Transvaginal Ultrasound-Guided Oocyte Retrieval (OPU: Ovum Pick-Up) in Cows and Mares

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Abstract

For about three decades, transvaginal ultrasound-guided oocyte retrieval (OPU, ovum pick-up) has been successfully adapted from human reproductive medicine to the use in cattle and later on in the horse. Over time, it turned out to be a reliable and minimally invasive method to collect (immature) oocytes from genetically high valuable donors on a repeated basis. While a large part of the success of this procedure relies on the availability of a reliable in vitro embryo production system, a major prerequisite remains the collection of good-quality oocytes. The current chapter will focus specifically on oocyte retrieval technology. Following a detailed description of OPU equipment, the technical and biological factors affecting oocyte retrieval in living donors are discussed extensively with particular interest on the need of donor preparation by hormonal stimulation. Attention will also be given to donor health issues related to repeated oocyte retrieval. Finally, a state of the art of OPU in the mare is given describing additional physiological aspects of the equine oocyte and embryo implying additional challenges both for oocyte retrieval and in vitro embryo production.

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10.1 Introduction

For several decades, puncture and aspiration of bovine (immature) ovarian follicles has been used to retrieve oocytes for in vitro embryo production (IVP). Several comprehensive reviews on IVP and embryo transfer (ET) in domestic animals have highlighted the availability of 'good'-quality oocytes as the primary prerequisite for success (Hasler 1998; Galli et al. 2001; Merton et al. 2003; Merton 2014). Cumulus oocyte complexes (COCs) can be recovered from the ovaries of both slaughtered cows and living donors. Traditionally, post-mortem oocyte recovery was accomplished by follicle dissection or aspiration with a needle and syringe. However, this resulted in considerable variation in oocyte number and quality, largely as a result of differences in recovery techniques (Takagi et al. 1992; Hamano and Kuwayama 1993). The method of oocyte retrieval has an impact on COC morphology and subsequent developmental capacity in vitro, and, in this respect, the importance of an intact cumulus cell investment for oocyte maturation and in vitro development has been described extensively (Konishi et al. 1996; Tanghe et al. 2002). Immature bovine oocytes can be divided into different quality categories based upon light microscopic evaluation of the compactness of the cumulus investment and the transparency of the cytoplasm (de Loos et al. 1989; Hazeleger et al. 1995). Intimate contact between cumulus cells and the ooplasm is established through cumulus cell process endings (CCPEs) that extend through channels into the zona pellucida (transzonal processes). In the highest oocyte quality category (category 1), these CCPEs penetrate the zona pellucida and establish functional gap junctions with the oolemma (de Loos et al. 1991), which are absent in category 4 oocytes. Following in vitro maturation, the category 4 oocytes exhibit consistently low developmental capacity.

Understanding the relationship between follicle diameter and the quality of the enclosed COC during follicle development (Aerts and Bols 2010) is of vital importance for successful follicle and oocyte selection. The follicle constitutes a specific and defined micro-environment for the oocyte. Growth of the dominant follicle is associated with an increasing concentration of estradiol-17β in the follicular fluid, which therefore becomes gradually more estradiol dominated (Assey et al. 1994). Subordinate follicles either have a lower estradiol-17β/progesterone ratio or are progesterone dominated. Moreover, after ultrasonographically tracking follicle growth and regression, Price et al. (1995) noted that estradiol-17β concentrations were significantly lower in regressing and histologically atretic compared to non-atretic follicles. With respect to the influence of follicle size on oocyte quality, Arlotto et al. (1995) reported oocyte growth in all bovine follicle sizes studied, whereas Fair et al. (1995) demonstrated only a small positive correlation between oocyte diameter and follicle size. Overall, it appears that the increase in oocyte diameter plateaus at about 120 μm, when the follicle reaches 3 mm, whereas full meiotic competence is achieved at an oocyte diameter of 110 μm. Nevertheless, since Lonergan et al. (1994) obtained more grade 1 COCs (with many layers of cumulus cells) and a higher number of blastocysts per oocyte from follicles with a diameter >6 mm, it is probable that full cytoplasmic competence is only reached somewhat later during follicle growth.

From a practical reproductive perspective, aspiration of immature follicles is particularly interesting when performed on living donors, because the procedure can be repeated and is highly repeatable. In addition, the physiological status of the donor at the time of oocyte recovery can be assessed and manipulated, e.g. by the injection of hormones. This chapter will concentrate on follicle aspiration methods in living donors, with an emphasis on transvaginal ultrasound-guided follicle aspiration, also known as ovum pick-up (OPU), in the cow and to a lesser extent the mare. Following a brief description of the OPU technique per se, we will concentrate on the technical and biological factors that influence the success of OPU.

10.2 Oocyte Retrieval from the Living Donor Cow

The ability to puncture immature follicles within the ovaries of living donors and harvest the oocytes has opened new perspectives in assisted reproduction programs because additional female gametes can be made available for in vitro embryo production (IVP) over an extended time period, which is not the case if the donor animal is slaughtered. In addition, OPU permits hormonal modulation of the donor's ovarian activity prior to oocyte retrieval and thereby an opportunity to influence the quantity and quality of the retrieved COCs. A few important differences exist between post-mortem and in vivo oocyte retrieval. Firstly, transrectal manipulation of the ovary is necessary during oocyte retrieval in the living donor, to facilitate follicle visualization by laparoscopic or ultrasonographic imaging. By contrast, when follicles in the ovaries of slaughtered cows are punctured, a specific follicle can be selected and punctured under direct visual control. Secondly, different mechanical forces play a role when puncturing follicles in vitro, compared to in vivo follicle aspiration with an adjustable aspiration vacuum pressure (Hashimoto et al. 1999).

Different methods have been used to repeatedly collect oocytes from living donor cows; these include puncturing the follicles under laparoscopic guidance (Schellander et al. 1989), which results in high recovery rates but has the disadvantage of being relatively laborious and carries the risk of adhesions developing at the site of puncture. Callesen et al. (1987) were the first to use ultrasonography to collect oocytes from living cattle, using an ultrasonographic transducer equipped with a needle guide via a transcutaneous approach. A transvaginal laparoscopic technique was described by Reichenbach et al. (1994), during which a sterile trocar and cannula were directed into the abdominal cavity through the vaginal wall under rectal guidance; laparoscopy allowed the aspiration of the follicles to be accurately monitored. Pieterse et al. (1988) modified a transvaginal ovum pick-up technique, originally developed for use in human reproduction (Dellenbach et al. 1984), for use in cattle. A big advantage of the transvaginal approach in cattle is that it is possible to both secure and manipulate the ovary per rectum so that it can be moved around the ultrasound transducer and needle, to present the most optimal position for puncture. As a result, a minimally invasive method with high repeatability (Pieterse et al. 1991) for oocyte retrieval from living donor cows became available. Becker et al. (1996) compared

transvaginal OPU under ultrasonographic guidance with oocyte retrieval guided by endoscopic instruments. They concluded that the use of ultrasound resulted in better-quality cumulus oocyte complexes, although it is not entirely clear why endoscopic aspiration should cause more damage to the COCs. As a consequence, ultrasound-guided transvaginal oocyte pick-up (abbreviated to 'OPU' for the rest of this chapter) was developed as a successful technique for repeatedly retrieving oocytes from selected heifers and cows of high genetic merit (Kruip et al. 1994), to produce large numbers of calves with known production traits and to shorten the generation interval in cattle breeding programs. Indeed, the ultimate aim was to produce more embryos and pregnancies per donor cow than was possible through multiple ovulation and classical embryo transfer (MOET) programs (Pieterse et al. 1991).

10.2.1 OPU Equipment and Procedure

An OPU system consists of three major components: an ultrasonographic scanner with an appropriate transducer (probe), an aspiration pump, and a needle guidance system connected to an oocyte collecting tube (Figs. 10.1 and 10.2). The transducer and the needle guide are commonly constructed as a single operational unit to enable accurate manipulation of the needle from outside the cow while bringing the transducer into close contact with the ovaries. Mounted alongside the transducer, the puncture needle can be visualized on the ultrasound screen when it is advanced into the sonographic field to enter a follicle; to facilitate visualization, it is helpful to have a biopsy guide on the ultrasound screen and to use needles with a roughened area just behind the tip that is echogenic by dint of trapping air ('echogenic tip'). The needle is in turn connected to a vacuum pump by silicone or Teflon tubing such that follicular contents are aspirated as soon as aspiration pressure is applied via the vacuum pump. The follicular fluid and oocytes are collected into a collection device positioned between the needle and the pump. This oocyte collection device can be a regular embryo filter or a simple Falcon tube sealed with a stopper, into which an afferent tube delivers the follicle aspirate and from which an efferent line is connected to the vacuum pump that applies the aspiration pressure (Figs. 10.1 and 10.2). Although not compulsory, prior to OPU cows can be sedated with detomidine hydrochloride and treated with hyoscine-N-butylbromide to induce relaxation of the intestines. Subsequently, the faeces is removed from the rectum, and epidural anaesthesia is induced using 2% lidocaine to combat excessive straining during the transrectal manipulation. After the tail has been fixed to one side, the vulva and perineum are thoroughly cleaned and disinfected before the OPU device, containing the transducer and the needle guidance system, is inserted into the vagina (Fig. 10.3). While the OPU handle can be manipulated with one hand outside the cow, the head of the ultrasound transducer is positioned cranio-dorsally to the left or right of the cervix, depending on which side oocytes are to be collected. Using the other hand per rectum, the operator fixes the ovary and holds it against the head of the transducer (Fig. 10.4) such that

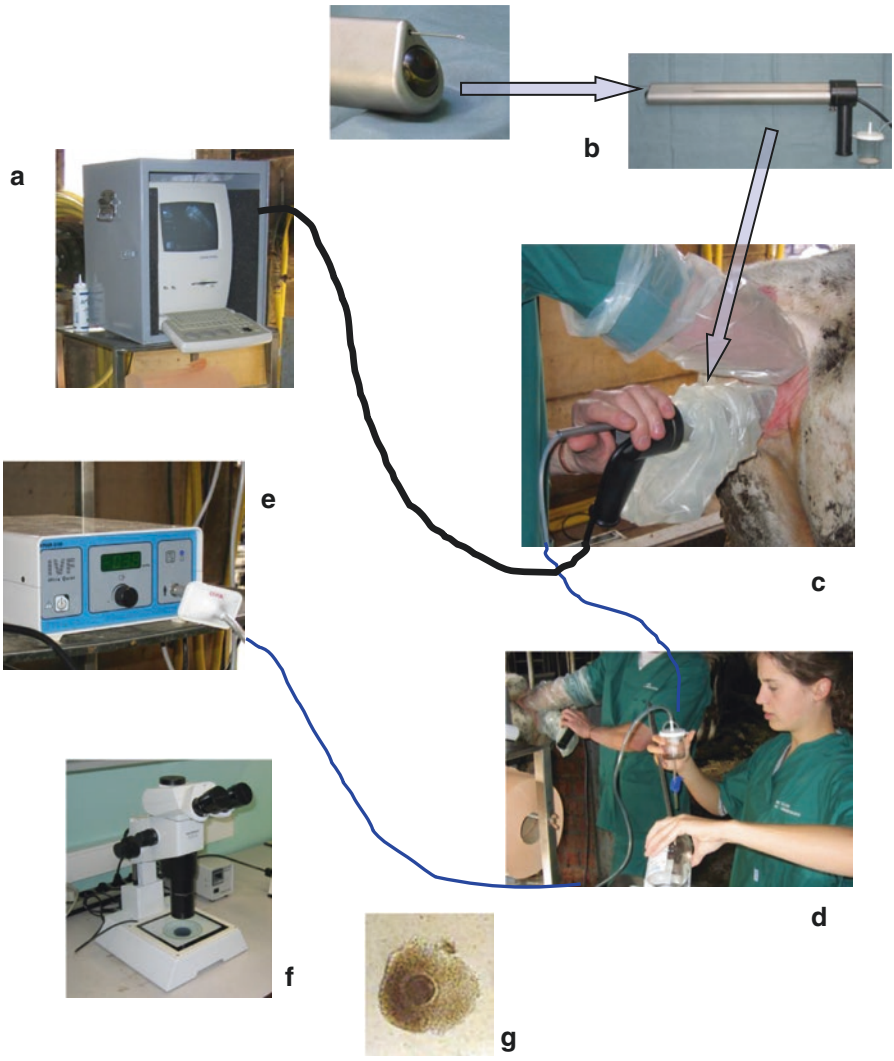

Fig. 10.1 Components of an OPU set up: (**a**) ultrasound scanner (Esaote/Pie Medical, Maastricht, the Netherlands) with (**b**) transducer and needle guidance system, inserted in the vagina of the donor (**c**). The needle is connected to the embryo filter (**d**), which is connected to the aspiration pump (**e**). Cumulus oocyte complexes (**g**) are looked for in the aspirated fluid by means of a stereo microscope (**f**)

the ovary and follicles can be visualized on the ultrasound screen (Fig. 10.5). A biopsy line programmed into the scanner's software is displayed on the screen and indicates where the follicle needs to be positioned for successful puncture. The operator then advances the needle slowly forward until the vaginal wall is pierced and the needle is visualized entering the ultrasound field. By monitoring the needle's position and simultaneously manipulating the ovary per rectum, the needle

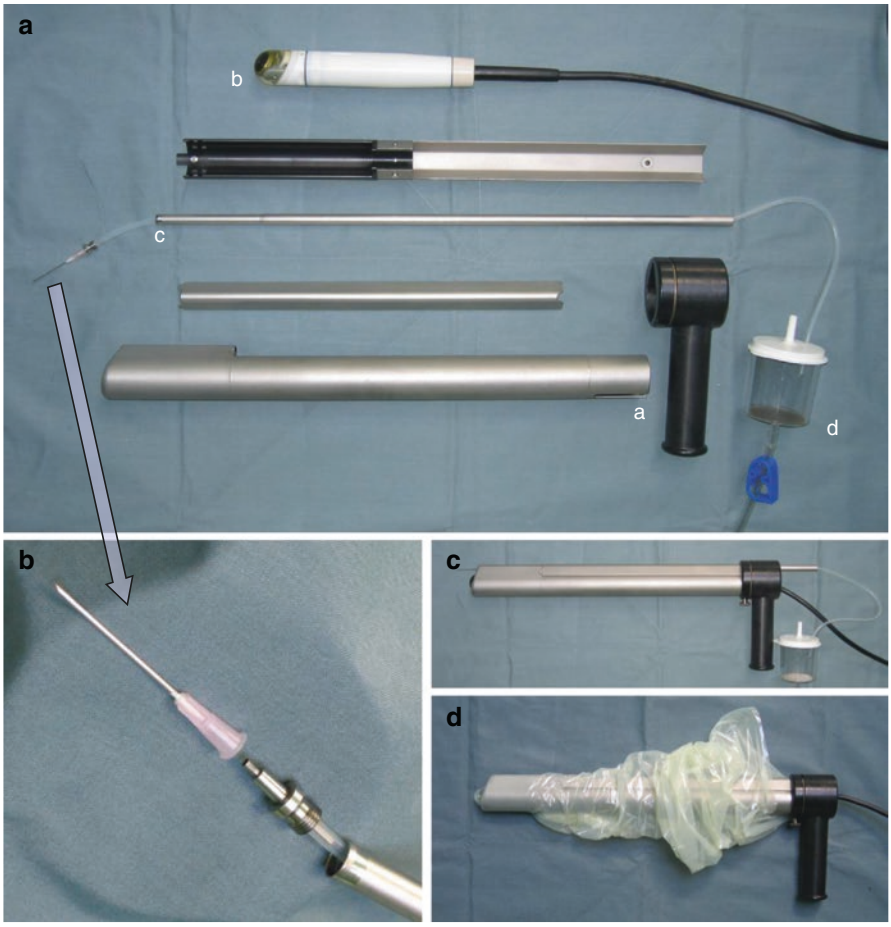

Fig. 10.2 (**a**) OPU device disassembled (**a**) and mounted ready for use (**c** and **d**) with a) intravaginal OPU handle, b) mechanical multiple angle sector transducer – MAP (Esaote/ Pie Medical, Maastricht, the Netherlands), c) needle guidance system and d) oocyte collection filter. (**b**) Detail of puncture needle connected to silicone tubing

can be directed into a follicle. Once the needle enters the follicle, the aspiration pump is activated using the foot pedal and the follicular fluid, and COCs are collected into the embryo filter which contains the oocyte collection medium. Subsequently, the filter contents are washed and transferred to a petri dish, and the oocytes are identified using a stereomicroscope, captured using a glass pipette and placed into maturation medium. After 24 h of maturation, they will be fertilized and cultured for 7 days in vitro to reach the blastocyst stage. The final outcome of OPU, in terms of numbers and quality of retrieved COCs, is influenced by both technical and biological factors (Bols 1997), both of which will be discussed in more detail.

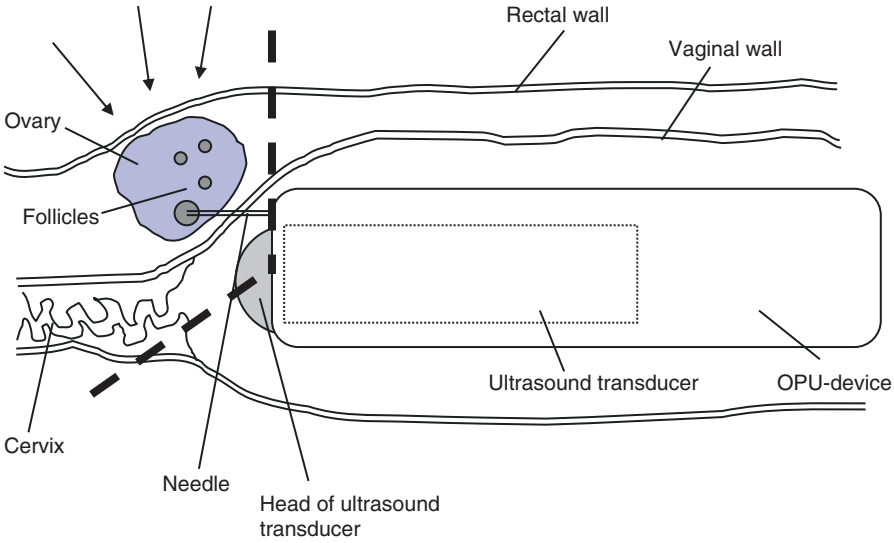

Fig. 10.3 Positioning of the ovary during transrectal palpation. Pressure is exerted on the ovary with the hand positioned intrarectally, from the direction indicated by the arrows. The bold dashed line delineates the scanned area

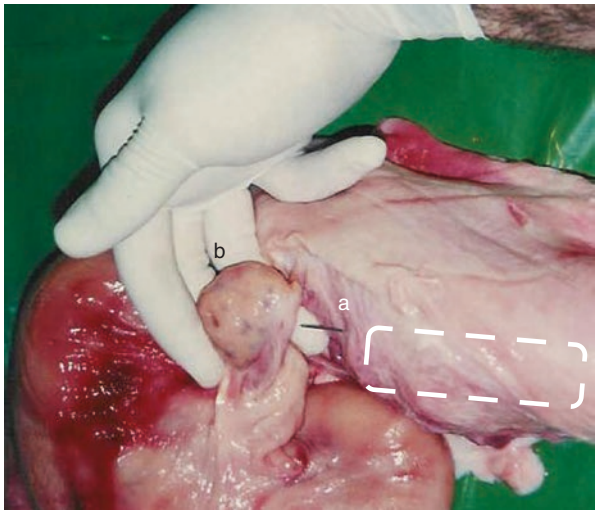

Fig. 10.4 Positioning of the ovary during transrectal palpation. The puncture needle penetrates the vaginal wall (**a**) when pushed forward. In vivo, the rectal wall lays between the hand of the operator and the ovary (**b**). The white dashed line delineates the position of the intravaginal OPU device

10.3 Technical Factors Influencing OPU Results

Since continuing advances in ultrasound technology have improved image resolution and the accuracy with which ovarian structures can be visualized (Hashimoto et al. 1999; Seneda et al. 2001; Singh et al. 2003; Bols et al. 2004), the 'weakest

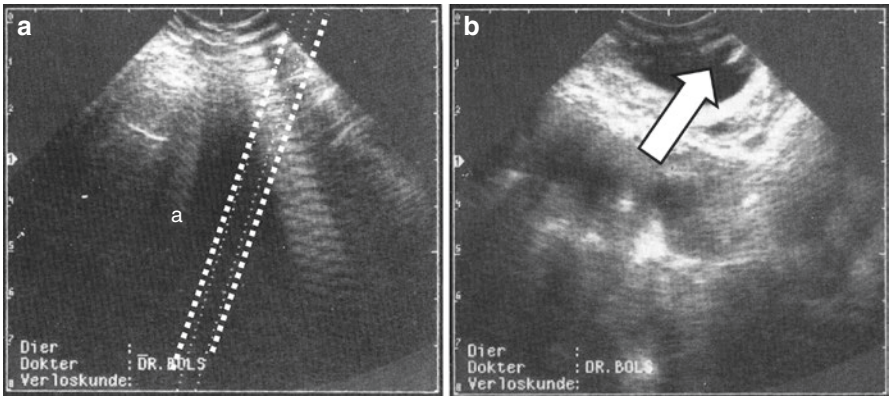

Fig. 10.5 Ultrasound images taken during the OPU procedure. (**a**) The biopsy or puncture line is fixed on the ultrasound screen (a), indicating where the needle will appear within the scanned area; (**b**) needle point penetrates the follicular wall (white arrow)

link' or component of highest concern is now the puncture needle because a sharp needle is a prerequisite for successful OPU (Scott et al. 1994). Traditionally, most operators used 50–60-cm-long needles, with an outer diameter of 1–1.5 mm, which are relatively simple to construct and easy to handle (Looney et al. 1994; Bols 1997). A major disadvantage of these needles is that they become blunt quite quickly and, even with regular resharpening, never regain their original sharpness. In addition, these long, non-disposable needles are relatively expensive and contain a large dead space. Alternative OPU systems have been developed that use disposable 18 gauge epidural needles (Rath 1993) or cheaper, regular hypodermic injection needles (Bols et al. 1995). These needles have the additional advantages of being sterile and available in different diameters and lengths and easy to change.

OPU success rate is quantified firstly in terms of the oocyte recovery rate (RR = number of COCs per 100 follicles punctured), which is influenced by factors including needle diameter, aspiration pressure and operator experience (Bols 1997). As a result, RRs have been reported to vary between 7% and 70% for different OPU teams. Over the years, many different needle diameters and aspiration pressures have been used in either experimental or commercial bovine OPU programs (Bols 1997), which makes it difficult to directly compare recovery rates. In addition, the exact aspiration pressure exerted through the tip of the needle depends on the aspiration device, the length and diameter of the tubing the size and type of collection vessel, as well as on the needle diameter. To make comparisons possible, the aspiration pressure needs to be expressed in terms of the amount of fluid (in ml) that can be aspirated per minute, rather than in mm Hg exerted from the vacuum pump. Indeed, a modest change in needle diameter can triple the rate of fluid aspiration without any change in aspiration pressure (Bols et al. 1996). Given the importance of an intact cumulus cell investment for oocyte maturation and future developmental capacity, any damage to the COC caused by the aspiration procedure has to be assessed for a given system so that preventive measures can be taken. Ideally, the

optimal aspiration pressure for a given OPU system should be established by puncturing a substantial number of follicles on ovaries from slaughtered cows. While various vacuum pressures and needle diameters can be tested, COC morphology should be evaluated following aspiration with special attention to the integrity of the cumulus cell investment. In this way a threshold value, or an optimal range, for aspiration pressure can be established that will not result in too much damage to the aspirated COCs but still maintain an acceptable RR. Systems that use simple disposable injection needles allow such an in vitro calibration (Bols et al. 1996). The percentage of retrieved intact COCs usually decreases progressively as the aspiration pressure increases, which is associated primarily with an increase in the number of denuded oocytes, as reported by Ward et al. (2000). As would be expected, higher numbers of good-quality COCs will translate to a higher number of cultured blastocysts produced. Aspirating selected top-quality COCs, which were initially retrieved following slicing of ovaries recovered from slaughtered cows, to assess the net damage that the aspiration procedure can cause, revealed an overall RR of 79% (Bols et al. 1997). In other words, one out of five oocytes was lost during the aspiration process. Fortunately, an average of 82% of the recovered COCs was still surrounded by a compact cumulus investment following aspiration. Thus, on average, around 20% of the initially good-quality COCs were microscopically damaged by the OPU procedure, by (partial) stripping of cumulus cells in a manner likely to impair the oocyte's in vitro developmental potential (Cox et al. 1993). A final very important factor determining OPU outcome is the experience of the operator or the team that is retrieving the oocytes, as evidenced by an in-depth analysis of 7800 OPU sessions performed in a commercial setting by Merton et al. (2003).

10.4 Biological Factors Influencing OPU Results

A substantial body of literature is available on biological factors that might influence the likelihood of blastocyst formation when in vitro embryo production (IVP) is based on COCs recovered via OPU. While there is no doubt that the highest blastocyst rates will be obtained with the best-quality COCs (as stated above), one should bear in mind that the IVP procedure 'as such' is an extremely complex process that critically influences the final blastocyst rate. Since discussing non-OPU factors that affect the success rate of IVP is beyond the scope of this chapter, we will concentrate on a few factors that are directly related to the OPU procedure per se.

10.4.1 Frequency and Timing of Follicle Puncture

The OPU technique has the advantage of being highly repeatable. Pieterse et al. (1991) punctured follicles during different oestrous cycle stages in the same donors, over a 3-month period. However, the presence of a dominant follicle appears to reduce the in vitro developmental competence of oocytes from the subordinate follicles, even at a relatively late stage of dominance (Hendriksen et al. 2004). This is

why the dominant follicle is often removed by aspiration prior to a regular oocyte retrieval session 48 h later (DFR). While some studies report no effect of collection frequency on the number of follicles aspirated or the number of COCs collected per session (Garcia and Salaheddine 1998), most researchers agree that a twice-weekly oocyte collection schedule has a positive effect on the number of follicles available for puncture and the number of blastocysts that results (Bols 1997). Indeed, it can be assumed that the developing dominant follicle will be ablated during each session when a cow is punctured twice a week, thereby stimulating an additional wave of smaller follicles to grow (Bergfelt et al. 1994).

10.4.2 Physiological Status and Body Condition of the Donor

In cattle breeding programs, OPU is generally performed on selected healthy heifers with excellent genetic potential for production traits that could in themselves be predictive for oocyte yield and the number of blastocysts produced (Merton et al. 2009). However, OPU can be performed at various stages of a cow's reproductive life; even pregnancy does not exclude OPU, since oocytes can successfully be retrieved during the first 3 months of gestation (Meintjens et al. 1995; Bungartz et al. 1995; Reinders and Van Wagtendonck-de Leeuw 1996). Argov et al. (2004) saw an increase in the number of oocytes recovered when a higher proportion of aspiration sessions were performed in cows in early lactation. On the other hand, undernutrition has a negative effect on the developmental competence of recovered oocytes in vitro, as illustrated by the decreasing percentage of blastocysts associated with decreasing body condition score of the donor (Lopez Ruiz et al. 1996) and an increasing proportion of good-quality oocytes with increasing body condition score (Dominguez 1995).

10.4.3 Breed and Age of the Donor

Early reports suggested that European breeds had significantly more large follicles than zebu or crossbred cows (Dominguez 1995), whereas no differences in the proportion of normal oocytes recovered were apparent. However, over the past 10 years, the use of OPU-IVP has rocketed in Latin-America and in particular in Brazil where the high fecundity of a single breed, the Nelore, has been the foundation for the production of hundreds of thousands of embryos. Indeed, a single OPU session in an average Nelore donor cow can yield up to 50–60 oocytes, resulting in up to 30 in vitro embryos per puncture session (Pontes et al. 2011). Strikingly, these results are obtained without any hormonal stimulation and have led some researchers to conclude that repeated OPU alters follicular dynamics and might increase follicle growth rate in zebu donor cows (Viana et al. 2010). Highly contrasting results have been reported in Belgian Blue donors with impaired fertility, which yielded an average of only 3.1 oocytes and 0.5 embryos per puncture session (Bols et al. 1996).

The use of OPU in young donors is limited by the smaller dimensions of the pelvis. Holstein Friesian heifers can be subjected to OPU from around the age of 6–8 months, depending on the dimensions of the intravaginal handle and transducer used (Rick et al. 1996; Bols et al. 1999). Follicles in calves can also be punctured, but this requires a different approach to access the ovaries (Brogliatti et al. 1995). The major problem with prepubertal donors is the impaired in vitro developmental capacity of the recovered oocytes (Taneja et al. 2000), resulting in a lower overall efficiency of the procedure.

10.4.4 The Role of Hormonal Stimulation to Prepare Donors for OPU

An enormous amount of research has been done on how potential donors can be prepared to maximize oocyte and subsequent embryo yields. An important general remark before describing a few of the possibilities is the fact that long-term, repeated use of OPU in an individual donor cow is possible without any hormonal stimulation (Pieterse et al. 1991). In the long term, the absence of hormonal stimulation offers many advantages because when using hormones to stimulate follicle growth, the blood flow to the ovaries increases enormously, rendering the cows useless for OPU for a few weeks after the initial puncture. Low or suboptimal follicular activity can be remedied in some potential donors, mostly by using FSH-LH combinations or equine chorionic gonadotrophin (eCG = PMSG, pregnant mare serum gonadotrophin). While these hormones have been widely used in ET programs, modifications in the dose and timing of treatments are necessary, because the final aim of stimulation prior to OPU is to generate additional follicles rather than to initiate multiple ovulations. Pieterse et al. (1988) achieved the highest oocyte recovery rates in PMSG-treated donors, which developed larger ovaries and had more follicles than non-stimulated animals. However, a later study (Pieterse et al. 1992) showed that while stimulation resulted in a larger number of aspirated follicles per cycle, it had the opposite effect on oocyte recovery rate (RR), which was lower in stimulated than non-stimulated donors. Positive effects of FSH on the number of follicles with a diameter >6 mm and the number of viable blastocysts have, however, also been reported (Looney et al. 1994; Goodhand et al. 2000). Unfortunately, the increase in the number of follicles, oocytes recovered and embryos produced is often inconsistent and might depend on the cycle stage at which treatment is initiated (Paul et al. 1995). Vos et al. (1994) were able to retrieve five times as many COCs 22 h after, compared to shortly before, the LH surge (in PMSG-treated donors). Stubbings and Walton (1995) found no differences in the mean number of follicles suitable for puncture between non-stimulated cows punctured twice a week and FSH-stimulated cows punctured only once. Subtle changes in FSH dose influenced the sizes, but not the number of follicles, which was mainly a factor of individual donor and OPU session variation (De Roover et al. 2005). Some authors have also used intravaginal progesterone-releasing devices (CIDR) in combination with FSH and LH to prepare oocyte donors, with varying results (Chaubal et al. 2007). It should be noted that

FSH (and probably also other hormonal) treatments might result in asynchrony between the maturation of the oocyte and its surrounding follicle (de Loos et al. 1991) or between nuclear and cytoplasmic maturation (Bousquet et al. 1999), resulting in reduced developmental competence.

As can be expected, hormonal stimulation and OPU puncture frequency together can affect the final embryo yield. De Ruigh et al. (2000) concluded that FSH treatment prior to OPU once every 2 weeks resulted in significantly more COCs and more embryos produced in vitro (expressed per OPU session) than a twice-per-week non-stimulated OPU schedule. However, total embryo production over a 2-week period turned out to be higher with the twice-weekly puncture scheme (four non-stimulated sessions in 2 weeks) than for one FSH-stimulated OPU session every 2 weeks. Goodhand et al. (1999) reported that the puncture of FSH-treated donors once a week produced a similar number of transferable embryos per 'donor week' as aspiration twice a week without FSH treatment. Chaubal et al. (2006) reported that a protocol combining dominant follicle removal and FSH stimulation with a subsequent single OPU per week seemed to be the most productive and cost-effective approach over a 10-week period. When calculating total costs of the procedure, one needs to keep in mind the price of the hormonal treatment, and its administration, which often requires animal handling twice a day for several days.

10.5 OPU-IVP to Treat Bovine Infertility

Compared to ET, where cows can typically be flushed three to four times a year, yielding around five embryos per flush, OPU can be performed as often as twice a week. In healthy donor cows, two embryos per donor per week can be produced, equating to four to five times the average ET yield (Kruip et al. 1994). An important additional advantage of using OPU-IVP is greater flexibility in choice of sire-dam combinations in vitro, i.e. using different bulls on oocytes from the same OPU session, which can accelerate the genetic selection process. In addition, OPU-IVP can be used to produce additional offspring from valuable cows that no longer respond to embryo flushing treatments. The first OPU-IVP calves in Belgium were born in 1995, following oocyte retrieval from Belgian Blue donors with impaired fertility (Bols et al. 1996). Following the transfer of 56 IVP embryos, 12 viable pregnancies were obtained, leading to at least 1 extra calf for 7 out of 12 high genetic merit donors considered to have reached the end of their breeding career. Looney and co-workers (1994) reported OPU in 200 mostly beef cattle donors, of which 50% had a history of good embryo production. An average of 6.3 oocytes were retrieved per session, and 16.4% yielded a blastocyst. Transfer of 813 embryos resulted in 325 pregnancies (40%). Hasler et al. (1995) carried out similar work on 155 infertile dairy cows. An average of 4.1 oocytes suitable for IVF were retrieved per session. Following transfer of 2268 fresh embryos, 1220 pregnancies (53.8%) were obtained. Large data sets like these illustrate that OPU-IVP has evolved to become a routine procedure to produce reliable numbers of embryos in vitro, albeit with a dependency on the breed of cow and the efficacy of the IVP system (Bousquet et al. 1999).

When comparing embryo yields and pregnancy rates between in vivo (classical ET) and in vitro (OPU-IVP) methods using the same donors, the in vitro approach turned out to yield the most embryos (Pontes et al. 2009). Because the ultimate success rate of assisted reproduction is determined by the number of calves produced, a well-synchronized, healthy, recipient herd into which fresh embryos can be transferred is a major prerequisite for success. When fresh transfers cannot keep up with embryo production, reliable embryo cryopreservation methods need to be available, increasing the complexity of the whole operation.

10.6 Donor Health and Repeated OPU

Reports on the impact of the OPU procedure on donor animal health and future reproductive performance are scarce. Pieterse et al. (1991) could not detect any adhesions following OPU, and the procedure did not seem to affect the donor's future fertility. Dairy heifers were closely monitored during two periods of 4–5 weeks while enrolled in a twice-weekly OPU schedule (Petyim et al. 2000). They only occasionally showed signs of oestrus, and corpus luteum-like structures often developed from punctured follicles, which concurred with earlier findings that, based on progesterone profiles, repeated OPU appeared to induce a degree of acyclicity (Bols et al. 1998). At the end of their first OPU period, heifers returned to normal cyclicity (Petyim et al. 2000). Post-mortem findings following the second OPU period included a thickening of the ovarian tunica albuginea and a slight hardening of the ovaries. The authors concluded that OPU did not have major negative effects on ovarian structure or on subsequent ovarian function. Additional research on the effects of OPU revealed a significant rise in FSH levels on the day following puncture (Petyim et al. 2001). In addition, heart rate and cortisol concentrations increased significantly following restraint and epidural injection. However, both parameters returned to normal within 10 min after completion of the OPU procedure.

10.7 Transvaginal Ultrasound-Guided Oocyte Retrieval in the Mare

As with other assisted reproductive technologies, the development and uptake of OPU-IVP in commercial horse breeding has been slower and driven by different primary goals to those that apply to cattle breeding (Galli et al. 2007). While initial reports of transvaginal ultrasound-guided oocyte retrieval in mares (Brück et al. 1992) followed closely behind those in cattle, interest in the technique waned for a number of practical reasons. Most important were the disappointing rates of oocyte recovery from immature follicles (<25% in early studies: see Hinrichs 2012 for review) and the absence of commercially available gonadotrophins capable of stimulating the development of multiple mature follicles from which to harvest in vivo-matured oocytes; taken together this meant that recovering enough high-quality

oocytes from living donors to run a viable IVP program appeared an insurmountable challenge. Since conventional in vitro fertilization using equine gametes also proved to be very poorly successful (Hinrichs 2012), commercial interest in equine IVP remained understandably low. However, interest in OPU was rekindled by the development of oocyte transfer (OT) as a tool to examine oocyte developmental competence (Carnevale and Ginther 1995) and to treat severe acquired infertility in mares (Carnevale 2004). Development of OPU was given further impetus by the first reports of intracytoplasmic sperm injection (ICSI) as a technique for successfully producing foals after fertilizing equine oocytes ex vivo (Cochran et al. 1998; McKinnon et al. 2000). Nevertheless, progress remained slow, largely because blastocyst production rates following IVP were much lower (<10% compared to approximately 35%) than those obtained after transfer of sperm-injected oocytes into the oviduct of either synchronized recipient mares (Choi et al. 2004) or progesterone-treated sheep (Tremoleda et al. 2003). The development of DMEM/Hams F-12-based equine IVP systems capable of supporting blastocyst production rates >35%, at least within an experimental set-up (Choi et al. 2006), was the final breakthrough required for equine IVEP to become a viable clinical technique. Indeed, when Galli et al. (2014) reported producing 0.6 blastocysts per OPU in a commercial OPU-IVP program, it became clear that OPU-IVP could be competitive with commercial embryo transfer, given that embryo recovery rates of 0.3–0.5 per cycle are the norm in commercial sport horse mares inseminated with frozen-thawed or chilled-transported semen (Stout 2006). Most recently, reports of blastocyst production rates of 15–20% per injected oocyte and > 1 per OPU (Hinrichs et al. 2014) even after overnight shipping of oocytes at 20 °C (Galli et al. 2016) have led to a surge in interest in equine OPU-IVP.

10.7.1 Clinical Applications of OPU in the Mare

OPU is the basis for two clinical procedures in horses, oocyte transfer (OT) and in vitro fertilization by intracytoplasmic sperm injection (ICSI) (Fig. 10.6). To date, the main reasons for wanting to use OPU in clinical equine practice has been subfertility. Indeed, OT was developed primarily as a technique for treating subfertility in mares that were not, or only infrequently, able to produce embryos by conventional AI and embryo flushing, due, for example, to repeated failure of normal ovulation or severe pathology of the oviducts, uterus or cervix (Carnevale 2004). OPU-ICSI was similarly introduced initially as a treatment for subfertile mares; however, given its original development as a technique for addressing ‘male factor infertility’ in human infertility, ICSI also rapidly became an attractive option for addressing stallion subfertility and/or limited availability of semen. Finally, significant improvements in in vitro blastocyst production rates and the realization that OPU-ICSI combined with blastocyst cryopreservation significantly improves the efficiency of recipient mare use have seen OPU-IVP emerge as a desirable method for producing embryos from actively competing sport horse mares (e.g. show jumpers and dressage horse) whose competitive peak overlaps with their most fertile

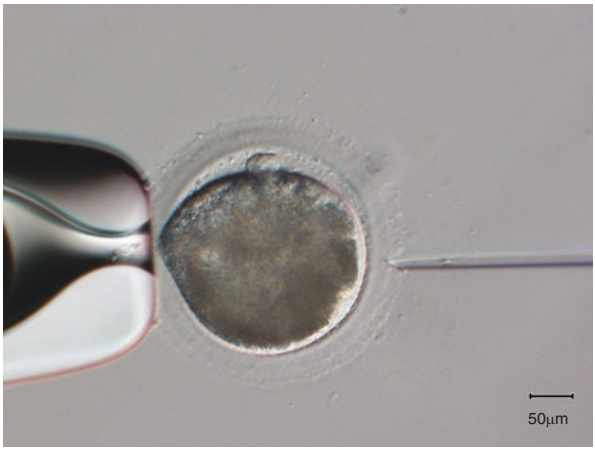

Fig. 10.6 An in vitro-matured MII horse oocyte immediately prior to intracytoplasmic sperm injection. The oocyte is immobilized with a holding pipette with the polar body orientated to 12 o'clock, to minimize the risk of injecting the sperm into the metaphase plate. A sperm is positioned in the tip of a conventional injection needle. The high lipid content of the horse oocyte makes it difficult to visualize cytoplasmic structures

years (Galli et al. 2014). OPU-IVP has the additional advantage over conventional ET that it can be performed as a single outpatient procedure with minimum impact on the training or competition schedule and without the need for any hormonal manipulation of the oestrous cycle; many owners and riders do not like their mares being returned to oestrus since it can negatively affect performance in some mares.

10.8 Oocyte Retrieval from Living Donor Mares

The equipment required for, and procedures involved in, recovering oocytes from living donor mares is essentially the same as those used in cattle, although some modifications are required to account for behavioural and anatomical differences between the species. The most important difference is the fact that immature equine COCs are surrounded by a cumulus investment with fewer cell layers that is attached more firmly to the follicle wall by a broader cumulus cell hillock with projections into an underlying thecal cell pad (Hawley et al. 1995). The practical consequence of this more tenacious attachment of the immature COC to the follicle wall is that simple aspiration of follicular fluid is not sufficient to reliably recover the oocyte. Instead repeated aspiration and flushing of the follicle accompanied by scraping of the follicle wall with the bevel of the aspiration needle is required to achieve a clinically acceptable oocyte recovery rate (Galli et al. 2007). In general, a 60 cm 12 gauge (approx. 2.75 mm outer diameter) double lumen needle is used for equine OPU. Aspiration is performed via the inner stylet which is connected, via a collecting vessel, to the vacuum pump; the vacuum pressure is adjusted to achieve fluid aspiration of roughly 20–25 ml per minute, since higher pressures increase the risk of denuding the already

relatively thin equine cumulus cell investment. Once the follicle has been evacuated, it is flushed repeatedly with commercial embryo flushing medium, supplemented with heparin (5–20 i.u. per ml) to prevent clotting of any blood or the gelatinous fluid commonly recovered from large or atretic follicles, and introduced via the outer needle. Using a double lumen needle significantly reduces the risk of an oocyte remaining in the needle's dead space and being repeatedly flushed into and out of a follicle.

10.8.1 Aspirating Immature Follicles

For immature oocyte recovery, follicles from approximately 8–10 mm in diameter are flushed 6–12 times, where larger numbers of flushes are used when few follicles are available for aspiration, to maximize the likelihood of recovering the oocyte. The need to repeatedly flush follicles means that the OPU can be a prolonged procedure (15–45 min) in the mare; epidural anaesthesia using 2% lidocaine is therefore recommended to prevent the mare straining in response to the presence of the ultrasound probe in the vagina and the manipulation of the ovaries via the rectum. In addition, fairly profound sedation with an alpha-2 agonist (e.g. detomidine hydrochloride) potentiated with an opioid analgesic such as butorphanol is recommended to ensure that the mare remains quiet throughout the procedure, while hyoscine-N-butylbromide can be used to further relax the rectum, thereby facilitating manipulation of the ovaries and reducing the risk of damaging the rectum wall. It is also advisable to administer a non-steroidal anti-inflammatory drug (NSAID) to combat pain during and immediately after the OPU procedure and perioperative antibiotics to cover the possibility of contaminants being introduced into the abdominal cavity during OPU. In our experience of >500 OPUs, the procedure is (surprisingly) well tolerated, even in young inexperienced mares, and post-procedure complications have been limited to mild pyrexia and/or abdominal discomfort of short duration (12–36 h) that responds well to NSAIDs. Others have reported occasional rectal bleeding associated either with needle puncture of the rectum wall or as a result of vigorous ovarian manipulation and emphasize the ever-present risk of more serious damage such as a rectal tear or ovarian abscess (Velez et al. 2012); fortunately, the incidence of serious complications appears to be low, and even repeating OPU at 2-week intervals over a period of months appears to have little or no lasting effects on subsequent ovarian structure, cyclicity or fertility (Velez et al. 2012). Recent reports on oocyte recovery rates suggest that, with an established team and system, average RRs from immature follicles of between 50 and 70% can be achieved (Jacobson et al. 2010; Galli et al. 2014, 2016; Hinrichs et al. 2014), although recovery during individual OPU attempts can vary from as little as 20% and up to 100%.

10.8.2 Harvesting In Vivo-Matured Oocytes

The major alternative to harvesting immature oocytes is oocyte recovery from the pre-ovulatory follicle of a donor mare at a set time after hormonal induction

of ovulation; indeed, this is the protocol of choice for OT and is also used in some OPU-IVP programs both because oocyte recovery rates from pre-ovulatory follicles are high (>70%: Carnevale et al. 2005; Foss et al. 2013) and because oocytes that undergo in vivo maturation have higher developmental competence, with blastocyst formation rates as high as 40–70% reported albeit on small numbers of oocytes (Jacobson et al. 2010; Foss et al. 2013). OT also aims to utilize the anticipated high developmental competence of in vivo-matured oocytes as a treatment for subfertility of female origin and involves the surgical transfer of a mature (metaphase II) oocyte to the oviduct of an inseminated recipient mare that has had her own oocyte removed by aspiration of the pre-ovulatory follicle (Carnevale 2004). In either situation, oocyte recovery involves aspiration of the single (occasionally 2–3) pre-ovulatory follicle between 20 and 35 h after induction of ovulation using either a long-acting GnRH analogue (e.g. deslorelin acetate), hCG (1500–2500 i.u.) or a combination of the two, in an oestrous mare with a follicle exceeding 35 mm in diameter (Carnevale 2004; Foss et al. 2013). Waiting until 35 h after ovulation induction has the advantage of ensuring that the oocyte has reached MII, i.e. is fully mature, and that the attachment of the COC to the underlying thecal pad has begun to loosen, thereby improving the likelihood of oocyte recovery. On the other hand, a small proportion of mares will ovulate before the 35-h time point and that cycle will therefore be lost. When recovery is performed at 20–24 h after ovulation induction, there is less risk of premature ovulation, but the oocyte will be at approximately the metaphase I stage of maturation and require a further 12–16 h of culture in vitro to complete maturation before transfer into the recipient's oviduct (Carnevale 2004; Galli et al. 2014).

10.8.3 Technical and Biological Factors Influencing OPU Results

As in the cow, the success of OPU-IVP can be divided into two interrelated components, oocyte recovery rate (RR) and blastocyst production rate, where the latter and the pregnancy and foaling rates following transfer of resulting embryos are ultimately most relevant. Historically, RR from immature follicles was poor at around 25% (for review see Hinrichs 2012). However, it is now clear that a RR of >50% can be achieved when aspirating and repeatedly flushing follicles ≥8–10 mm in diameter (Galli et al. 2007; Jacobson et al. 2010; Galli et al. 2014). While this may not quite reach the RR of oocytes from pre-ovulatory follicles (>75%; Carnevale et al. 2005), it is more than compensated by the larger number of oocytes and the fact that in vitro oocyte maturation rates of OPU-derived oocytes is high (>65%: Foss et al. 2013; Galli et al. 2014). One critical technical factor is needle size, with the RR falling when smaller diameter needles are used, e.g. Velez et al. (2012) reported a RR of 38% for a 15 gauge double lumen as compared to 48% for a 12 gauge double lumen needle. While it is not entirely clear exactly why a larger needle is better, it presumably relates either to more rapid flow and greater turbulence during flushing or more effective scraping of the inside of the follicle.

Currently, there is too little data to make firm conclusions about factors influencing the ultimate results of OPU-IVP; indeed, there is very little published data about pregnancy and foaling rates. Nevertheless, the recent upsurge in the use of OPU-IVP is beginning to yield some interesting data. For example, preliminary reports indicate that pregnancy rates exceeding 75% following transfer of fresh (Hinrichs et al. 2014) and exceeding 60% after transfer of cryopreserved (Galli et al. 2007, 2016) OPU-IVP embryos are possible; on the other hand, early pregnancy loss rates appear to be higher than after conventional breeding, AI or ET (>20% versus 5–10%). In addition, mare age, breed, timing of an OPU attempt and time of season all seem to affect aspects of the OPU-IVP process. For example, performing OPU at a fixed interval of 14 days results in a fall in the number of follicles available for puncture (7–9 yielding 3.5–4.5 oocytes; Jacobson et al. 2010; Velez et al. 2012) compared to monitoring mares and delaying the subsequent OPU until follicle numbers have increased. Using the latter approach, Galli et al. (2014) reported aspirating 14–17 follicles during repeated OPU attempts, yielding 9–12 oocytes per OPU. In the clinical program at Utrecht University, the policy is to advise owners to wait until a mare has at least 15 follicles >10 mm, while accepting that some mares will never develop more than 6–10 follicles and need to be aspirated at this point; this policy has resulted in means of 23.5 follicles yielding 12.8 oocytes during 252 commercial OPUs (Claes et al. 2016). With respect to time of season, the autumn and spring transitional periods appear to be optimal for the collection of immature oocytes because mares develop more mid-sized follicles than during the breeding season (e.g., 11.5 versus 6 follicles exceeding 12 mm; Donadeu and Pedersen 2008). Mare age also significantly affects follicle number with mares older than 20 years having significantly fewer follicles during the transitional period than 17–19-year-old mares, which in turn had fewer follicles than 3- to 7-year-olds (Carnevale et al. 1997). These two observations explain why oocyte recovery in a commercial OPU program decreased with increasing mare age and was higher during spring and autumn than in the summer (Claes et al. 2016).

Equine blastocyst production by ICSI is currently a highly operator-dependent process, and, to date, only a handful of laboratories worldwide have been able to generate commercially acceptable embryo production rates (Hinrichs 2012) Even so, it is becoming apparent that there are breed effects on blastocyst production rates with Galli et al. (2014) reporting embryo production rates of 0.84 (11.3%), 0.6 (10%) and 0.29 (4.1%) per OPU (per injected oocyte) for Warmblood, Quarterhorse and Arab mares, respectively. In addition, Claes et al. (2016) recently reported significant effects of antral follicle count (follicles >4 mm at the time of OPU) and donor mare reproductive history on blastocyst production, with fewer blastocysts resulting from mares with lower follicle numbers and with a history of subfertility/fertility using other techniques, irrespective of mare age (Fig. 10.7).

Since OT is a more established technique than OPU-IVP, more information is available about factors affecting pregnancy rates following OT than for OPU-IVP. While operator experience also clearly plays an important role in results, the other principle factor influencing success is age of the donor mare. Indeed, in an experimental setting, OT of oocytes from young mares yielded a 92% pregnancy rate compared to 31% for aged mares (Carnevale and Ginther 1995). Similarly, in a

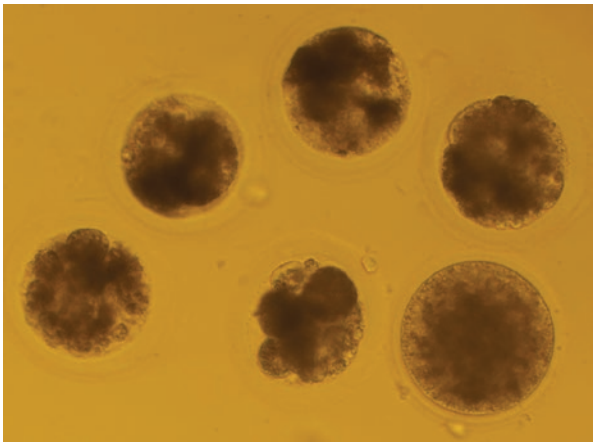

Fig. 10.7 Developing horse embryos 8 days after ICSI. The embryo in the bottom right has developed to the blastocyst stage as evidence by expansion, thinning of the zona pellucida (ZP) and development of a palisading trophoblast layer. In vitro-produced embryos do not produce a confluent blastocyst capsule, which explains the absence of a capsular layer between the trophectoderm and the ZP. The other embryos all underwent cleavage and early cell divisions but are now in various stages of degeneration. It can be challenging to definitively differentiate between blastocysts and degenerate embryos/oocytes

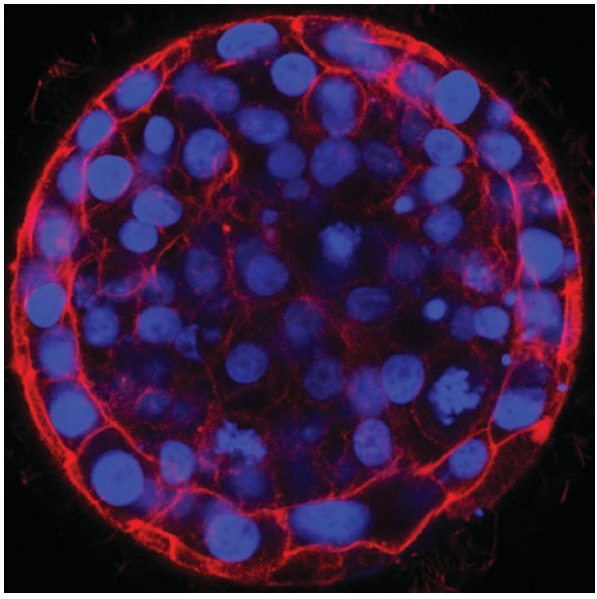

Fig. 10.8 An in vitro-produced horse blastocyst stained with Hoechst 33,342 (Blue) to visualize the nuclei and phalloidin to demonstrate the actin cytoskeleton. Because it takes practice to reliably differentiate viable IVP horse blastocysts from degenerating embryos, staining to demonstrate the presence of numerous cells and formation of a trophoblast layer can be essential to the establishment of an equine IVP program

commercial setting, day 15 pregnancy rates averaged 50% for mares <15 years old compared to only 16% for mares >23 years (Carnevale et al. 2005). As for OPU-IVP, pregnancy loss rates in a clinical OT program exceed 20%, presumably reflecting the bias in the donor mare population to aged mares with reduced intrinsic oocyte developmental competence (Hinrichs 2012) (Fig. 10.8).

Conclusion

OPU is now a routine, widely performed procedure in both commercial cattle practice and research into the developmental competence of bovine oocytes. In a commercial setting, the technique offers greater flexibility, in terms of bull use, and is capable of generating more embryos per unit time than conventional multiple ovulation and embryo transfer protocols. In horses, OPU was first introduced into the clinic as a vital component of oocyte transfer, where success is limited by the bias towards aged subfertile mares as donors; nevertheless, OT has allowed production of foals from mares that would otherwise have been considered infertile. Equine OPU-IVP has only very recently become a commercially viable proposition, as a result of significant improvements in immature oocyte recovery and in vitro blastocyst production; nevertheless, OPU-IVP is already proving to be very competitive with AI-ET in terms of numbers of embryos generated per unit time, can be used in cases of both male and female (acquired) infertility and is attracting increasing interest from the owners of competing mares because of its flexibility, availability as an outpatient treatment and lack of any requirement for hormonal manipulation of the oestrous cycle.

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