, the regulation of IGF-I and II through the controlled expression and degradation of IGF binding proteins could be one of the major processes of control of late follicular growth (Mazerbourg *et al.* 2003).

Another important aspect of COC environment during IVM is the antioxydative potential of the IVM medium (Ali *et al.* 2003). As all living cells, oocyte is sensitive to reactive oxygen species (ROS) that could induce detrimental chemical reactions (lipid or protein peroxydation). To overcome possible ROS detrimental effects during culture, antioxydative molecules (β -mercaptoethanol, cystein, vitamin A C, E), divalent cations chelators (EDTA, taurine, hypotaurine, transferrine) or ROS scavengers (superoxyde dismutase, catalase) could be added to maturation medium (Blondin *et al.* 1997; Ikeda *et al.* 2005; Kobayashi *et al.* 2006). However, COC are capable to produce their own protection system by secreting ROS scavenger enzymes like superoxyde dismutase and catalase (Cetica *et al.* 2001) or through the production of intracellular reducers molecules like glutathion (de Matos and Furnus, 2000). Stimulating these endogeneous protection systems may help oocyte to survive challenging in vitro conditions. The addition into IVM medium of cysteamine, a cell permeable precursor of cysteine, itself involved in glutathion (GSH) synthesis, has been shown to increase GSH concentration in oocytes and, in turn, to increase the resulting developmental competence after IVM in different species (Cognie *et al.* 2004; de Matos and Furnus, 2000; de Matos *et al.* 2002; Gasparrini *et al.* 2003; Khatir and Anouassi, 2006). Surrounding cumulus cells are also contributing to establishment of high GSH content in the oocyte (Luciano *et al.* 2005). Therefore, stimulating cumulus cells may also help to protect oocytes against environment ROS.

It has been established for a long time that the presence of cumulus cells is required for the success of oocyte cytoplasmic maturation and fertilization (Gilchrist *et al.* 2004). Therefore, maintaining the viability and functionality of cumulus cells is likely to be another important challenge for IVM success. Oocyte secreted factors (ESF) are known to drive cumulus cell differentiation during follicular growth (Li *et al.* 2000) and to allow cumulus expansion during oocyte maturation (Dragovic *et al.* 2005). The precise identification of the active factors is not fully established. However, GDF-9 and BMP-15, two members of the TGF- family of growth factors, are specifically produced by the oocyte and their effect on cumulus cells, and in turn on matured oocyte developmental competence, have been recently established (Hussein *et al.* 2006). Since these factors are able to protect cumulus cells against apoptosis that appear in culture (Hussein *et al.* 2005), their addition to IVM medium may be recommended, at least to help at maintaining cumulus functionality in culture.

IN VITRO FERTILIZATION

The techniques used for in vitro fertilization are well established in cattle as well as in small ruminants. The media used for IVF are Tyrode derived saline supplemented with BSA, lactate and pyruvate and with a capacitating agent (Parrish *et al.* 1986). Sperm capacitation occurs physiologically in the female genital tract. This phenomenon involves modification of spermatozoa membrane as well as changes in its mobility properties that lead to spermatozoa fertilization ability. Many protocols support sperm capacitation in vitro. In cattle, heparin is widely used for this purpose, usually without pre treatment (Parrish *et al.* 1989). In small ruminant, spermatozoa are usually capacitated by preliminary treatment with serum collected from females in estrus (Cognie *et al.* 2004). Frozen semen is generally used and a selection step is required to enrich the sperm suspension in living and motile spermatozoa. In the swim-up method, spermatozoa are deposit at the bottom of a culture medium containing tube and allowed to swim in the medium. The top fraction, containing motile sperms is then collected after a given incubation time. Alternatively, motile sperms may be purified by centrifugation in a discontinuous density gradient (Percoll). The motile fraction is collected at the bottom of the tube after centrifugation.

The sperm concentration used for IVF ranges between 0.5 and 2×10^6 spermatozoa per ml in fertilization medium. This concentration should be optimized for each male and even for each ejaculate from a given male. The fertilization rate increases together with sperm concentration. However, after a given value, an increasing proportion of the oocytes become polyspermic. A compromise has to be established for high fertilization rate with low polyspermy rate. For better convenience, sperm and oocytes are usually co-incubated for 18-24h, although a six hours incubation time seems to be sufficient to reach the higher level of normal fertilization.

A strong male effect has been reported on the success rate of IVF and, in turn, IVF may be used as a tool to evaluate male (or ejaculate) fertility (Marquant-Le Guienne *et al.* 1990; Papadopoulos *et al.* 2005). In addition, heterologous oocytes may be used to evaluate ejaculate fertility of males from rare species (Comizzoli *et al.* 2001a). Different males may provide different cleavage rates. Additionally, males providing similar fertilization rates may also differ in their ability to promote the development to the blastocyst stage. These differences between males may be related to their respective ability to up regulate glucose metabolism during G1 phase at pronuclear stage (Comizzoli *et al.* 2003) and consequently to allow earlier onset of the S phase in the zygote, translating into earlier cleavage and higher development rate (Comizzoli *et al.* 2000a). Therefore, each male to be used in IVF has to be tested for optimized fertilization conditions and for its ability to promote embryo development. In the case of OPU-IVP with high genetic merit females, the bulls' ejaculates to use should previously be tested on slaughterhouse oocytes.

IN VITRO DEVELOPMENT

In standard cell culture conditions, ruminant embryo development blocks at the 8-16 cell stage. This block was first overcome through the use of co-culture systems involving oviduct epithelial cells (Eyestone *et al.* 1991) or other cell lineage (Myers *et al.* 1994) in standard tissue culture media (TCM199). The effect of cells has not been clearly established. They may produce some growth factors positively regulating embryo development or remove toxic media components (Killian 2004). In a second step, new culture media have been designed to more exactly fit embryo requirements without the need for co-culture. One of the first media

designed for this purpose was synthetic oviduct fluid or SOF (Fukui *et al.* 1991). The composition of SOF was established from the composition of bovine oviduct fluid. However, SOF or SOF modifications have also been successfully used in small ruminant species. More recently, sequential media have been proposed to fit the evolution of embryo requirements during early development (Gardner *et al.* 2000). For example, glucose seems to be toxic to early cleaving embryos, whereas morula need some glucose in the medium for further development (Donnay *et al.* 2002; Gardner *et al.* 2000).

Semi defined media represent an easy way to produce mammalian embryos *in vitro*. However, embryo quality (viability after cryopreservation and/or transfer to recipient) appears to be low after development in such systems (Garcia-Garcia *et al.* 2006). On the other hand, co-culture systems may be more complicated to manage but allow the production of higher quality embryos. Therefore, some commercial bovine IVP teams still use co-culture system or embryo development in the oviduct of transient recipient ewes. In this case, the embryos are transferred in the oviduct of synchronized ewes just after IVF and surgically collected seven days later (Galli *et al.* 2004). This IVP system allows the production of embryos with similar quality and cryotolerance as *in vivo* collected ones. Recently, as we were trying to produce deer embryos, we observed that these embryos were not able to develop beyond the morula stage in the conventional SOF development system we use in our laboratory for different domestic ruminant species. To allow sika and red deer embryos to reach the blastocyst stage, we have been obliged to develop a co culture system involving ovine oviduct epithelial cell (oOEC) monolayers in SOF medium (Table 2). This culture systems allows the production of viable embryos since we obtained kids after transfer of fresh or frozen/thawed IVP red deer embryos produced in theses conditions (Locatelli *et al.* 2005; Locatelli *et al.* 2006). This observation in deer species highlight the function of the oviduct in the regulation of early embryo development and encourage research in view to identify and use oviduct originated embryotrophic factors to increase the efficiency of IVP (Killian, 2004).

Table 2. Cleavage on Day 2 and development on Day 8 of *in vitro* produced red and sika deer embryos in SOF medium with or without ovine oviduct epithelial cell (oOEC) support (adapted from Locatelli *et al.* 2005; Locatelli *et al.* 2006).

Species	oOEC	Oocytes (n)	Cleavage (%)	Blastocysts (%)
Red deer	-	64	66	3
	+	93	63	25
Sika deer	-	62	84	0
	+	68	82	28

Blastocyst rate was calculated from total oocyte entering into IVM.

The development usually takes place at 39°C in droplets of culture medium overlaid with oil for 7 to 9 days. A high density of embryos per volume unit (one embryo per µl) seems beneficial to development (Ferry *et al.* 1994). When embryos are cultured without cell support, the oxygen concentration in atmosphere should be reduced to 5% to avoid induction of oxidative stress on the embryos (Olson and Seidel, 2000; Voelkel and Hu, 1992). As

during IVM, addition of antioxydant molecules may increase rate or quality of IVP embryos (Bing *et al.* 2003; Dalvit *et al.* 2005; Takahashi *et al.* 2002). Serum is usually added to the culture medium from the beginning or after the first cleavages (48h post insemination). The presence of serum accelerates the development of embryos but seems to negative effects on their quality (Rizos *et al.* 2003) translating into fetal and placental abnormalities (Large Offspring Syndrome, see below) reported during pregnancy (Thompson *et al.* 1995). The rate of cleavage is usually high (over 80% of the total oocytes), when appropriate bull and fertilization conditions are used. However, the rate of development to the blastocyst stage plateaus at around 40% of the total oocytes. It has been clearly established that oocyte quality (intrinsic quality of primary oocyte coupled to maturation conditions used) determine the rate of blastocyst production, whereas development environment is determinant for embryo viability (Rizos *et al.* 2002).

The pregnancy rates obtained after transfer of fresh IVP embryos are usually good (40 to 50% of calves born). Some problems of calf abnormality and perinatal mortality have been reported (Farin *et al.* 2004; Farin *et al.* 2006; Kruij *et al.* 2000). However, these problems are less frequent than those observed with cloned embryos and seem to be linked to in vitro environment and to the presence of serum in the culture medium that induces chromosomes aberrations, modifications of embryo gene expression pattern and metabolism (Lazzari *et al.* 2002; Lonergan *et al.* 2004; Rizos *et al.* 2003).

The main limitation to a wider use of OPU – IVP technique in breeding schemes is the low cryoresistance of the resulting embryos. Although well established techniques of embryo freezing are described in cattle, sheep and goat and provide good results of pregnancy rate after transfer of the thawed embryos, these techniques appeared sub-optimal for the cryopreservation of IVP embryos. Several alternative methods of embryo cryopreservation have been proposed to overcome the low viability of these embryos. Some vitrification procedures (high speed cooling with high concentration of cryoprotectants leading to amorphous solidification without ice crystals formation) appeared to partially solve the problem. However, the quality of the embryo which is determined by the culture condition, appears to be one of the critical factors. Similar pregnancy rates have been observed after 10% glycerol slow freezing for in vitro fertilized embryos grown in sheep oviduct than for in vivo derived embryos (Galli *et al.* 2004). This indicates that culture conditions still remain to be improved to better mimic the in vivo conditions.

POTENTIAL INTERESTS OF IVP

Mass embryo production

The mass production of embryos from oocytes collected in the ovaries of slaughtered females may be of interest in some breeding schemes. For example, ovaries from the same breed may be pooled at slaughterhouse and used to produce embryos of a defined breed without precise knowledge of their parental origin, except breed. These embryos produced at low cost may be used to produced valuable beef calves in dairy herds (“beef from dairy”). This allows to increase the financial value of calves in these herds and may help solving fertility problems in high producing dairy herds (Galli *et al.* 2003; Hansen, 2006). Similar methods could also be used to produce average genetic value low cost dairy or beef embryos to help the diffusion of genetic progress toward developing countries. A similar approach has been used with success to reconstitute genetics of herds culled for sanitary reasons due to infections like tuberculosis or to BSE suspicion (Galli *et al.* 2003). In addition, IVP from

slaughterhouse oocytes represent the most convenient way to develop basic research on oocyte maturation and cell cycle regulation, gametes recognition and fusion and regulation of early embryo development. Most recent knowledge of gene expression patterns and gene function in these mechanisms have been made possible thanks to slaughterhouse material.

Production of embryos from selected donors

Oocyte collection by OPU coupled to IVP offers several advantages over more traditional *in vivo* embryo production after superovulation and artificial insemination as applied in MOET schemes (Bols, 2005; Galli *et al.* 2004). In 2003, the International Embryo Transfer Society (IETS, reported 106,220 transfers of IVP cattle embryos worldwide (Hansen, 2006). Indeed, the number of embryos produced through OPU-IVP technique from a given female over a given period of time is higher than the number that may be collected *in vivo*. Indeed, OPU could be performed twice a week for long period of time without altering the reproductive potential of the females whereas several weeks or months of rest are required between two treatments of ovarian stimulation. In addition, in small ruminants, the surgery required for embryo collection *in vivo* induces tissue adhesions that could impair future embryo production, whereas LOPU is less invasive and could be repeated during long periods of time (Baldassarre and Karatzas, 2004). OPU can be performed in young females (from 3 months in age through laparoscopy) as well as in old cows. In addition, OPU could be continued during the first trimester of pregnancy without risk for the fetus, and it could be also performed on animals with some pathologies of the ovary (ovarian cysts), oviduct or uterus that prevent *in vivo* embryo production or even compromise fertility (Galli *et al.* 2003). OPU-IVP also allows the use of different males for producing the embryos (different males could be used for the oocytes from one aspiration session or from different OPU sessions). Furthermore, OPU-IVP could be beneficial in the case of females which are not responding to ovarian stimulation.

Wild species

Residual population of wild species maintained in captivity often encounter reproductive disorders that impair the amplification of the herd in view of reintroduction of animals in their natural environment or for the simple maintenance of these captive residual populations (Comizzoli *et al.* 2000b; Pukazhenthii *et al.* 2006). In addition, techniques of assisted reproduction like artificial insemination or embryo transfer may help to maintain proper management of scarce genetic diversity of few remaining individuals scattered between different locations. In this view, LOPU-IVP presents several advantages over MOET, like in domestic species and, in addition, it allows to reduce stress generating manipulations of wild animals (Locatelli *et al.* 2006).

MAJOR LIMITATIONS AND PERSPECTIVES

There are still some limitations to the extended commercial use of IVP (Faber *et al.* 2003; Hansen, 2006). The low success rate of development to the blastocyst stage is increasing the cost of each embryo produced. Additionally, some females appear to be poor oocyte donors in terms on both quantity and quality (developmental potential). The embryos produced by this technique have a low viability following cryopreservation and should be transferred fresh to get an optimized pregnancy rate. This limitation implies the disposition of a large recipient herds and the synchronization of recipients at each oocyte collection to prepare fresh transfer

of resulting embryos, and consequently increases the overall cost of the technique. In addition, the possibility of embryos transportation is limited and embryo storage is not recommended.

Active research in several directions may help to overcome these limitations in a near future. Manipulations of ovarian stimulation protocols have shown that increasing late intrafollicular differentiation of the oocyte allows to increase its developmental potential. For example, introducing a coasting period after FSH stimulation of ovarian activity increases the rate of oocyte development to the blastocyst stage up to 80% (Blondin *et al.* 2002). This late differentiation appears to be critical to oocyte competence (Lequarre *et al.* 2005) and several authors tried to mimic this differentiation in vitro. Since meiotic resumption appears spontaneously in vitro, after suppression of follicle induced meiotic inhibition, and rapidly blocks transcriptions suspected to support developmental competence, mimicking late oocyte differentiation in vitro requires artificial maintenance of meiotic block (Mermillod *et al.* 1999). Attempts to recreate in vitro a follicular-like environment by adding follicular cells together with COC, provided limited successes (Sirard, 2001; Sirard and Bilodeau, 1990). More recently, it has been established that kinase inhibitors specifically blocking the kinase activity of M-phase Promoting Factor (MPF, key regulator of G2 – M transition of the eukaryotic cell cycle) were able to reversibly maintain cattle oocyte at the GV stage in culture (Lonergan *et al.* 2000; Mermillod *et al.* 2000). However, these techniques of meiotic inhibition failed to increase competence after a 24 h pre maturation culture of the oocytes at the GV stage. Nevertheless, these approaches allowed to establish that numerous signaling pathways are involved in oocyte cell cycle control (Vigneron *et al.* 2003; Vigneron *et al.* 2004a; Vigneron *et al.* 2004b). The increasing knowledge of this control will allow to derive more valuable methods of oocyte meiotic inhibition in the future. In addition, future studies will benefit from the increasing knowledge of the expression of genes related to developmental competence of the oocyte (Albertini *et al.* 2003).

Finally, some abnormalities have been reported during pregnancies after transfer of IVP embryos that have been called large offspring syndrome or LOS (Farin *et al.* 2006). This translates into abnormally large offspring after longer gestation time and higher rate of perinatal mortality. In sheep, this syndrome has been clearly related to the presence of serum in embryo culture medium (van Wagtendonk-de Leeuw *et al.* 2000) and may result from the maintenance of gene expression alterations induced by culture environment during early development stages (Lazzari *et al.* 2002). These problems may be overcome by using more physiological conditions for IVD. This may be achieved by developing the embryos into the oviduct of transient recipient ewes (Galli *et al.* 2004). However, recent progress in the comprehension of oviduct physiology (Killian, 2004) may allow to develop in vitro culture conditions mimicking in vivo environment to increase embryo quality. In addition, the increasing knowledge of embryo gene expression pattern and its regulation will allow to use marker genes to follow more precisely the effect of modification of embryo environment (Lonergan *et al.* 2006).

CONCLUSIONS

In conclusion, IVP is a very promising technique that could find numerous applications in assisted reproduction of ruminant domestic and wild species. It also offers unique occasion to study early mechanisms controlling reproductive physiology and open the way to advanced biotechnology such as nuclear transfer cloning and transgenesis. The low quality of oocytes recovered from small growing follicles as well as the low resistance of IVP embryos to conventional freezing methods still limit the use of IVP in breeding schemes. Improving IVP

conditions in the future will require strong research efforts in view to improve our knowledge of oocyte and early embryo physiology and gene expression and to use this knowledge to develop in vitro treatments more adapted to precise requirements of oocytes and early embryos.

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