# In Vitro Production of (Farm) Animal Embryos

12

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**Abstract**

Over the past decades, in vitro production (IVP) of bovine embryos has been significantly improved, and in particular bovine IVP is now widely applied under field conditions. This in vitro technique provides new opportunities for cattle producers, particularly in the dairy industry, to overcome infertility and to increase dissemination of animals with high genetic merit. Improvements in OPU/IVP resulted in large-scale international commercialization. More than half a million IVP embryos are generated on the yearly basis demonstrating the enormous potential of this technology. These advances and the fact that bovine and human early development is remarkably similar have prompted the use of bovine embryos as a model system to study early mammalian embryogenesis including humans. In horses, OPU/IVP is also an established procedure for breeding infertile and sports mares throughout the year. It requires ICSI because conventional IVF does not work in this species. In small ruminants, application of IVP on the commercial and research basis is low compared to other livestock species.

Despite all the improvements, embryos generated in vitro still differ from their in vivo-derived counterparts. Embryos must adjust to multiple microenvironments at preimplantation stages. Consequently, maintaining or mimicking the in vivo situation in vitro will aid to improving the quality and developmental competence of the resulting embryo.

The successful clinical application of the techniques in reproductive biotechnology requires both species-specific clinical skills and extensive laboratory experience.

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## 12.1 Introduction

The birth of the first IVF calf derived from an in vivo matured oocyte in 1981 (Brackett et al. 1982) and the discovery of heparin as capacitating agent for bull sperm in 1986 (Parrish et al. 1986) were two key events, ultimately resulting in efficient IVP systems for bovine preimplantation embryos, including in vitro maturation (IVM) of the immature oocyte to the matured metaphase II stage, in vitro fertilization (IVF), and subsequent in vitro culture (IVC) of embryos to the desired stage of development, preferably the blastocyst stage. The first calves produced entirely from IVM-IVF-IVC were born in 1987 (Fukuda et al. 1990).

While artificial insemination (AI) is an effective way to disseminate the genetics of valuable sires, with the implementation of embryo biotechnologies, female genetics can also be distributed worldwide. In the last decades, major advances were made in multiple ovulation and embryo transfer (MOET), ovum pickup (Pieterse et al. 1991; Kruip et al. 1991) combined with in vitro production of embryos (OPU/IVP) and cryopreservation (Colleau 1991).

The current technology of OPU/IVP harvesting immature oocytes from living cows can routinely be performed twice a week for an extended period of time without any detrimental effects on the donor's cow fertility (Chastant-Maillard et al. 2003). At present, the application of IVP combined with ovum pickup (OPU) from valuable donors is increasing (again) due to new breeding strategies based on genomic selection using SNP (single nucleotide polymorphism) chips. Depending on the chip used, thousands of these SNPs can be analyzed even in a biopsy taken from an embryo. This technology is now reaching routine usage for genomic selection (GS) in cattle (Ponsart et al. 2014).

With regard to IVP efficiency (Table 12.1), approximately 80–90% of immature bovine oocytes undergo nuclear maturation in vitro, about 80% undergo fertilization, 30–40% develop to the blastocyst stage, and around 50% of the transferred embryos establish and maintain a pregnancy (Galli et al. 2014; Lonergan et al. 2016; Wrenzycki et al. 2007).

According to the International Embryo Technology Society (IETS) statistics (Fig. 12.1), the number of embryos produced in vitro and transferred into recipients has increased more than 10 times in the last century and is now approaching the numbers of embryos produced in vivo by superovulation. This indicates that OPU and IVP are considered a reliable and cost-effective technique and have acquired a significant role in cattle breeding. Detailed data stemming from the year 2016 are shown in Tables 12.2a and 12.2b.

**Table 12.1** Efficiencies of bovine IVP

| | |
|---|---|
| Maturation rate[a] | 90% |
| Fertilization rate[a] | 75–80% |
| Cleavage rate[a] | 60–70% |
| Blastocyst rate[a] | 30–40% |
| Pregnancy rate[b] | 50–60% |
| Calves born[c] | 70–90% |

[a]calculated on the number of immature oocytes
[b]calculated per embryo transfer
[c]calculated per pregnancy

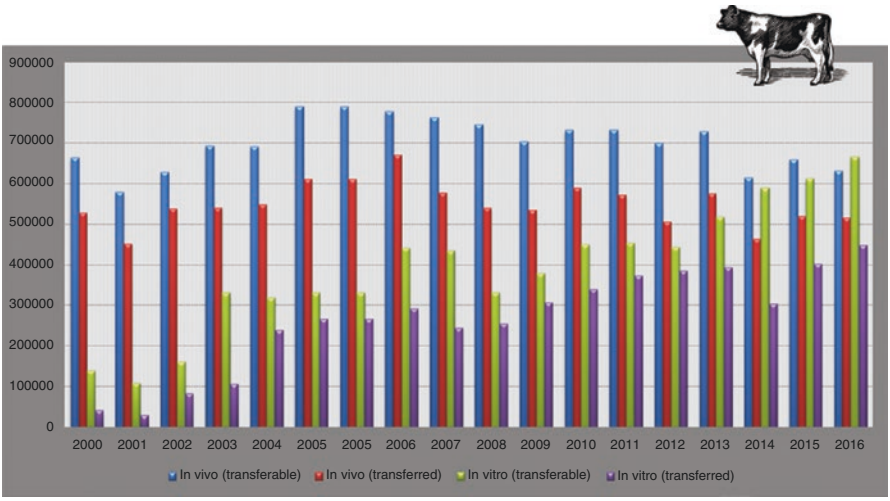


**Fig. 12.1** Bovine embryo transfer statistics published annually by the International Embryo Technology Society (IETS)

**Table 12.2a** Collection and transfer of bovine OPU/IVP embryos by regions (year 2016)

| | Collection | | | Transfers | |
|---|---|---|---|---|---|
| Region | Donors | Oocytes | Embryos | Fresh embryos | Frozen embryos |
| Africa | 619 | 19,062 | 2167 | 379 | 246 |
| Asia[a] | 3177 | 59,224 | 9438 | 3250 | 1164 |
| Europe | 10,651 | 94,407 | 18,879 | 10,424 | 3635 |
| North America | 45,918 | 805,072 | 260,574 | 80,825 | 50,672 |
| Oceania | 2241 | 21,587 | 6304 | 4732 | 1702 |
| South America | 49,739 | 1,138,302 | 378,291 | 230,263 | 65,235 |
| Grand Total | 112,345 | 2,137,654 | 675,653 | 329,873 | 122,654 |

[a]Data from 2015

**Table 12.2b** Collection and transfer of bovine abattoir-derived IVP embryos by regions (year 2016)

| | Collection | | | Transfers | |
|---|---|---|---|---|---|
| Region | Donors | Oocytes | Embryos | Fresh embryos | Frozen embryos |
| Africa[a] | 156 | 2033 | 235 | 0 | 0 |
| Asia[a] | 35,335 | 714,783 | 56,740 | 10,685 | 7831 |
| Europe | 256 | 18,317 | 1095 | 40 | 133 |
| North America[a] | 5 | 9117 | 1037 | 273 | 418 |
| Oceania | 4 | 60 | 16 | 8 | 4 |
| South America | 1345 | 5017 | 1511 | 0 | 0 |
| Grand Total | 37,101 | 749,327 | 60,634 | 11,006 | 8386 |

[a]Data from 2015

## 12.2 General Steps of In Vitro Production of Embryos

Production of embryos in vitro is a three-step process involving IVM, IVF, and subsequent culture of the presumptive zygote to the blastocyst stage (IVC). The procedure of IVP is best developed in cattle. A schematic drawing is shown in Fig. 12.2. Figure 12.3a illustrates the processes occurring during oocyte growth and maturation, ovulation, fertilization, and early embryonic development within the oviduct, finally leading to the blastocyst which has already entered the uterus. Representative pictures of oocytes and early bovine embryos of in vivo and in vitro origin are shown in Fig. 12.3b.

### 12.2.1 Collection of Cumulus-Oocyte Complexes (COC)

COC can be collected from ovaries of slaughtered or euthanized animals or from those of live animals. With slaughterhouse ovaries, COC are usually isolated by aspiration or slicing, less often via isolation and dissection of follicles. With these methodologies, cumulus-enclosed immature oocytes at the germinal vesicle (GV) stage can be harvested. Immature oocytes are also collected from antral follicles of living animals via the OPU technology. This is a noninvasive and repeatable technique which can also be used to collect MII oocytes shortly before ovulation with or without hormonal stimulation similar as in human-assisted reproduction.

OPU without hormonal pre-stimulation can be routinely done twice a week. Twice a week OPU can be done for an extended period of time without detrimental

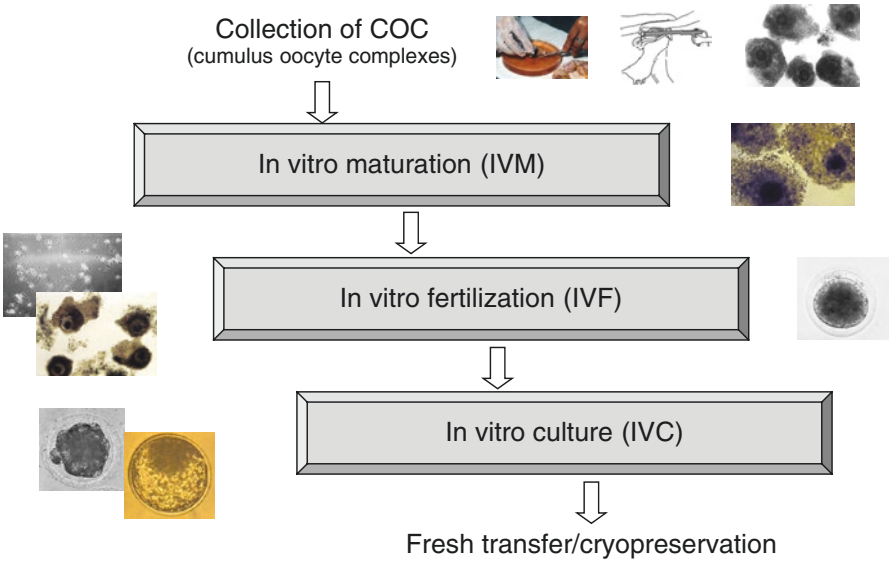


**Fig. 12.2** In vitro production of embryos (for further information, see text)

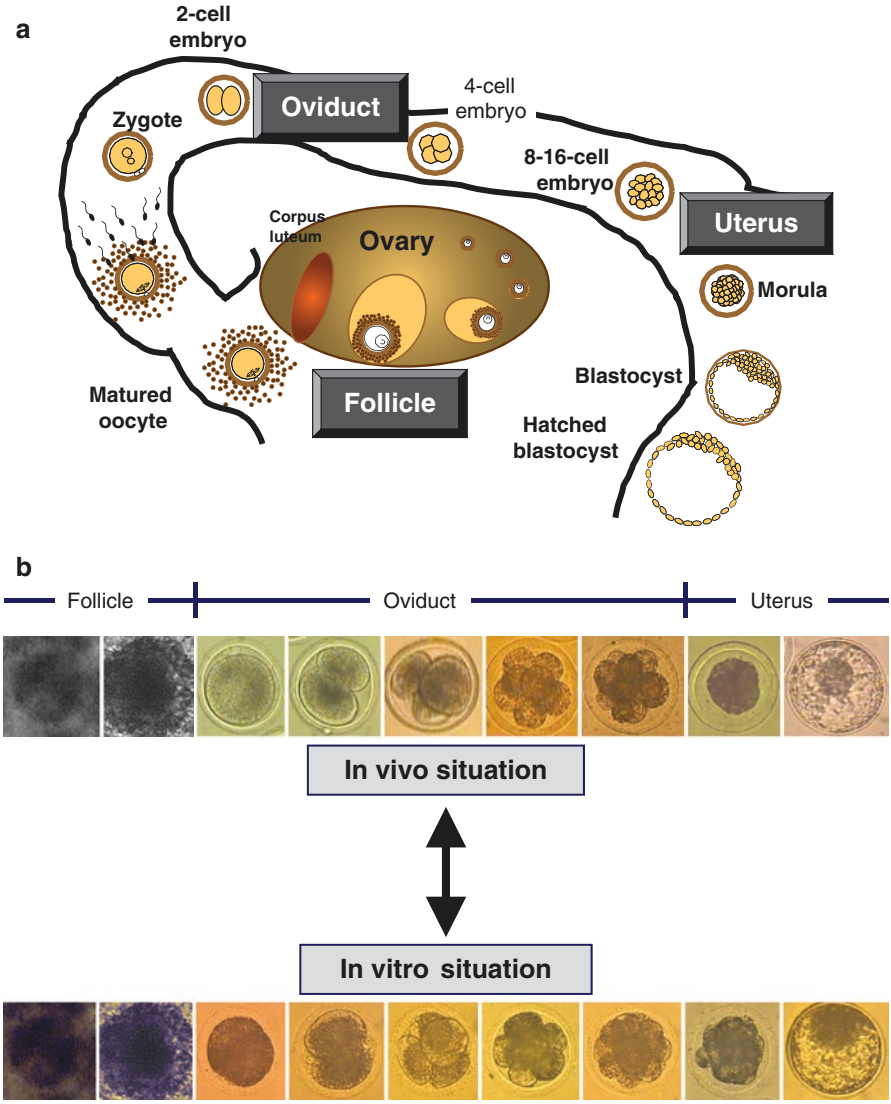


**Fig. 12.3** (**a**) Oocyte growth and maturation, fertilization, and early embryonic development (see text). (**b**) Oocyte and embryo development in vivo and in vitro

effects on the donor cow's fertility (Chastant-Maillard et al. 2003) and will result in a higher number of embryos produced per cow per time unit compared to the once a week OPU schedule. Best results are obtained when a 3- and 4-day or 2- and 5-day interval is maintained between OPU sessions (Merton et al. 2003). OPU was initially applied on problem cows that did not respond to superovulation (Kruip et al. 1994; Looney et al. 1994). It can be used to collect COC also from pregnant cows and heifers, including prepubertal heifers (Presicce et al. 1997). The number of

follicles can be increased by the use of a hormonal pre-stimulation. The disadvantage of pre-stimulation is that it involves the use of hormones, whereas the lack of requirements for hormones in the case of OPU has been generally considered an advantage compared to MOET (Merton et al. 2003). In contrast to oocytes recovered from abattoir ovaries, the genetic merit and health status of the donor animal including its oocytes are known. The number of oocytes collected from an animal during a single session of OPU depends on a variety of technical and biological factors (Merton et al. 2003). Usually, all follicles between 3 and 8 mm in diameter are aspirated.

OPU has become possible due to the development of ultrasound-guided transvaginal oocyte aspiration in humans and the adoption in the bovine in 1988 (Pieterse et al. 1988). Attempts were undertaken to combine OPU with color Doppler ultrasonography which is a useful, noninvasive technique for evaluating ovarian vascular function, allowing visual observation of the blood flow in the wall of preovulatory follicles (Brannstrom et al. 1998). Blood flow determination of individual preovulatory follicles prior to follicular aspiration for human IVF therapy provides important insight into the intrafollicular environment and may predict the developmental competence of the corresponding oocyte (Coulam et al. 1999; Huey et al. 1999). In cattle, it has been shown that the time interval between the individual OPU sessions had an effect on the quality of oocyte and embryos at the molecular level, whereas differences in the perifollicular blood flow did not (Hanstedt et al. 2010). An increase in blood supply to individual follicles appears to be associated with increased follicular growth rates, while a reduction seems to be closely related to follicular atresia (Acosta et al. 2003; Acosta 2007).

There are various systems used for grading bovine COC by visual assessment of morphological features, including compactness and quantity of surrounding follicular cells and homogeneity of the ooplasm as well as the size of the oocyte. An example is given in Fig. 12.4. Oocytes smaller than 110 μm are transcriptionally active and have a reduced ability to resume meiosis (Fair et al. 1995).

Taken together, OPU can be considered a mature technique and no major improvements should be expected in the technology and its results in the near future.

### 12.2.2 In Vitro Maturation (IVM)

Cumulus-oocyte complexes (COC) collected from ovaries of slaughtered or euthanized animals or from living animals via OPU require IVM as they are arrested at the GV stage. Maturation involves a series of events that already begin in fetal life with the initiation of meiosis. At birth, the oocytes are arrested at the diplotene stage. After puberty when they are exposed to preovulatory surges of LH and FSH, they proceed with meiosis and are arrested again at the metaphase II, the stage at which they are ovulated (Monniaux et al. 2014). In addition, optimal conditions for cumulus cells surrounding the oocyte need to be considered as there is a complex bi-directional communication between these two cell types (Monniaux 2016; Gilchrist 2011).

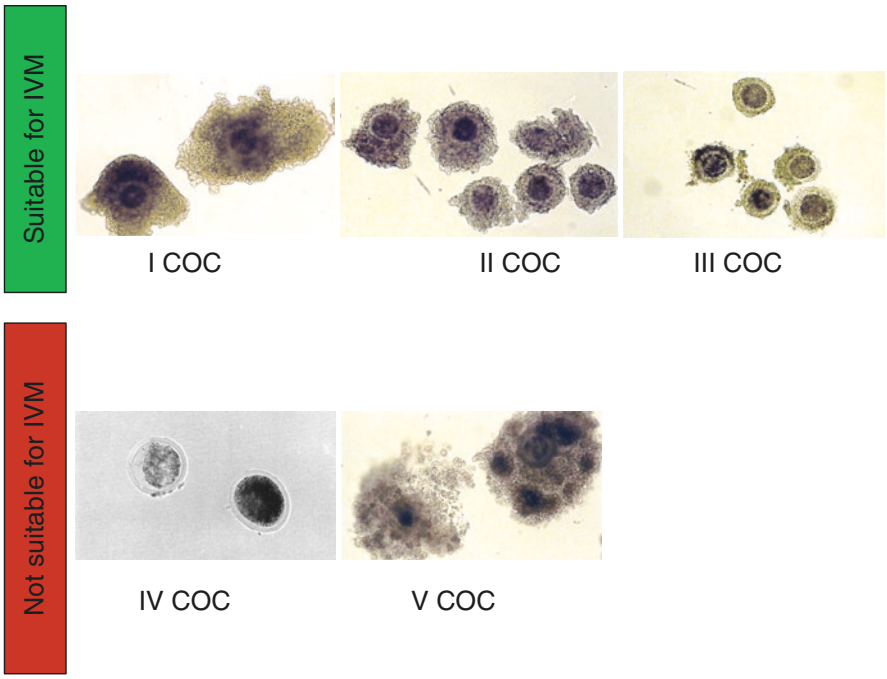


**Fig. 12.4** Different categories of COC (I, II, and III COC are suitable for IVM, IV and V COC not; see text)

Maturation is subdivided in nuclear and cytoplasmic maturation. Nuclear maturation refers to the resumption of meiosis, from germinal vesicle breakdown (GVBD), through metaphase I, anaphase, telophase, and rearrest at metaphase II (MII), and can be assessed noninvasively by determining extrusion of the first polar body. Nuclear maturation does not necessarily ensure that cytoplasmic maturation has been completed and should not be used as the sole determinant of oocyte quality. The oocyte must undergo a complex array of cytoplasmic rearrangements that allow the oocyte to support subsequent fertilization and initiate embryonic development. Multiple processes are involved in cytoplasmic maturation, including carbohydrate and lipid metabolism, mitochondrial function and location, reduction of oxygen radicals, accumulation of follistatin, epigenetic programming, communication between cumulus cells and the oocyte, and secretion of oocyte-derived growth factors (Sirard 2016).

Proper maturation of the bovine oocyte from the GV stage to metaphase II takes place within a period of 20–24 h and is a prerequisite for fertilization and preimplantation development. It is possible to achieve blastocyst rates of up to 70% if in vivo matured oocytes are used. In contrast, if oocytes are matured in vitro, blastocyst rates are usually only half that of those matured in vivo. This rather limited success may be attributed to the heterogeneous population of oocytes which are retrieved from follicles of 3–8 mm rather than from preovulatory follicles. In

contrast to in vivo ovulated oocytes, these oocytes usually would not make it up to the preovulatory stage and are matured in vitro. Therefore, substantial efforts have been devoted to the establishment of noninvasive and non-perturbing means for selecting the most competent oocytes (Fair 2010; Krisher 2013; Wrenzycki and Stinshoff 2013).

Maturation is initiated immediately following the removal of the immature oocyte from small antral follicles, and such oocytes may have neither the time nor the correct environment to complete the necessary changes required for subsequent successful development (Lonergan and Fair 2016; Krisher 2013; Wrenzycki and Stinshoff 2013).

Much of the success of IVP critically depends on the quality of the starting material, the oocyte. Developmental competence of the oocyte also termed oocyte quality is defined as the oocyte's ability to mature, to be fertilized, and to give rise to normal and healthy offspring (Duranthon and Renard 2001). The success of IVM is also dependent on the composition of the culture media which are usually quite different from in vivo conditions (Roberts et al. 2002; Sutton et al. 2003). A better understanding of intrafollicular conditions is critical for successful oocyte selection and maturation regardless of the species. In this context, profiling bovine follicular fluid revealed important clues about the composition of bovine follicular fluid (Sanchez-Guijo et al. 2016). Therefore, metabolomic analysis of follicular fluid may be a useful tool for characterizing oocyte quality (Sinclair et al. 2008; Bender et al. 2010).

Although substantial progress has been made to improve the efficiency of IVM protocols, there is a lack of consistency in the success rates of conventional in vitro maturation protocols compared to the in vivo situation. Multiple factors likely contribute to the overall poorer quality of in vitro matured oocytes. One of the important factors may be oxidative stress (OS). The generation of prooxidants such as reactive oxygen species (ROS) is an important phenomenon in culture conditions (Khazaei and Aghaz 2017).

The so-called simulated physiological oocyte maturation (SPOM) system had been introduced a few years ago (Albuz et al. 2010). It prevented spontaneous resumption of meiosis after mechanical oocyte retrieval and thereby improved in vitro embryo development. However, due to the fact that these first results were not repeatable, a revised version has been reported (Gilchrist et al. 2015). At the moment, most laboratories practicing IVM of cattle oocytes use a relatively simple oocyte maturation system.

Given that oocyte maturation in vivo takes place in the follicle prior to ovulation, and given the difficulties to mimic the situation in the preovulatory follicle in vitro, it is attractive to mature oocytes within the follicle. To achieve this, a modified ovum pickup equipment has been used to transfer in vitro matured oocytes into the preovulatory follicle of synchronized heifers (follicular recipients), enabling subsequent ovulation, in vivo fertilization (the animals have been artificially inseminated prior to oocyte transfer), and in vivo development. On average, 35% of embryos were recovered in excess after uterine flushing at Day 7. This technique is called intrafollicular oocyte transfer (Kassens et al. 2015). Transfer of frozen-thawed

IFOT-derived blastocysts to synchronized recipients (uterine recipients) resulted in 8 pregnancies out of 19 transfers. In total, seven pregnancies presumed to be IFOT-derived went to term, and microsatellite analysis confirmed that five calves were indeed derived from IFOT. These were the first calves born after IFOT in cattle. The present study established the proof of principle that IFOT is a feasible technique to generate high-quality embryos capable of developing to apparently high-quality blastocysts as well as healthy calves. Blastocysts (Spricigo et al. 2016) and calves (Michael et al. 2017) could also be produced after full in vivo development of immature slaughterhouse derived oocytes transferred to the preovulatory follicle. Therefore, the embryo production by IFOT of immature oocytes represents an alternative for the production of a large number of embryos without requiring hormones and basic laboratory handling only. As an alternative procedure, gamete intrafallopian transfer (GIFT, transfer of in vitro matured COC simultaneously with capacitated spermatozoa) is a viable compromise between in vitro and in vivo conditions and provides an option for reducing impacts of in vitro effects (Wetscher et al. 2005).

A better understanding of the in vivo regulation of follicular development and in particular of final maturation, in concert with oocyte differentiation by intrafollicular factors and intercellular communication, will lead to improved in vitro protocols for final maturation, thus compensating for the lack of preovulatory development of immature oocytes (Wrenzycki and Stinshoff 2013).

### 12.2.3 In Vitro Fertilization (IVF)

IVF is a complex step whose success requires appropriate oocyte maturation, sperm selection, sperm capacitation, and IVF media. In vivo, fertilization rates are around 90% for heifers and cows (Diskin and Sreenan 1980).

Semen samples contain a heterogeneous population of sperm cells. In vivo, sperm cells are thought to be selected by various mechanisms within the female reproductive tract, with the result that a small number of spermatozoa found near the oocyte are typically those best capable to penetrate the zona pellucida and fertilize the oocyte. However, when using IVF, these natural selection mechanisms are circumvented.

Treatment of bull sperm prior to IVF generally involves the selection of cells with the highest progressive motility. Furthermore, seminal plasma, cryoprotectants, and other factors are removed. One of the most common methods for preparing spermatozoa for IVF is to centrifuge them through a concentration gradient, such as a 45% Percoll mixture layered on a 90% solution.

A variant of colloid centrifugation using only one layer of colloid (in which case there is no gradient) has been developed. Single-layer centrifugation (SLC) through a species-specific colloid has also been shown to be effective in selecting spermatozoa with good motility, normal morphology, and intact chromatin (Thys et al. 2009; Goodla et al. 2014; Morrell et al. 2014; Gloria et al. 2016). An alternative method is the swim-up procedure. The disadvantages of swim-up are that it lasts

approximately 45–60 min and allows only 10–20% of the spermatozoa in the sample to be recovered. For colloid centrifugation, only a 25-min preparation time is needed (including the centrifugation), and a recovery rate of >50% is commonly achieved (Thys et al. 2009), which is critically depending on the sperm quality of the original sample.

The changes a sperm cell has to go through before it can successfully fertilize an oocyte are summarized under the term capacitation (Brackett and Oliphant 1975). Capacitation is recognized as a complex series of biochemical and physiological reactions (Breitbart et al. 1995), including the expression of hyperactivated patterns of motility and the acquisition of the capacity to respond to signals originating from the oocyte. Media have been developed to support this process, e.g., TALP medium. As mentioned earlier, the primary capacitation agent in current IVF systems is heparin. The majority of semen samples used for IVF is frozen-thawed. Apparently, fresh semen requires a longer capacitation period than the frozen one. Frozen-thawed semen has been intensively screened prior to cryopreservation. Furthermore, there are numerous studies indicating that the selection of bulls producing sperm cells with a high IVF capacity is an important factor in achieving successful and reproducible IVP results. The marked variability that occurs among bulls in the suitability of semen for IVF may be due to the penetration of the zona and/or ooplasm or processes taking place in the ooplasm. The most common final sperm concentration used in the IVF drop is $1 \times 10^6$ sperm/mL, whereas in the cow's oviduct, fertilization is likely to occur in a sperm/oocyte ratio close to 1 to 1 (Hunter 1996).

Once IVM is complete, oocytes are ready to be fertilized. This involves the co-incubation of oocytes with sperm cells. Most laboratories allow for 18–19 h of co-incubation. During this time period, sperm pass through the cumulus cell layers enclosing the oocyte, attach and bind to the zona pellucida, undergo the acrosome reaction, and finally penetrate the zona pellucida. The fertilizing sperm cell will then bind to and fuse with the oolemma, followed by activation of the oocyte and formation of male and female pronuclei. After co-incubation, attached cumulus cells are removed from the zonae. Successful fertilization is characterized by the extrusion of the second polar body and the formation of the female and male pronucleus. In cattle, the pronuclei are not visible due to the amount of lipid vesicles which is in contrast to the picture in for example humans and mice. Among the abnormalities seen after IVF are polyspermy and parthenogenesis (Parrish 2014).

Beside the conventional co-incubation of matured COC and sperm cells, there are four approaches available to assist fertilization when sperm numbers are reduced or when sperm motility is compromised; these approaches are partial zona dissection, zona thinning/zona drilling, subzonal sperm insertion (SUZI), and intracytoplasmic sperm injection (ICSI). ICSI involves the fertilization of MII oocytes by direct injection of a spermatozoon (Goto and Yanagita 1995). However, even haploid parthenogenetic embryos injected with sperm can result in full-term development in mice suggesting that sperm reprogramming sufficient to support full and healthy development can occur not only when M II oocytes are fertilized and in mitotic embryos well after meiotic exit (Suzuki et al. 2016).

ICSI is performed with the aid of a pair of glass pipettes adjusted to an inverted microscope, where the embryologist holds the oocyte with one pipette and injects a selected sperm cell with the second pipette straight into the ooplasm as shown in Fig. 12.5. The clinical use of ICSI was developed many years ago as a solution for some sperm-related male infertility in human-assisted reproduction (Palermo et al. 1992). The use of ICSI in cattle has never attracted much interest and has mainly been used for research purposes, essentially because IVF after heparin capacitation works very efficiently with the majority of bulls.

The success of ICSI in cattle is usually poor (Arias et al. 2014; Sekhavati et al. 2012) and appears to be limited by the low-activating stimulation of the injected spermatozoon (Catt and Rhodes 1995; Malcuit et al. 2006) and asynchronous pronucleus formation (Chen and Seidel 1997). Furthermore, bovine sperm are especially resistant to nuclear decondensation by in vitro matured oocytes, and this deficiency cannot be simply overcome by exogenous activation protocols. Therefore, the inability of a suboptimal ooplasmic environment to induce sperm head decondensation limits the success of ICSI in cattle (Aguila et al. 2017). In addition, the size of bovine sperm cells requires a pipette with a relatively large outer diameter of 10 μm that could be responsible for the damage on the cytoskeleton, thus reducing the developmental potential of the resulting embryos (Galli et al. 2003). Taken together, the efficiency of intracytoplasmic sperm injection (ICSI) in cattle is low compared to other species due in part to inadequate egg activation and sperm nucleus decondensation after injection.

### 12.2.4 In Vitro Culture (IVC)

IVC of bovine embryos is the last step in IVP and involves approximately 6 days of culture from the presumptive zygote onward. During the early post-fertilization period, several major developmental events occur in the embryo including (1) the first cleavage division, (2) the activation of the embryonic genome, (3) the compaction of the morula, and (4) the formation of the blastocyst.

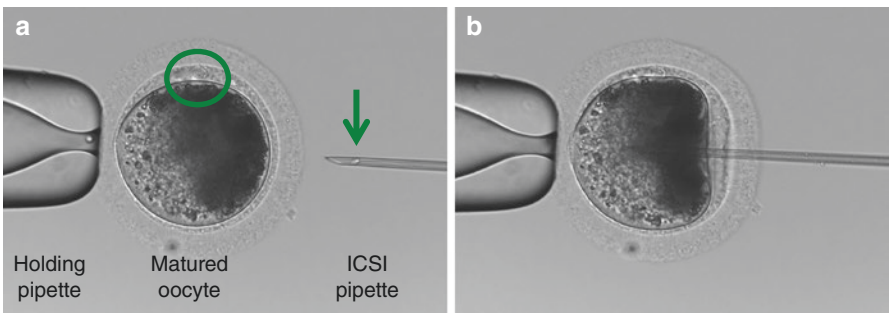


**Fig. 12.5** Intracytoplasmic sperm injection (green circle, polar body; green arrow, sperm cell)

Previously embryo requirements were not known, and a temporary in vivo culture in surrogate oviducts of sheep was performed (Lazzari et al. 2010). Oviducts of several other mammalian species including rabbits and mice have also been used to culture bovine embryos in vivo. The isolated mouse oviduct has also been employed as a system to culture bovine embryos successfully (Minami et al. 1988; Rizos et al. 2010). The success of in vitro embryo production techniques demonstrates that it is possible to bypass the oviduct during early development and, to a certain extent, replicate the conditions in vitro. However, overall they do not adequately mimic well enough the complex series of development-specific steps for which the oviduct has evolved a unique and dynamic microenvironment (Kenngott and Sinowatz 2007; Besenfelder et al. 2012).

The most common media for culturing bovine embryos are variations of the original synthetic oviduct fluid (SOF) medium (Tervit et al. 1972). SOF is now part of most routine bovine IVP systems with/without serum. Embryos can be cultured either in only one medium throughout the entire time or in a sequential system in which the medium formulation changes at certain time points in the culture period, i.e., so-called stage-specific media. These sequential media try to mimic the physiological changes that embryos encounter in vivo when they travel down the oviducts into the uterus. Parameters vary from lab to lab, e.g., the volume of medium and the atmosphere in the incubator.

IVC conditions have been improved in the last years, mainly by adjustment of media formulations. However, while more than 30% blastocyst formation could be achieved in most culture systems, it soon became obvious that quantity did not always match quality (Lonergan et al. 2006; Niemann and Wrenzycki 2000; Wrenzycki et al. 2005, 2007) and that serum supplementation was detrimental to embryo/fetal development as one main causal factor of the so-called large offspring syndrome (LOS), characterized by altered embryonic and fetal growth, deviant embryonic and fetal gene expression patterns, and high perinatal losses (Young et al. 1998; Lazzari et al. 2002). A large field study demonstrated that the incidence of LOS was greatly reduced by in vitro culture in cell-free and serum-free SOF media (van Wagtendonk-de Leeuw et al. 2000). These observations highlight the importance of the post-fertilization culture environment for the quality of the resulting blastocysts. However, the existence of diverse embryo culture media and methods makes it very challenging to define the optimal components of embryo culture media. Undefined media supplemented with serum and somatic cells as monolayer have been replaced by semi-defined media culturing embryos in the absence of cells with no serum supplementation. Completely defined media, developed by modifications of the SOF medium with glutamine or citrate and nonessential amino acids (Holm et al. 1999; Keskintepe et al. 1995), and by replacing BSA with polyvinyl alcohol (Keskintepe and Brackett 1996), also support embryo development to the blastocyst stage. Defined conditions not only facilitate the improvement of culture media to provide a better, more consistent environment for the developing embryo but are also critical in disease control (Stringfellow and Givens 2000).

Recently, a culture system for the formation of an in vivo-like oviduct tissue substitute from primary oviduct epithelial cells has been developed. This air-liquid

interphase (ALI) culture is fully functional in terms of morphological differentiation (polarization, columnar shape, ciliary activity), generates oviduct fluid surrogates, and enables embryonic development up to the blastocyst stage without addition of embryo culture medium (Chen et al. 2017).

The success of an IVP laboratory may stem not only from improvements of the IVC per se but from the entire IVP system (Baltz 2012; Gardner 2008; Leese 2012). The latter includes incubation conditions, gas phase, culture media, oil overlay, plastic ware, and embryo density and the volume of the medium. In addition, the skills of the staff involved in the entire process are critically important for the success of the system.

The use of noninvasive strategies, such as analysis of follicular fluid and culture media (after culture), also appears to be useful for the search for molecular biomarkers indicative of oocyte competence. The presence of cytokines and growth factors in follicular fluid is crucial for determining oocyte quality (Dumesic et al. 2015). In this context, the metabolic characterization of the culture media, in which IVP embryos are maintained for many hours, may represent an important noninvasive tool to either indicate possible predictive biomarkers of viability or to explain IVP outcome afterward (Munoz et al. 2014).

In general, bovine IVP is at an advanced stage of development. However, an aspect that may change in the future is automation and miniaturization of the IVP process by better mimicking in vivo environment, e.g., using microfluidics (Wheeler et al. 2007) or an encapsulation technology (Blockeel et al. 2009) to obtain IVP embryos of similar quality as the in vivo-derived counterparts. Such systems would facilitate the gradual change of the culture medium to meet the precise requirements of the developing embryo and overcome substantial limitations of conventional culture systems. Nevertheless, commercial production of bovine embryos via IVP is now successful in many laboratories around the world.

## 12.3 IVP of Embryos in Other Animals

### 12.3.1 Farm Animals

As mentioned earlier, IVP is best developed in cattle. Nevertheless, there are often species-specific differences, even with cattle (Sartori et al. 2016).

#### 12.3.1.1 Bos indicus/Buffalos

*B. indicus* animals have greater numbers of retrieved oocytes, due to higher antral follicle counts, resulting in higher percentages of viable oocytes, number of blastocysts, and blastocyst rates when compared with *B. taurus* ones. The better blastocyst rates obtained with *B. indicus* cows and heifers could be attributed to an intrinsically better quality of the oocytes (Viana et al. 2012). OPU/IVP is the only option for embryo production to advance the implementation of embryo-based biotechnologies in buffalo production, although it is still in an early developmental phase (Galli et al. 2014). OPU works better, and it is more cost-effective than superovulation in

*Bos indicus* females. In this species, OPU has great potential because superovulation (multiple ovulation and embryo transfer) yields poor results compared with cattle (Carvalho et al. 2002). The procedure is similar to the one used in *Bos taurus*. Oocyte recovery, embryo production, and offspring obtained have been described (Galli et al. 2014), but only about 10%–15% of the oocytes recovered develop to transferable embryos. In general, the ovaries of buffalo cows and heifers are small, and, in addition, the follicles tend to be fewer and of small diameter.

#### 12.3.1.2 Horses

In the horse, OPU/IVP is now an established procedure for breeding from infertile and sporting mares throughout the year. It requires ICSI that in the horse, contrary to cattle and buffalo, is very efficient and the only option because conventional IVF does not work (Galli et al. 2014).

To collect in vivo matured oocytes from living mares, the most practical, less invasive, efficient, and repeatable technique used is the ultrasound-guided transvaginal follicular aspiration using a double-lumen needle (Carnevale et al. 2005). Oocytes can be collected from the preovulatory follicle that has reached at least 35 mm in diameter, 24 h after hCG injection with the donor showing signs of uterine edema. The recovery rates are much higher than those for the recovery from immature follicles (see below), because the COC is expanding and is detached from the follicle wall just prior to ovulation. After recovery from the donor mare and sometimes after a few hours of culture to complete maturation, oocytes can either be surgically transferred to the oviduct of inseminated recipients called oocyte transfer (OT) (Carnevale et al. 2005) or can be subjected to intracytoplasmic sperm injection (ICSI). OT has been developed for clinical and research purposes because of frequent failures of conventional IVF. In addition, after ICSI, the fertilized oocytes can be transferred surgically to the oviduct of a synchronized recipient or cultured in vitro up to the blastocyst stage. Nowadays, ICSI and transfer after IVC are the methods of choice.

As only one preovulatory follicle is present at any cycle and only during the breeding season, OPU of immature equine oocytes has emerged as useful alternative. All follicles that are at least 1 cm, also in the nonbreeding season, are flushed. Because of the large size of the follicles and the firm attachment to the follicle wall, it is necessary to use double-lumen needles with separate in- and outflow channels that allow for repeated flushing up to eight to ten times for each follicle (Galli et al. 2007). As mentioned above, due to the firm attachment of the immature oocyte to the follicular wall, follicle flushing is a prerequisite. Mares can be subjected to repeated collections without side effects (Mari et al. 2005).

Immature cumulus-oocyte complexes could also be obtained from slaughterhouse ovaries or after ovariectomy. Because of the tight attachment of the oocyte to the follicular wall, follicle scraping is the method of choice (Hinrichs and Digiorgio 1991) rather than slicing or aspiration as done in cattle. Oocytes and their surrounding cumulus cells are classified by their cumulus morphology as either compact or expanded. Oocytes classified as compact are more likely to have a homogenous cytoplasm which is associated with a lower meiotic competence (Hinrichs and Williams 1997).

The optimum duration of maturation is between 24 and 30 h for oocytes with expanded and between 30 and 36 h for those with compact cumulus. Once matured, there was no difference in developmental competence (rate of blastocyst development) between both types of oocytes (Hinrichs 2005). Evaluation of oocytes after in vitro maturation and preparation for ICSI requires removal of the cumulus; this is more difficult in horses than it is in many other species. The transzonal processes of the equine cumulus are extensive.

Only two foals have been reported from conventional IVF of in vivo matured oocytes (Palmer et al. 1991; Cognie et al. 1992). The first foal, derived from an in vitro matured oocyte fertilized by ICSI, was born in 1996 (Squires 1996). To date, several laboratories have consistently and reproducibly reported the birth of ICSI foals both from in vivo and in vitro matured oocytes. It has been reported that conventional IVF is successful after treatment of sperm with procaine to induce hyperaction (McPartlin et al. 2009). However, recent data showed that procaine induces cytokinesis in equine oocytes associated with elevated levels of parthenogenetic development (Leemans et al. 2015).

Good blastocyst rates (20% to 40% of injected oocytes) are achieved using the Piezo drill (Hinrichs 2005; Galli et al. 2007). No exogenous activation of the oocytes is required. For IVC, a variety of media can be used, and supplementation with 17–19 mM glucose is important (Hinrichs 2005; Herrera et al. 2008). Equine embryos start to develop to the blastocyst stage at Day 7 of culture. In vivo, an acellular capsule forms inside the zona pellucida after entry of the equine embryo into the uterus (Betteridge et al. 1982). The equine capsule is composed of mucin-like glycoproteins produced by the trophectoderm, containing a high proportion of sialic acid (Oriol et al. 1993). Formation of this capsule is not observed in vitro.

#### 12.3.1.3 Pigs

The pig has been a particularly difficult species to obtain high rates of fertilization and subsequent blastocyst development in vitro. Insufficient oocyte cytoplasmic maturation in vitro, high rates of polyspermy, and low embryonic development rates are the major obstacles that still need to be overcome (Gil et al. 2010; Romar et al. 2016).

The developmental potential of porcine IVM oocytes is typically much poorer than that of cattle and sheep IVM oocytes. Pigs are generally slaughtered at 6 or 7 months of age to meet market demands for pork. Therefore, abattoir-derived ovaries are usually collected from prepubertal gilts that have not yet experienced regular estrous cycles. Porcine oocytes are typically matured in vitro for about 40–44 h, much longer than oocytes of other livestock species. Only during the first half of IVM porcine COC are exposed to gonadotropins (Funahashi and Day 1993).

The use of BSA and caffeine as capacitating agents was an important breakthrough and was instrumental for successful in vitro fertilization in the pig. However, the rate of polyspermy has been reported to be over 50% in some laboratories (Mugnier et al. 2009). The degree of polyspermy was found to be closely associated with the number of sperm per oocyte at fertilization (Rath 1992). Unfortunately, simply reducing the number of sperm per oocyte within the insemination droplet has not been useful to overcome the polyspermy problem, because this also results

in a decrease of the overall penetration rates. An issue that confounds the polyspermic fertilization in porcine embryo IVP systems is that polyspermic embryos are still able to develop to the blastocyst stage.

Despite these difficulties, IVP blastocyst development rates are in the range from 30% to 50% from monospermically fertilized oocytes in most laboratories (Gil et al. 2010). The addition of particular components such as porcine follicular fluid into the IVM media has been shown to improve the quality of porcine IVM (Algriany et al. 2004). Hormone supplementation at particular stages of maturation and adding insulin-transferrin-selenium to the media have also contributed toward improved maturation rates (Hu et al. 2011). Due to a refinement of techniques, IVM rates now vary from 75% to 85% (Gil et al. 2010). Embryo culture (IVC) has been developed extensively in the pig, and two media compositions are used widely today, including NCSU23 and NCSU-37 (Petters and Wells 1993). The first piglets born from IVP procedures including IVM/IVF/IVC up to the blastocyst stage were reported in 1989 (Mattioli et al. 1989). The rate of live offspring resulting from IVP is relatively low compared to the number of transferred IVP embryos, and, to date, successful production of offspring depends on the transfer of large numbers of blastocysts or earlier embryos, to produce large enough litter sizes. The need to transfer relatively large numbers of embryos to achieve even a comparatively low litter size and the lack of stable non-surgical procedures for embryo transfer remain significant obstacles toward the practical implementation of IVP. However, today, the in vitro procedures used to mature oocytes and culture embryos are integral to the production of transgenic pigs by SCNT (Grupen 2014) or gene editing (Niemann and Petersen 2016).

#### 12.3.1.4 Small Ruminants (Sheep and Goat)

There is less research on assisted reproductive technologies including IVP in small ruminants compared to other livestock species. Results on IVP are still unpredictable and variable which is an important limitation for its commercial application (Paramio and Izquierdo 2016, 2014). This large variation is also seen in in vivo embryo production after superovulation treatment. Oocytes are usually recovered from the follicle of females via laparoscopic ovum pickup (LOPU) which is less traumatic than laparotomy.

The first kid born from IVF of an ovulated oocyte was reported in 1985 and the first lamb in 1986. In 1993 the birth of the first kid from an IVM-IVF oocyte and in 1995 the first kid from IVM-IVF and IVC oocytes was announced (Paramio and Izquierdo 2014). Despite all the improvements to increase blastocyst numbers and blastocyst quality by modifying the different components of IVP, results are still unpredictable and variable with significant differences between laboratories and experiments (Paramio and Izquierdo 2016). The most commonly used medium for IVM is tissue culture medium (TCM199). Oocytes are usually cultured for 24–27 h to achieve maturation. Sperm capacitation is obtained by using heparin and estrus sheep serum (ESS) for fresh and frozen buck and ram semen, respectively. IVF is usually carried out in SOF medium in sheep and in Tyrode's albumin lactate pyruvate medium supplemented with hypotaurine in goats. Sperm and oocytes are

co-incubated for 16–24 h. At present, ICSI is not very efficient in small ruminants. Chemical activation of oocytes is an essential part of ICSI protocols in sheep, yielding similar cleavage rates in IVF and sperm-injected oocytes (Shirazi et al. 2009). Goat blastocysts have been obtained by ICSI without further chemical activation of the oocytes (Keskintepe et al. 1997). Currently, the most commonly used IVC medium for culturing small ruminant embryos is SOF with amino acids and the addition of 5%–10% of fetal calf serum or BSA.

It has been suggested that the procedures used in bovine IVP can be applied in small ruminants after minimal modifications (Souza-Fabjan et al. 2014).

#### 12.3.1.5 Camelids

The camelid family includes dromedary and Bactrian camels, llamas, alpacas, vicunas, and guanacos. The first two are Old World camelids, whereas the last four are known as New World camelids or South American camelids.

The development of IVP techniques has been slow in South American camelids (SAC). As for other species, a high number of oocytes can be collected from slaughterhouse ovaries. Oocytes can also be obtained from living animals via laparotomy [llama (Trasorras et al. 2009); alpaca (Ratto et al. 2007)] or ovum pickup [llamas (Brogliatti et al. 2000; Berland et al. 2011)]. There are currently no reports on ultrasound-guided transvaginal follicle aspiration in alpacas (Trasorras et al. 2013). IVM conditions for SAC oocytes are similar to those for ruminants. South American camelid semen has very particular characteristics, such as high structural viscosity (Casaretto et al. 2012). This characteristic feature renders semen handling difficult in the laboratory (Tibary and Vaughan 2006), hindering separation of spermatozoa from the seminal plasma and the isolation of motile from immotile sperm (Giuliano et al. 2010). In addition, spermatozoa from these species show oscillatory movements in the ejaculate rather than progressive motility.

Embryos have been produced in vitro using spermatozoa from the epididymis or from ejaculates. The advantages of using epididymal spermatozoa are that these cells are progressively motile and sample management is easier because of the absence of seminal plasma. The rate of development of IVF produced embryos to the blastocyst stage after 5 days of culture is only 10% in llamas. Although pregnancies by ET using in vivo produced embryos (Trasorras et al. 2010) have been generated, no pregnancy has been achieved until now after transcervical transfer of IVP embryos to the uterus of previously synchronized females.

All research done so far proves that it is possible to produce llama embryos in vitro, but it remains necessary to find an adequate culture medium and conditions to favor embryo development to stages that are compatible with establishing pregnancies (Trasorras et al. 2013).

For Old World camelids, the developmental rate up to the blastocyst stage is 20% in dromedary camels. Only in vivo matured/in vitro fertilized and in vivo matured/fertilized produced embryos continued normal development until term and resulted in the birth of normal and healthy live calves (Tibary et al. 2005).

## 12.4 Quality Assessment of Preimplantation Embryos

The ultimate test of the quality of an embryo is its ability to produce live and healthy offspring after transfer to a recipient. Morphology and the proportion of embryos developing to the blastocyst stage are important criteria to assess developmental competence. Evaluation of embryo morphology remains the method of choice for selecting viable embryos prior to transfer. It is the most practical and clinically useful approach to assess of embryo viability (Van Soom et al. 2003). A bovine embryo grading system developed previously (Lindner and Wright 1983) is, with minor modifications, still widely applied in this field and published in the IETS Manual. However, embryo morphology alone may not accurate enough to serve as the sole criterion for the prediction of embryo developmental potential in vivo.

Identification of the embryo with the highest potential to implant, establish, and maintain a pregnancy is a primary goal in assisted reproduction techniques. Culture by incubation in a time-lapse imaging system does not harm embryos compared with the standard IVP protocol and results in similar overall embryo development (Holm et al. 2002). Morphokinetic embryo analysis by monitoring the changes in embryo morphology over time is by far the most important noninvasive embryo selection tool today and is routinely used in human IVF laboratories (Pribenszky et al. 2017). However, due to its high costs, it has not entered the commercial veterinary field.

Embryo selection is based on methods that can give a direct or indirect clue regarding the potential of a given embryo to implant. These methodologies are either invasive or noninvasive and can be applied at various stages of development from the oocyte to cleavage-stage embryos and up to the blastocyst stage. In view of the shortcomings of invasive embryo selection, the use of noninvasive selection methods seems to be a better strategy toward identification of viable embryos without the risk of possible impacts due to the investigation itself.

Better noninvasive markers and improved techniques for assessing embryo quality are required. These techniques can provide more information on embryo viability (Rocha et al. 2016). For examples, measurement of oxygen consumption using the nanorespirometer (Lopes et al. 2007) and amino acid profiling (Sturmey et al. 2010) can be employed to predict developmental competence and embryo viability of in vitro produced embryos. Although noninvasive approaches are improving, invasive ones have been extremely helpful in finding candidate genes to determine embryo quality (Wrenzycki et al. 2007; Rizos et al. 2008; Graf et al. 2014).

As epigenetic changes can be induced by environmental factors, understanding how vitro production conditions can interfere with these processes is of critical importance. The embryonic epigenome will continue to be an important area of research, especially to gain a better comprehension how the epigenome influences short- and long-term health. A better understanding of the mechanisms and the role of epigenetics during early embryogenesis will likely improve in vitro protocols and ultimately animal health and productivity. Epigenetic remodeling during preimplantation development is complex and dynamic, including changes in DNA methylation and histone modifications that occur both on a global scale but also

differentially at specific loci. Uncovering the bases of these mechanisms will improve our understanding of early development and are promising for improving animal fertility and diagnosing and treating infertility problems (Urrego et al. 2014; Canovas and Ross 2016).

Despite the large number of publications in the field, it is still a long way to better understand and manipulate the mechanisms controlling oocyte maturation and early embryonic development. Overcoming these gaps may allow us to improve ART results. In particular, studies aiming at finding effective biomarkers for oocyte and embryo competence are urgently needed. The field of OMICs seems to be quite promising, especially regarding new findings in transcriptomics, proteomics, and lipidomics in oocytes, CCs, embryos and EVs within the follicular fluid (Krisher et al. 2015; Scott and Treff 2010; Benkhalifa et al. 2015). Future studies on this subject might enable the design of more complex, defined, and efficient culture conditions for oocytes and embryos.

## 12.5 Challenges and Future Developments

### 12.5.1 Culture of Post-Hatching Stages

Post-hatching bovine embryonic development in vitro would allow for the establishment of better tools for evaluating developmental potential without the need for transfer to recipient animals. In vitro development of bovine embryos beyond hatching has been reported with the establishment of the hypoblast and contemporary signs of shedding of the polar trophoblast, i.e., Rauber's layer. Moreover, elongation may be achieved upon physical constraints in agar tunnels (Brandao et al. 2004). However, further development of the epiblast is compromised, and embryonic disk formation is lacking (Vejlsted et al. 2006). Further studies are needed to determine the appropriate physical, chemical, and hormonal environment required to pass through this block of development and reach subsequent developmental stages in vitro.

In humans, an in vitro system to culture embryos through implantation stages in the absence of maternal tissues has been established (Shahbazi et al. 2016; Deglincerti et al. 2016). In contrast to cattle where implantation takes place around day 20, the human embryo must implant into the uterus of the mother to survive at the seventh day of development.

### 12.5.2 Genomic Selection (GS)

In the last decade, significant advances in molecular genetics and bioinformatics have made it possible to establish genomic selection as a new tool to increase the genetic gain in animal breeding (Stock and Reents 2013). The success of genomics in animal breeding begins with the use of whole-genome information (Meuwissen et al. 2001).

A chip was developed already in 2007 that allows to genotype >54,000 single nucleotide polymorphisms (SNP) simultaneously. These markers represented only a small proportion of all discovered SNPs, but they were highly polymorphic in several breeds and evenly spaced over the genome. This chip has immediately been used to genotype existing progeny-tested bulls. With these first reference populations, genomic breeding values (GEBV) were accurate enough to rapidly replace progeny testing. They were made official in 2009 in different countries, allowing the dissemination of semen of young bulls with genomic evaluation. This revolutionized selection (Boichard et al. 2016). In order to decrease genotyping costs, a low-density chip was designed with good imputation accuracy (Boichard et al. 2012), i.e., with excellent prediction of missing markers.

Since the introduction of OPU/IVP, substantial efforts have been made to improve embryo production efficiency. GS provides new tools for improving the efficiency of OPU/IVP programs by selecting donors with high in vitro production results. Heritability for qualitative traits (quality of oocytes, cleavage, and developmental rates) seems to be lower than for quantitative traits (total number of oocytes, number of embryos (Merton et al. 2009)). Different components of reproductive biotechnologies have already been associated with SNPs in cattle and could be added as new traits to improve fertility as well as efficiency of ARTs in donor cows. In particular, SNPs associated with the number of viable oocytes, fertilization, cleavage, and developmental rates have been recently highlighted (Ponsart et al. 2014).

Furthermore, GEBV can already be determined from preimplantation embryo biopsies. Taking embryo biopsies requires highly skilled and trained operators and specific equipment, such as an inverted microscope combined with micromanipulators. Three methods, the microblade biopsy, the aspiration biopsy, and the needle technique, have been described (Cenariu et al. 2012). Usually, five to ten blastomeres are collected as shown in Fig. 12.6. As they do not contain enough DNA for SNP chip analyses, a DNA amplification step (whole-genome amplification, WGA) has to be included. The mean call rate (proportion of called markers) from WGA of genomic DNA stemming from an embryo biopsy should be higher than 85%

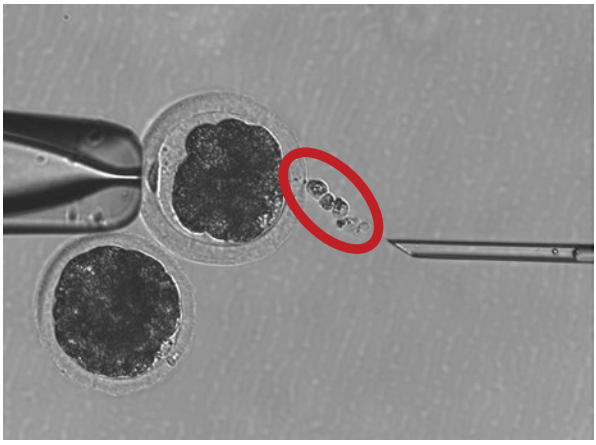

**Fig. 12.6** Biopsy taken from a bovine morula (red circle, biopsied blastomeres)

ensuring a low amount of allele drop out (ADO) and replication errors. Another bottleneck for genotyping techniques combined with IVP remains the cryopreservation of IVP biopsied embryos (Ponsart et al. 2014). To overcome this problem, blastocentesis (collection of blastocoel fluid) which is less invasive might be performed as already done in horses (Herrera et al. 2015) and humans (Gianaroli et al. 2014). Furthermore, instead of freezing, biopsied embryos could undergo liquid preservation (Ideta et al. 2013).

Taken together, GS has revolutionized cattle breeding schemes. Strategies to genotype embryos for multiple markers were developed and combined with embryo biopsy.

### 12.5.3 Exosomes/miRNAs

Recently, a new mechanism of cell communication within the ovarian follicle has been discovered which is performed by extracellular vesicles (EVs). Initially, EVs were described in ovarian follicular fluid of mares (da Silveira et al. 2012). These EVs are lipid bilayer structures secreted by many cell types into the extracellular fluid, serving as a vehicle for membrane and cytosolic proteins, lipids, and RNA (Raposo and Stoorvogel 2013)). Several articles identified miRNAs in bovine (Miles et al. 2012), equine (da Silveira et al. 2012), and human (Santonocito et al. 2014) follicular fluid, suggesting EVs could be a potential mediator of cell-to-cell communication, impacting oocyte and follicle growth (da Silveira et al. 2015). Profiles of miRNAs isolated from EVs present in follicular fluid were described and associated with proper cytoplasmic oocyte maturation; hence, these miRNA profiles can be used to predict oocyte competence (Sohel et al. 2013). The presence of extracellular miRNAs in various biological fluids, including follicular and seminal fluid, their stability, and the relatively easy procedures required to obtain these samples make miRNAs excellent candidates for the use of biomarkers of female and male reproduction and fertility (Pratt and Calcatera 2016; Tesfaye et al. 2016).

The well-orchestrated expression of genes that are derived from the maternal and/or embryonic genome is required for the onset and maintenance of distinct morphological changes during the embryonic development. Optimum regulation of genes or critical gene regulatory events in favor of early embryonic development has been shown to be under control of miRNAs (Hossain et al. 2012). Recently, it has been shown that the addition of extracellular vesicles from oviductal fluid from the isthmus to in vitro culture of bovine embryos in the absence of serum improves development and quality of the embryos (Alminana et al. 2017; Lopera-Vasquez et al. 2017; Pavani et al. 2016).

### 12.5.4 Microfluidics

During IVP gametes and embryos are exposed to changes in pH, osmolarity, and mechanical stress, which can interfere with successful blastocyst production. Most

of the research efforts to reduce these stress factors have been focused on the media rather than finding systems that could reduce the stress introduced by the operator. One possible solution to reduce many of these types of stress on gametes/embryos may be the application of micro- and nanotechnologies, particularly microfluidic technologies. Microfluidic technologies first emerged in the late 1980s and early 1990s (Masuda et al. 1987). The full potential of microfluidic technology has yet to be realized for ARTs in livestock. Major advances in automation and robotics combined with microfluidics have the potential to revolutionize livestock IVP and animal breeding. New technologies in 3D printing may improve our ability to develop physical systems that closely mimic the in vivo environment. Sophisticated pumping and fluid handling methods will allow for the alteration of the fluid milieu that surrounds the gametes and embryos. Subtle changes in media composition delivered at specific time points are achievable using microfluidic devices (Wheeler and Rubessa 2017).

### 12.5.5 Follicle Culture

For bovine and other domestic species, the development of culture systems capable of supporting the growth of immature follicles to a stage where they could be matured and the oocyte fertilized would ensure a constantly large supply of oocytes for experimental and applied purposes. Development of a successful culture system for preantral follicles with immature oocytes is dependent upon efficient procedures to recover the follicles from the ovary and culture them as well. Basically, there are two ways to culture bovine preantral follicles: (1) enclosed in ovarian tissue fragments (slices or strips), also called "in situ," or (2) using isolated follicles (Araujo et al. 2014). Significant advances have been described, and a major achievement was the in vitro development of secondary follicles up to early antral stages and the production of very low number of embryos (Silva et al. 2016). Although antrum formation was reached, culture of bovine secondary follicles did not lead to the production of meiotically competent oocytes (Gutierrez et al. 2000; McLaughlin and Telfer 2010). A two-step culture system for bovine preantral follicles has been described (McLaughlin and Telfer 2010).

The growth of primordial follicles up to maturation in domestic species is a long process, and a better understanding of the physiological and pharmacological requirements of the various stages of follicle development is inevitable. This technique will be of great value for experimental or diagnostic purposes. Profound similarities in the dynamics of follicle development exist between the menstrual cycle in women and the estrous cycle in cattle and horses (Ginther et al. 2004). In this regard, research using animal models for studying human ovarian function is important to provide a hypothetical basis for further studies in women (Baerwald et al. 2009) as is for the entire IVP procedure (Menezo and Herubel 2002; Santos et al. 2014; Langbeen et al. 2015; Wrenzycki et al. 2007). Further characteristics of early embryo biology are shown in Table 12.3 indicating that bovine and human early development is remarkably similar.

**Table 12.3** Comparative aspects of early embryonic development

| | Human | Cattle |
|---|---|---|
| **Oocyte diameter** (μm) | 150–180 | 150–180 |
| **Time (h) to reach** | | |
| 2-cell stage | 30 | 36 |
| Blastocyst stage | 120 | 150 |
| Hatching stage | 150 | 200 |
| **Stage of EGA**[a] | 4-cell | 8-cell |

[a]Embryonic genome activation

### 12.5.6 Artificial Gametes

Artificial sperm and artificial oocytes generated from pluripotent germline stem cells (GSCs), embryonic stem cells (ESCs), or induced pluripotent stem cells (iPSCs) have resulted in the birth of viable offspring. Artificial sperm and artificial oocytes have also been generated from somatic cells directly, i.e., without documentation of intermediate stages of stem or germ cell development or (epi)genetic status. Albeit embryos showed reduced development, haploidization by transplantation of a somatic cell nucleus into an enucleated donor oocyte has led to fertilized artificial oocytes (Hendriks et al. 2015). Complete restoration of gametogenesis in culture will be important to our understanding of biological events at the cellular and molecular levels that are important for germline development. Understanding these processes will have enormous impact on applications in the biomedical and agricultural communities (Zeng et al. 2015). Fully potent mature oocytes were generated in culture from embryonic stem cells and from induced pluripotent stem cells derived from both embryonic fibroblasts and adult tail tip fibroblasts. Moreover, pluripotent stem cell lines were re-derived from the eggs that were generated in vitro, thereby reconstituting the full female germline cycle in a dish. This culture system will provide a platform for elucidating the molecular mechanisms underlying totipotency and the production of oocytes of other mammalian species in culture (Hikabe et al. 2016).

### 12.5.7 Mitochondrial Transfer

A promising advancement in human ART involves the replacement of mutant mtDNA in unfertilized oocytes or zygotes by healthy donor mitochondria, thereby allowing women carrying mtDNA mutations to circumvent passage of the condition to their children (Craven et al. 2010). Two microsurgical nuclear transfer procedures termed spindle transfer (ST) and pronuclear transfer (PNT) have been developed. The first approach is conducted at the MII oocyte stage. The spindle is isolated and transplanted into the cytoplasm of a donated unfertilized oocyte that, itself, has been enucleated. The reconstructed oocyte, now free of mutated mtDNA, can be fertilized and subsequently transplanted to the patient (Mitalipov and Wolf 2014). The first baby from this approach has been born in 2016 (Zhang et al. 2017). Human

pronuclear transfer has also been reported (Craven et al. 2010; Zhang et al. 2016). Potential concerns with pronuclear and spindle transfer include the impact these procedures might have on the risk of mtDNA carryover during karyoplast transfer, embryogenesis, epigenetics, and genome integrity (Craven et al. 2011). These issues need to be carefully assessed more basic in-depth research (Reznichenko et al. 2016).

### 12.5.8 Gene Editing

Molecular scissors (MS), including zinc finger nucleases (ZFN), transcription activator-like endonucleases (TALENS), and meganucleases, possess long recognition sites and are thus capable of cutting DNA in a very specific manner. These molecular scissors mediate targeted genetic alterations by enhancing the DNA mutation rate via induction of double-strand breaks at a predetermined genomic site. Compared to conventional homologous recombination-based gene targeting, MS can increase the targeting rate 10,000-fold, and gene disruption via mutagenic DNA repair is stimulated at a similar frequency. The successful application of different MS has been shown in mammals, including humans (Petersen and Niemann 2015). Recently, another novel class of molecular scissors was described that uses RNAs to target a specific genomic site. The CRISPR/Cas9 system is capable of targeting even multiple genomic sites in one shot and thus could be superior to ZFNs or TALEN, especially by its rather simple design. However, careful sequencing of the targeted locus is required for all mutagenesis projects, including the ones by CRISPR/Cas9 (Mianne et al. 2017). Confirmation of the desired on-target mutation and the detection of off-target events is of utmost importance (Zischewski et al. 2017).

The ability to generate gene knockouts is a powerful tool for analysis of gene function and for the generation of animals with novel biotechnological or breeding applications (Fahrenkrug et al. 2010). In livestock species this process traditionally involves the generation of a knockout cell line by utilizing homologous recombination followed by somatic cell nuclear transfer (SCNT). This remains the method of choice for many applications (Kurome et al. 2013); however, application of SCNT strategies requires a high level of technical expertise, a reliable supply of oocytes, and a large recipient herd, features not available in many areas where gene editing might have the greatest impact (Proudfoot et al. 2015). However, in vitro produced embryos can be used for rapid introgression of gene edits into defined populations.

Recently, CRISPR/Cas9-mediated gene editing has been applied to human zygotes (Liang et al. 2015; Kang et al. 2016), and the correction of a pathogenic gene mutation has been reported in 2017 (Ma et al. 2017). Efficiency, accuracy, and safety of the approach suggest that it has the potential to be used for the correction of heritable mutations in human embryos by complementing preimplantation genetic diagnosis. However, much remains to be investigated before clinical applications, including the reproducibility of the technique (Ma et al. 2017).

**Conclusion**

In vitro production (IVP) of livestock embryos follows a well-developed procedure that is commercially available for most species. Albeit all the improvements in oocyte and embryo culture, at best only 30–35% of immature bovine COC develop to the blastocyst stage. Nevertheless in light of the underlying ovarian mechanisms with the high degree of atresia, this represents a reasonable efficiency. But the in vivo situation cannot yet be mimicked sufficiently well. The quality of the embryos produced is still impaired in comparison with their in vivo-derived counterparts. This suggests that there are still improvements to be made in increasing oocyte and embryo developmental competence. More basic research is needed to unravel the molecular mechanisms, e.g., epigenetic reprogramming during early embryonic development as well as detailed studies on the composition and interactions of culture media. By altering the conditions of oocyte maturation and embryo culture, respectively, to mirror more closely the in vivo conditions, it may be possible to produce not only more blastocysts but, more importantly, blastocysts of better quality.

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