Arch. Tierz., Dummerstorf **45** (2002) 1, 99-108

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Transvaginal ultrasound-guided oocyte aspiration for production of embryos *in vitro*

Summary

There is still much to be studied and learned in the use of assisted reproductive technologies to maximize reproductive potential in genetically valuable animals (BEAL et al., 1992). Now that repeatable oocyte retrieval methods are being fine-tuned, it is likely these procedures will become routinely used to obtain oocytes for further gamete and embryo research and also by seedstock producers for *in vitro* embryo production from farm animals in the commercial sector. The use of transvaginal ultrasound-guided oocyte aspiration and IVF procedure does offer an alternative to cattle producers who have genetically valuable cows that for some reason are unable to produce viable embryos through standard embryo collection procedures. This technology can be used on oocytes harvested from older ovulating or nonovulating cows, females with physical injuries (e.g., fractured leg) and problem cows having an abnormal cervix. Good success has been reported using IVF procedures on oocytes obtained from supplemental follicles of cows with cystic ovarian disease. With IVF the potential exists for more embryos to be produced in a shorter period of time, since the procedure can be repeated on the same cow 3 to 4 times or more a month. At this station, we are harvesting oocytes from early postpartum (<40 days) beef and dairy cattle, before the female begins cyclic activity. The approach allows the opportunity to produce one or more extra calves from the cow before she is mated for a natural pregnancy. Currently, transvaginal ultrasound-guided oocyte aspiration is now being used to harvest valuable oocytes from minor farm animal breeds, from domestic females representing rare bloodlines, clinically infertile females and reproductively senescent cows. Research continues to find applications for this technology, including harvesting oocytes from young prepubertal heifers and early postpartum beef cows for *in vitro* embryo production. The use of ultrasound-guided oocyte aspiration should not be overlooked to obtain oocytes for *in vitro* embryo production and to aid in germplasm preservation of endangered exotic species.

Key Words: oocyte collection, transvaginal, ultrasound, cattle, goats, horses, swine

Zusammenfassung

Titel der Arbeit: **Transvaginale ultraschall-geleitete Oozytenaspiration für die Embryonenproduktion in vitro**

Für die effektive Nutzung assoziierter Reproduktionstechniken zur Maximierung des Reproduktionspotentials genetisch wertvoller Tiere sind noch viele Untersuchungen notwendig (BEAL et al., 1992). Nachdem die Methoden der wiederholten Oozytengewinnung erarbeitet sind, ist es wahrscheinlich, dass sie routinemäßig für die weitere Gameten- und Embryonenforschung eingesetzt werden und so das Kernstück der Embryonenproduktion bei landwirtschaftlichen Nutztieren im kommerziellen Bereich bilden. Die Anwendung der ultraschallgeleiteten Oozytenaspiration (OPU) und die in vitro Fertilisation (IVF) bieten eine Alternative für Züchter mit genetisch wertvollen Rindern, von denen durch die konventionelle Embryonengewinnung keine Embryonen produziert werden können. OPU kann angewendet werden bei älteren oder nicht ovulierenden Kühen, bei weiblichen Tieren mit Verletzungen (z.B. ein gebrochenes Bein) und bei Problemtieren mit abnormer Cervix. Mit Hilfe der IVF können mehr Embryonen in einem kürzeren Zeitraum produziert werden, wenn die Methode 3 bis 4 mal oder mehr pro Monat und Kuh wiederholt werden kann. Die Methode der ultraschallgeleiteten Oozytengewinnung kann auch bei präpuberalen Tieren, bei tragenden Tieren und bei Exoten für die Embryoproduktion angewendet werden.

Schlüsselwörter: Oozytengewinnung, transvaginal, Ultraschall, Rind, Ziege, Pferd, Schwein

Introduction

Assisted reproductive technologies will allow producers to make continued genetic advancements in farm livestock. One success story is artificial insemination (AI), where the use of this technology in the 1950s through the 1990s in dairy herds in the United States increased the average milk production per cow over 300% from the mid-1940s average milk production per cow. During this same period the number of cows in the United States were reduced by more than one half. Improve genetic selection for milk production using frozen semen for AI and improved herd management dramatically changed the dairy industry in North America. This success story occurred because researchers developed the technology and progress producers used this technology to stay competitive in the market place.

Another assisted reproductive technology that received a great deal of interest by the cattle producers in the late 1970s and 1980 was embryo transfer (ET). Although the first embryo transfer producing a live calf was reported in 1951 at the University of Wisconsin, it was not until 1976 that the nonsurgical collection and transfer procedures were developed for cattle to lead the way commercial for in-field use of this technology. ET was the first realistic procedure that became available to help enhance the reproductive potential of the female. With the standard ET procedure, females are superovulated to produce multiple ova for *in vivo* fertilization.

Over the years, ET technology has been used to increase the number of possible offspring from selected females, however, there are still drawbacks associated with this approach. For example, some females simply do not respond or stop responding to the stimulatory agents, or develop physiological conditions that make it difficult retrieving the embryos. One of the major concerns with the overall ET procedure, however, is that cows are most often kept "open" so that they may be hormone-stimulated for subsequent embryo collections. Cows that are superovulated and collected usually take 60 days or longer to become pregnant either with AI or natural mating (BAK et al., 1989). There are often complaints that some of these hormone-treated donors also develop cystic follicles during the process.

Although high beef cattle prices, industry promotion and producers' keen interest enhanced the use of ET technology in the late 1970s and early 1980s, embryo transplantation in North America today is more often used by the dairyman than by the beef cattle producer.

Background

It has been said that males of an animal species tend to have an advantage over females in the propagation of their genes to future generations. For example, in wild populations it is common for males of many of the hoofstock species to mate with females in seasonal breeding groups, as long as the male is strong enough to ward off subordinate males. Also, males can constantly renew their supply of gametes to pass on their genes to subsequent offspring, as sperm cells can be produced by the testes throughout the male's life, in almost unlimited quantities.

In contrast, the farm animal female is born with all the oocytes stored in her ovaries she will ever produce in her lifetime. For example, a beef heifer may have 150,000 or more primordial follicles containing oocytes in their ovaries at the time of birth. Since that beef female might only produce 10 calves in her lifetime, what happens to the remaining 149,990 oocytes of the pool? First, nonpregnant cyclic cows ovulate at 20- to 22-day intervals removing one ovum from this oocyte pool each cycle. Follicles in cattle develop in waves of 4 to 12 follicles at a time, and a cow usually has two to three waves of these growing follicles during a typical estrous cycle. Over this estrous cycle 12 to 36 follicles develop to varying degrees and most then regress during this interval, with usually only one follicle ovulating that cycle. Follicular waves have also been reported to occur during the early part of pregnancy, again removing oocytes from the pool. This difference in male and female gamete production presents a unique paradox for discussion, however, the answer the producers are most often seeking is how can they maximize utilization of the gametes from their favorite females?

In Vitro Fertilization

The advent of *in vitro* fertilization (IVF) techniques have now begun to change animal reproductive management. This *in vitro* technique was first successfully used to produce offspring in rabbits (CHANG, 1959), and the first farm animal produced by IVF was a healthy bull calf reported by BRACKETT et al. (1982). For over 16 years researchers have been developing IVF procedures for cattle. Oocyte collection and IVF technology has only been commercially available to dairy and beef cattle owners in the USA since the early 1990s.

IVF is a multi-step process that requires a well-equipped laboratory and a skilled technician. The IVF procedure involves harvesting the oocytes from the cow's ovaries and fertilizing them *in vitro*. The resulting embryos are held in an incubator for 7 or 8 days and then transferred nonsurgically to recipient females who are at the same stage of their estrous cycle. The pregnancy success rate for good quality IVF-derived bovine embryos is expected to range from 50 to 65%. Success rate is lower if the embryos have been frozen and then thawed prior to recipient transfer. Although years of research has been conducted in this area, today IVF methodology is still being tested and fine-tuned for the farm animals.

Initial *in vitro* embryo production (IVP) utilized oocytes collected from slaughterhouse ovaries. This worked well during early experimentation, when large numbers of immature oocytes were necessary to develop *in vitro* laboratory procedures and to train graduate students. In the 1980s it was proposed that the application of IVF in animals would likely be its use in rare exotic animals and more commonly in genetically valuable seed stock.

Transvaginal Ultrasound-Guided Oocyte Collection

Early attempts at retrieving oocytes from potential donor cattle included surgical and less invasive laparoscopic procedures, but there was a limit to how many procedures could be performed safely without causing injury to the donor animal. At this stage, there was essentially no safe, repeatable method of harvesting the oocytes from live farm animals. Then in the mid 1980s, a method was developed in humans for retrieving oocytes using ultrasonography to visualize the ovary while a needle was guided transvaginally into the follicle (DELLENBACH et al., 1985; and others). Thus, fresh oocytes could be aspirated from individual follicles and subjected to *in vitro* maturation, *in vitro* fertilization and then *in vitro* culture procedures. These efforts paved the way for the new reproductive technology developed to harvest oocytes from live cattle (CALLESEN et al., 1987; PIETERSE et al., 1988, 1991; KRUIP et al., 1991). This approach was then evaluated for its efficiency in harvesting oocytes from mature beef and dairy cows and young cyclic heifers.

Today, oocytes are routinely harvested from cattle donors by transvaginal ultrasound-guided collection procedures. To retrieve the oocytes for IVF, a trained professional, inserts an ultrasound-guided stainless steel needle through the wall of the vagina near the cervix to extract the oocytes from the follicles detected by ultrasonography. The aspiration procedure is usually conducted on the small, medium and large-size follicles on the ovaries of the donor female. Currently, transvaginal ultrasound-guided oocyte aspiration (TUGA), also known as ovum pick-up (OPU) in human medicine (see WIKLAND et al., 1993), is now under further evaluation in cows, mares and goats. More recently, TUGA is being developed for pigs, domestic sheep and exotic hoofstock species.

Cattle. In cattle, the donor female is restrained in a suitable holding chute and administered an epidural block. A convex ultrasound 5-MHz sector transducer is fitted onto the distal end of a specially fitted 500-mm plastic handle to visualize the ovaries on the ultrasound monitor. The plastic handle (with a latex protective covering) is inserted into the vaginal canal, and then the ovary is grasped *per rectum* and placed against the transducer. Follicles are identified as black (hypoechoic) circular shapes on the monitor screen. An 18-gauge, 550 or 600-mm long needle is inserted through the needle guide in the plastic handle. This needle is connected to a suction pump by means of polyethylene tubing, passing through an embryo filter or into a 50-ml conical-shaped test tube for collection of the follicular fluid containing the oocytes. The flushing medium used for this procedure is phosphate-buffered saline (PBS) with 10% bovine serum, antibiotics and heparin. Using this aspiration method, 60 to 70% of the medium to large-size follicles punctured result in oocytes recovered, with an average of 3 to10 oocytes per nonstimulated donor female.

Aspirations are usually performed once-a-week, but have been performed twice-a-week for up to 3 months in cows, with no adverse effects reported for these donor females (BROADBENT et al., 1997). *In vitro* production of embryos generally results in 1 to 3 embryos for transfer per oocyte collection procedure for nonstimulated donors. Oocytes can be harvested from donor cows at anytime of the estrous cycle, including the early growth phase of the first follicular wave (PAUL et al.,1995). The transvaginal ultrasound-guided aspiration procedure can be used to remove dominant follicles (luteal phase) prior to gonadotropin stimulation to enhance the super-stimulation response in donor cows (LINDSAY et al., 1994).

With IVF, the potential exists for more embryos to be produced in a shorter period of time, since the procedure can be repeated on the same cow 4 to 6 times or more a month. At this station, we are harvesting oocytes from early postpartum (<40 days) beef and dairy cattle, before the female begins cyclic activity (PEREZ et al., 2000, 2001). The approach allows the opportunity to produce one or more extra calves from the cow before she is mated to produce a natural pregnancy.

A concerted effort has been made to harvest oocytes by TUGA on prepubertal dairy and beef heifers (LOONEY et al., 1995; ADAMS et al., 1996; BROGLIATTI et al., 1996; and others). With modification of vaginal probes, oocytes have been recovered from these young females, however, the number of IVF offspring born to date has been less than encouraging. Currently, transvaginal ultrasound-guided oocyte aspiration is now being used to harvest valuable oocytes from domestic females representing rare maternal bloodlines, reproductively senescent females and clinically infertile cows (LOONEY et al., 1994).

Horses. The horse has presented a unique problem for researchers working in the assisted reproductive technology area. Even though embryo collection and transfer are relatively simple in the mare, attempts to superovulate horses have produced poor results. Due, at least in part, to the unique anatomical structure of the horse ovary and other factors, only one oocyte usually ovulates at the appropriate time during each estrous cycle. Also, for some yet unknown reason, typical *in vitro* procedures were not working consistently in the horse.

Although only one follicle normally ovulates during estrus, mares have been reported to have one or two waves of multiple follicles during that estrous cycle. Once again, this developing follicle population could make it possible to use transvaginal ultrasound-guided aspiration to harvest oocytes from live mares in an effort to develop an efficient IVF procedure. Thus, researchers then began to evaluate transvaginal ultrasoung-guided oocyte aspiration procedures on horse and pony mares to collect oocytes for IVF research (BRÜCK et al., 1992; COOK et al., 1992; BRACHER et al., 1993; MEINTJES et al., 1995a).

The aspiration setup is similar to that used in cattle, but with some modifications. Briefly, mares require sedation instead of an epidural block, and most often a 12-gauge needle is used to puncture the follicles. Extra rinsing of the follicle is necessary in the horse, since the oocyte is usually well embedded in the follicle wall. In this case, the needle recommended is a double-lumen needle, so that the follicular fluid can be aspirated, and the medium used to again rinse the follicle (two to four times per follicle). The follicular fluid is collected into a 500-ml glass bottle (Figure), and then later passed through the standard bovine embryo filter. Using this modified method, oocytes have been successfully recovered from mixed-breed cyclic mares and ponies (MEINTJES et al., 1995a) and free ranging zebras in South Africa (MEINTJES et al., 1997) by members from this laboratory. Oocyte recovery rate usually ranges between 40 and 75 % of follicles punctured per mare. If cleavage occurs after the IVF procedure, developing 2- to 4-cell stage embryos are transferred into the oviducts of recipient mares.

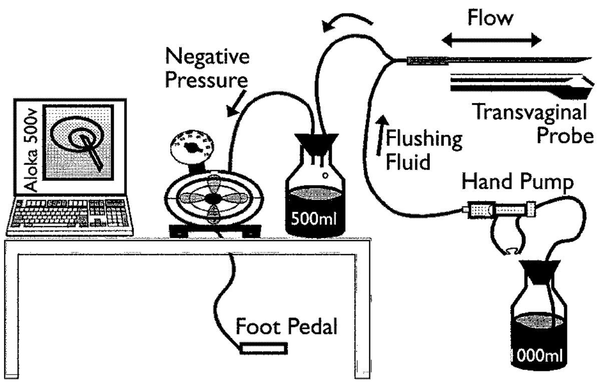

Figure: Using an Aloka 500-V ultrasound unit with a plastic hand pump and negative pressure to collect oocytes form developing follicles in both small and large mares (from MEINTJES et al., 1995a)

With TUGA now available, researchers intensified their effort to develop other assisted reproductive technologies to produce offspring from genetically valuable mares. Several groups began to develop a procedure termed intracytoplasmic sperm injection (ICSI) for horses, where individual sperm cells are injected into the oocytes to trigger the fertilization process and produced a pregnancy (SQUIRES et al.,1996; MEINTJES et al., 1996). The first foals produced by ICSI with oocytes aspirated from live mares were reported the same year in the United States and in Australia (COCHRAN et al., 1998; McKINNON et al., 1998). After ICSI, embryos are most often surgically transferred at the 2- to 4-cell stage into the oviducts of suitable recipients (COCHRAN et al., 2000), since the culture of IVF-derived equine embryos has still not been perfected for IVF-derived embryos. Although only a small number of test tube foals have been produced to date, the ICSI procedure appears to be the method of choice to produce IVF horse embryos in the laboratory.

Pregnant Donor Females. One problem with the larger farm animal donor females (e.g., cow, mare) is that their gestation intervals are lengthy in comparison with those of smaller domestic mammals, and that these donor females are out of *in vivo* embryo production during their gestation. However, both cows and mares have been reported to continue follicle wave development during early to mid-gestation. With this in mind, it was deemed logical to take advantage of having these developing follicles available for oocyte harvest during gestation and thus, an attempt was made to collect oocytes from bovine and equine females using TUGA during early pregnancy (MEINTJES et al., 1995a, 1995b).

The main concern was whether the oocyte aspiration procedure would affect the ongoing pregnancies. Successful transvaginal oocyte aspiration has been reported from first trimester pregnant cows (MEINTJES et al., 1995b) and first trimester pregnant mares (MEINTJES et al., 1995a, 1996). This oocyte aspiration approach proved not to be a problem, and pregnant donors were found to consistently produce more oocytes per collection than similar nonpregnant, cyclic females. The first offspring produced from oocytes collected by transvaginal ultrasound-guided aspiration from pregnant donor animals resulted from cows (MEINTJES et al., 1995b) and horses (COCHRAN et al., 1998) at this station.

Goats. Goats are another farm animal species in which *in vitro* embryo production has proven successful. Transvaginal aspirations have also been performed on cyclic and noncyclic adult goats with good success by members of this laboratory (GRAFF et al., 1999). Although the oocyte recovery rates usually range from 60 to 80% for the follicles punctured per donor female, there are some problems with aspiration of ovarian follicles from does using the ultrasound-guided transvaginal method. First, the ovaries cannot be grasped *per rectum* for optimum visualization with ultrasonography. Secondly, since the ovaries cannot be easily grasped, it is more difficult to puncture follicles and aspirate the oocytes.

Although the methodology for puncturing the follicle is similar to the cow and the horse, the goat is sedated, placed under anesthesia and then placed in dorsal recumbency for the procedure. Manual pressure is placed on the female’s abdomen in an effort to stabilize the ovaries for aspiration. The ultrasound probe, which in this case the probe is smaller like that used for humans, with the convex transducer at the distal end of the handle is inserted into the vagina. The aspiration itself proceeds with no need for extra rinsing of the follicles to recover the oocytes as in the mare.

Oocyte recovery is usually a little slower than desired because not all follicles can be visualized, and not all follicles visualized can be adequately punctured due to the difficulty of securing the ovary. Goat offspring have been produced using the transvaginal aspiration procedure together with IVP methods developed at this laboratory (HAN et al., 1996). Although this noninvasive procedure takes expertise and patients, it is an important assisted reproductive technology needed to reduce the risk of ovarian adhesions or death loss from using the standard surgical method to harvest oocytes from valuable donor goats. Efforts are now underway to modify the caprine TUGA procedure for use in mature ewes at this station.

Swine. The transvaginal ultrasound-guided oocyte aspiration technique has recently been used successfully to harvest oocytes from mature adult sows (BELLOW and DAVIS, 1999, unpublished data; BELLOW et al., 2001). In the latter study, the 15 sows that yielded oocytes had a mean of 4.53 oocytes per female. This procedure was found to be effective if the sow was properly restrained and as long as rectal manipulation of the ovaries was possible in the donor female. More research is needed improve the learning curve and validate this transvaginal approach to oocyte collection in the sow.

Exotic Animals. Ultrasound-guided follicle aspiration has also been used successfully in other animals, with modifications made primarily to account for anatomical differences of the donor animals. Transvaginal ultrasound-guided oocyte aspiration has also been used successfully to harvest oocytes in the Southeast Asian Swamp buffalo (PAVASUTHIPAISIT et al., 1995), in the Italian Mediterranean buffalo (BONI et al., 1996) and in llamas residing in North America (BROGLIATTI et al., 1996; BROGLIATTI et al., 2000). This procedure is also the same proposed or in use for other exotic hoofstock (LOSKUTOFF et al., 1995). More recently, TUGA has been used successfully to obtain oocytes in the rare Bongo antelope (POPE et al., 1999) and also the African eland (POPE et al., 1998; unpublished data; WIRTU et al., 2000; unpublished data).

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Received: 2001-07-24

Accepted: 2001-10-30

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