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BIOTECHNOLOGY IN SMALL RUMINANT REPRODUCTION

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SUMMARY

During the last two decades, new biotechnologies have been developed that are on the verge of revolutionizing reproductive processes in animal. The modern techniques in assisted reproductive technology (ART) are used for improvement livestock and enhancement of the animal reproductive efficiency. The artificial insemination in sheep has low fertilization rate; but by using intra uterine insemination (IUI) the fertilization rate increases. The ART technologies have facilitated the development of methods to transfer desired single gene or alternatively, the entire genome from desirable individuals or embryos. In addition rapid advances in techniques to manipulate embryos in the laboratory have permitted screening of embryos for genetic defects or highly desirable traits by using molecular markers.

ART in sheep and goats includes artificial insemination (AI), estrus synchronization, estrus induction, synchronization of parturition, superovulation, in vivo and in vitro embryo production, embryo collection, embryo transfer, embryo cryopreservation, embryo splitting, cloning and production of transgenic animals. The small ruminant are excellent models for the listed techniques and are used in basic and applied research.

Introduction

Sheep are considered to be one of the first domesticated animals and associated with man from early date. The world population is increasing at dramatic rates; therefore, the demand for meat and milk products will continue to grow (Cast, 1999). Consumers also are demanding a more uniform, higher quality, nutritious product at reasonable price, requiring producers to raise animals that meet these specific demands. Altering herd genetics is a slow and expensive process because of the limitation of having a set number of offspring produced by each animal. Through the use of ART; the breeder will be able to extend the use of genetically superior animals and perhaps increase the number of offspring. Assisted reproductive technologies ART are powerful tools for

genetic improvement and enhancing reproductive efficiency in the small ruminant industry. Sheep offer the potential of making an important contribution to providing food and fiber for growing world population. The fact that most ewes in the temperate countries are seasonal breeders and in general produce smaller lamb crops than the farmers wish, has made sheep an obvious target for the reproductive physiologist's attention (Shelton, 1995). Natural selection has provided sheep with signaling systems, which couple certain forms of environmental variation with the appropriate neuroendocrine responses to insure that reproductive activity occurs at the most favorable time of year, depending on the length of gestation. Such neuroendocrine responses continue to operate in the sheep despite the selection practices by man over several thousand years of domestication (Gordon, 1997). Through the use of ART each animal has the ability to produce an increased number of offspring, thereby allowing the producer to more easily alter herd genetics to meet the consumers demands. Using ART, such as induction of superovulation, IVF or embryo transfer, farmers will be able to increase the number of offspring produced by genetically superior parents.

The development of commercially acceptable controlled reproduction techniques in small ruminants started in the last century. Controlled reproduction covers several areas of reproduction including:-

a- Breeding animals toward the end of normal anestrus.
b- Breeding animals to permit an extremely compact lambing period in small flocks.
c- Breeding females to top quality males by artificial insemination.
d- Rapid build up of stocks of certain breeds by embryo transfer.
e- Induction superovulation.
f- Estrus and lambing or kidding synchronization.
g- In vivo and in vitro embryo production.
h- Cloning and production of transgenic animals (Gordon, 1997 and Cognie et al, 2003).

The technology for control of reproduction including estrus synchronization, fixed time artificial insemination, early pregnancy diagnosis and induction lambing and kidding are now available and offers possibilities in allowing lamb and kid production to be planned for small ruminant producers.

One of the goals of ART is genetic improvement of sheep and goats through the substitution of genetically superior animals for those of a little genetic merit. Cross breeding has helped in increasing milk, meat and fiber production to a great extent, but in cross breeding the benefit has been primarily from male germ plasm, ignoring the potential of the untouched female (Ishwar and Memon, 1996). The procedure of superovulation followed by recovery of embryos and transfer to appropriately synchronized recipients has proven to be an effective means of increasing the contribution of superior females to gene pool of the population. Nearly all technologies related to embryo transfer and manipulation in domestic ruminant, from estrus synchronization and artificial insemination to the production of Dolly, the first mammal cloned from the somatic tissue, have been developed in sheep before being transferred to its more economically important counterpart, the cow (Loi et al., 1998). The success of embryo transfer depends on management of donor and recipient animals, estrus synchronization in donors and recipients, superovulation in donors, embryo collection and evaluation, transfer of embryos and factors affecting survival of transferred embryos.

Artificial insemination

The ewe cervix restricts the passage of transcervical (**TC**) AI equipment (Fukui and Roberts, 1977; Bunch and Ellsworth, 1981), preventing common use of TC AI in sheep (Dun, 1955; Andersen et al., 1973; Halbert et al., 1990 and Wulster-Radcliffe et al., 2004). The size and shape of the external cervical canal and the tortuous nature of the cervical canal present the major physical barriers to TC AI (Dun, 1955; Andersen et al., 1973 and Halbert et al., 1990). A viable TC AI procedure for sheep must include a means of coping with the cervix without inducing trauma. Therefore, to overcome the physical barriers presented by the cervix developed a modified TC AI catheter. Previous studies indicated that TC AI is extremely difficult in ewes (reviewed by Eppleston and Maxwell, 1993). However, methods for managing the physiological and anatomical limitations that the ewe cervix imposes. Results indicate that using a modified TC AI catheter allows the intrauterine deposition of semen either directly into the uterine lumen, via laparotomy and a small incision through the uterine horn, or transcervically without decreasing fertilization and

pregnancy rates (Wulster-Radcliffe et al., 2004). Cervical manipulation associated with movement of an AI catheter through the cervix has been associated with reductions in pregnancy and lambing rates (Sayre and Lewis, 1997). These negative results have been attributed to the secretion of an unknown spermicidal compound in response to cervical manipulation (Hawk, 1983).

AI technique is mostly coupled with estrus synchronization and or superovulation. Regardless of the type of superovulation treatment, fertilization often fails, particularly in ewes showing a high ovulatory response. Fertilization failure is equally frequent in ewes bred naturally or artificially inseminated and appears to be due to faulty transport of spermatozoa through the cervix (Boland et al., 1983). This problem can be overcome by intra uterine deposition of semen through surgical procedure or by laparoscopic insemination (Ishwar and Memon, 1996). Intra uterine insemination is especially effective in overcoming the fertilization failure of donors exhibiting high ovulation rates. Intra uterine laparoscopic insemination should be carried out in the middle of the estrus period (Walker et al., 1986). In goats the cervix is more open than in sheep and transport of the spermatozoa through the cervix does not pose the problems found in sheep. However, cervical insemination results very low rates of fertilization (Moore and Eppleston, 1979). Superovulated does which are mated with proven ferile bucks produce a high proportion of fertilized ova.

Embryo Transfer

The success of embryo transfer depends on anagement of donor and recipient animals, synchronization of estrus in donors and recipients, superovulation of donors, breeding (natural or artificial insemination), embryo collection and evaluation, transfer of embryo and factors affecting survival of transferred embryos. The procedure of superovulation followed by recovery of embryos and transfer to appropriately synchronized recipients has aproved to be an effective means of increasing the contribution of superior females to the gene pool of the population. In farm animals, early embryos can be removed from the uterus or oviduct of their dam (donor) and transferred to the uterus or oviduct of other females (recipients) for development to term. The main use of embryo transfer is increased

productivity of selected females; others are identification of potential artificial insemination rams through contact mating, disease control, importation and exportation of livestock, rapid screening of AI males for genetically recessive characteristics. Embryo transfer also is a useful research tool for evaluating fetal and maternal interactions. Techniques and results in sheep and goats are basically similar to those in cattle, except that surgical or laparoscopic methods are almost always used for collection and transfer the embryos. Ewe and doe recipients are synchronized so that ovulation occurs ~12 hr before donor ovulation. Transcervical collection of embryos in the doe has been attempted with variable success. Per rectum manipulation of the tract is not possible, so flushes generally involve the whole uterus rather than individual horns as in the cow. Although this method is possible in goats, it is much less repeatable in sheep because the more convoluted cervix is difficult to cannulate.

1- Synchronization of estrus in donors and recipients

Synchronization of estrus may be achieved through the use of prostaglandin $F_{2\alpha}$ and / or progesterone, repeated progesterone injections for 16-17 days, intravaginal sponge {30-40 mg fluorogestone acetate (FGA) for 11-18 days or 50-60 mg of medroxy progesterone acetate (MAP) for 9-17 days}, or by using subcutaneous ear implants with a dose of 2-6 mg progesterone for 9-17 days (Robinson, 1965; Nuti et al., 1987; East and Rowe, 1989; Ishwar and pandey, 1990 and Stenbak et al., 2001 and 2003). It is possible to synchronize estrus through luteolysis, prostaglandin $F_{2\alpha}$ and its analogues have luteolytic action and two injections administered 11days apart in cycling ewes give satisfactory results (Trounson, 1976 and Ishwar and Memon, 1996). Progesterone may be considered as a drug of choice, because it is easily applied at low cost.

2- Superovulation

Tremendous progress has been made in female germ plasm use through multiple ovulation and embryo transfer (MOET).

Principles of inducing suprovulation in sheep and goats are the same as in cattle. The number of follicles and the number of oocytes and embryos obtained from animals must be optimized to maintain high efficiency of ART. Numerous studies have focused on hormonal treatments to optimize follicular development in sheep and goats

during the non-breeding season for successful embryo production and subsequent pregnancy rates (Gordon, 1997) increases number of mature follicles. Previous studies have shown that administration of exogenous FSH (Gordon, 1997; Reynolds et al., 1998 and Stenbak et al., 2001). Follicle stimulating hormone (FSH) has been shown to induce development of multiple follicles on each ovary when injected into ewes for two or more days at regular intervals during the normal breeding and non-breeding seasons (Gordon, 1997; Reynolds et al., 1998 and Stenbak et al., 2001). Additionally, several studies have examined the effects of exogenous melatonin and progesterone administration on ovarian activity and follicular development in vivo (Wheaton et al., 1990; Waller et al., 1988 and Carlson, 2000). A major role of melatonin is to coordinate seasonal changes in reproductive activity (Hazlerigg, 2001). Melatonin increases ovulation rate and litter size during the non-breeding season (Rajkumar et al., 1989 and Haresign, 1992). The use of progestogens has also promoted ovarian activity by increasing the number of follicles and rate of ovulation (Waller et al., 1988; Rajkumar et al., 1989 ; Wheaton et al., 1990; Safranski et al., 1992; Leyva et al., 1998 and Knights et al., 2001). Treatment with progestogens has been used in conjunction with high levels of FSH to promote the development of large number of follicles during the non breeding season (Reynolds et al., 1998 and Stenbak et al., 2001) and during the normal breeding season (Gordon, 1997 and Stenbak et al., 2001). Optimal levels of exogenous gonadotropins should be used to promote proper oocyte development and depending on regime of gonadotropin treatment, positive or negative effects on oocyte maturation and fertilization have been observed (Evans and Armstrong, 1984; Pugh et al., 1991; Assey et al., 1994; Greve et al., 1995 and Stenbak et al., 2001).

The follicle-stimulating gonadotropin is administered either near the end of the luteal phase of the estrous cycle (days 11-13) or around one or two days before the end of the synchronizing treatments (Jablonka Shariff et al., 1994; Ishwar and Memon, 1996; Stenbak et al., 2001 and Grazul-Bilska et al., 2003). A high ovulatory response is observed in sheep during superovulatory treatment, which hampers the process of fertilization. Embryo yield after superovulation is dependent on many factors that can be grouped as follows (Loi et al., 1998).

i- Inherent factors that are difficult to modify (breed, season and management). Reproductive biologists made a major effort in the past to fit suitable superovulation protocols into many domestic breeds under a broad environmental range (Gordon, 1997).

ii- Factors susceptible on improvement (gonadotropin and knowledge of ovarian physiology).The most widely used gonadotropin preparations for superovulation are equine gonadotropin (eCG) or pregnant mare serum gonadotropin (PMSG) and pituitary follicle stimulating hormone (FSH-P).

Equine gonadotropin is administrated as a single subcutaneous or intramuscular injection given one day prior to the last synchronization treatment. However FSH-P given at 12 hours intervals in equal or decreasing doses for about three or four days or days 12-16 of the estrous cycle increase the superovulation responses. Prostaglandin $F_{2\alpha}$ is administered intramuscular at the time of the fifth or seventh FSH injection (Senn and Richardson, 1992).

Exogenous gonadotropin interplay with somatic and germinal compartments of the follicle leads to greater than norm ovulation rates. Negative effects can occur during early embryonic development as a result of unbalanced hormonal profiles. Many strategies have been suggested for optimizing the yield of transferable embryos from superovulated donors including the administration of anti-PMSG antibodies, FSH-P instead of PMSG, association of the two gonadotropins, single versus multiple injection or inclusion of GnRH or growth hormone in the treatments (Bindon and Piper, 1986; Walker et al., 1986; Ryan et al., 1991 and Meinecke-Tillman, 1993). However, the well-known side effects of the superovulatory treatment such as unovulated follicles, low fertilization and recovery rates were not fully solved (Loi et al., 1998).

A novel approach to improve superovulation includes the use of a GnRH agonist or antagonist combined with a progestagen treatment to suppress endogenous gonadotropins and follicular development beyond 1-2 mm followed by exogenous gonadotropins adminsterad over 4 days. Thus a pre-treatment over a 2 week period with an agonist (Busereline, 40 μg/day,Receptal) or 10 days with an antagonist (Antarelix,0.5mg / day,

Teverelix) suppresses large follicles, doubles the number of small ones and improves the response to FSH by 50% (Cognie et al., 1999).

Once the superovulation protocol is established, the next step is to verify responsiveness of the same donor to repeated treatments. Multiple superovulations can be induced in sheep at one year interval without a significant reduction in ovarian response (Loi et al., 1998). Whether immunological responses induced by gonadotropins used for superovulation can reduce the ovarian response still remain an open question. However, side effects of repeated treatment with gonadotropins are not the major factors limiting multiple superovulation in sheep. The major problems related to repeated superovulation and frequent laparoscopic procedures of oocyte or embryo collections are adhesions caused by protrusions of the endometrium at the puncture site in laparoscopic recovery. The occurrence of adhesions may reduce the number of flushing obtainable from the donor (Nellenshulte and Nieman, 1992).

3- Artificial insemination

Regardless of the type of superovulation treatment, fertilization often fails particularly in ewes showing a high ovulatory response. Fertilization failure is the same in ewes bred naturally or artificially inseminated and appears to be due to faulty transport of spermatozoa through the cervix (Boland et al., 1983). This problem can be overcome by direct deposition of the semen into the uterus (Trounson and Moore, 1974) through surgical procedure in superovulated ewes or by laparoscopic (Ishwar and Memon, 1996). Intra-uterine insemination is effective in overcoming the fertilization failure of donors showing high ovulation rate. The intra-uterine insemination should be done in the middle of the estrus phase (Walker et al., 1986). However, if fertile rams or bucks are available, natural mating should be used in the superovulation program and ewes or does should be mated every six hours during the standing estrus (Ishwar and Memon, 1996).

4- Embryo collection

Sheep embryos are usually collected surgically under general anesthesia. All donor ewes are taken off feed for 24 hours and water for 12 hours prior the surgery. During the surgical embryos collection the uterus and oviducts are exposed to the midventral laparotomy and the reproductive tract flushed using sterile medium. Flushings are collected in sterile Petri dishes for immediate examination under a dissecting microscope.

Laparoscopic techniques were introduced for embryo recovery to reduce the extent of surgical intrusion (McKelvery et al., 1986). Despite lowering the level of surgical intrusion, laparoscopic techniques, like surgical collection may lead to adhesions of the reproductive tract and ovaries. This limits the number of times a ewe can be used as a donor. Attempts are made for cervical collection after ripening the cervix with hormones like prostaglandin E_2 and estradiol (Barry et al., 1990). Although some successes have been achieved using cervical embryos collection, more studies are necessary to make it a reliable option in sheep.

The embryos are usually recovered at the morula-blastocyst stage, six to seven days after fertilization then evaluated under a dissecting microscope before being frozen or immediately transferred either by laparpotomy or laparoscopy into synchronous recipients (Loi et al., 1998).

5- Embryo freezing

Successful methods for freezing sheep embryos have been available since the 1970's, when the first lamb was born following a transfer of frozen-thawed embryo (Willadsen et al., 1976). The embryos were incubated in an appropriate concentration of permeating cryoprotectors, cooled 3-7°C below freezing point, and then ice nucleation was induced in the medium by touching the vial with a pair of precooled forceps. The cryoprotectant was removed step-wise after thawing and embryo was ready for transfer. Research in the area of embryo freezing has led to the increase of embryo survival through the adoption of low toxicity cryoprotectants such as ethylene glycol and to incorporation of non-permeating osmotic buffer, sucrose, into the freezing medium, which allows direct transfer after thawing (Loi et al., 1998). Further research allowed the

development of cryopreservation protocols where embryos were cooled very rapidly in a high concentration of cryoprotectants which formed a glass structure without the formation of ice crystals. This procedure called vitrification, has been adapted for many species including sheep and the results in terms of survival rate and lambs born are continuously progressing (Szell and Windsor, 1994 and Dobrinsky, 2001 and 2002). Using open pulled-straw vitrification technique increased the embryonic survival in sheep (Dattena et al., 2001) and goats (El-Gayer and Holtz, 2001).

6- Embryo transfer

Embryos are transferred to the uterus or oviduct of recipients by laparoscopic technique or using laparotomy . Comparison between the simplified laparoscopic and surgical embryo transfer showed that the laparoscopic method can achieve high pregnancy rates (Stefani et al., 1990). The laparoscopic transfer is a safe minimally invasive surgical procedure and it should be encouraged for the transfer of embryos in small ruminants.

Limited studies have reported success for transcervical embryo transfer in small ruminants. However, Flores-Foxworth et al. (1992) did not observe any significant difference in terms of kidding between transcervical and laparoscopic embryo transfer in goats.

The developmental stages at which embryos or oocytes were transferred have ranged from follicular oocytes (transferred to mated recipients) and zygotes to elongated blastocysts on day 12 of the cycle (Gordon, 1997). Number of transferred embryos ranged from one to more than five per ewe. Transferring two embryos gives acceptable results and yields with increased number of twin pregnancies, more than 80% of the recipients of two embryos carried pregnancies to term and two-thirds of these giving birth to twins (Ishwar and Memon, 1996). The survival of embryos transferred as twins was significantly higher when both embryos were transferred to the same oviduct or uterine horn (unilateral transfer) than when one was transferred to each oviducts or uterine horns (bilateral transfer). Improved embryo survival following unilateral twins transfer suggests that there is some synergism between the two embryos, which

influencing each other's survival upon transfer. Such co-operation includes enhanced luteotrophic or antiluteolytic action resulting in improved luteal maintenance in recipients, or enhanced signals to the endometrium involved in the process of placental attachment (Ishwar and Memon, 1996).

The occurrence of estrus in donor and recipient animals must be closely synchronized if the survival rate of transferred embryos is to be optimized. Optimum results in terms of embryo survival were found among recipients in heat 12 hours before to 12 hours after donors (Shelton and Moore, 1966).

7- In vitro production of embryos

In vitro production of embryos from domestic animals is used to augment conventional genetic improvement programs in agriculture and for research. In vitro embryo production (IVP) in small ruminant provides an excellent source of low-cost embryos for basic research on developmental biology and physiology and for commercial application of the emerging biotechnologies such as nuclear transfer and transgenesis (Baldassare, et al., 2002). Embryos can be produced in the laboratory by four methods. First, using in vitro oocyte maturation (IVM), in vitro fertilization (IVF) and in vitro culture of the embryos (IVC). Second, using nuclear transfer and cloning. Third, using gene transfer and produce transgenic animals. Fourth, using imprinting technique. Using the in vitro production of embryo techniques may have major implications for the structure and organization of animal herds in future.

a- In vitro fertilization

In vitro fertilization is the creation of embryos by mixing the spermatozoa and ova in the laboratory. Embryos produced by this way can be transferred in to the uterus of the recipients, discarded frozen or experimented upon.

The scientists have used in vitro fertilization to solve infertility problems in human for many years. Animal scientists use this biotechnology in cows with blocked oviducts or that do not respond well to superovulation.

Recovery of sheep and goat oocytes for in vitro fertilization may be of interest to those involved in conventional small ruminant embryo applications for breeding improvement purpose. As in cattle, the repeated recovery of oocytes from live small ruminant may increase commercial interest in the development of effective breeding improvement programs. Oocyte recovery from live sheep by laparoscopic follicular aspiration procedures shows that such oocytes are capable of maturation and fertilization. Flores- Foxworth et al. (1995) found that laparscopic oocyte aspiration technique to be relatively simple and effective, as well as being less traumatic than normal embryo recovery procedures. During the last decade the first IVF lamb was born in Japan (Gordon, 1997). After the oocytes recovery from both large and small follicles three steps are required to produce in vitro fertilized embryos:

1- Oocytes are allowed to mature in vitro for about 24 hours in a maturation medium, which contaning hormones.
2- Oocytes are fertilized in vitro by using frozen or in vitro capacitated fresh semen for 18 to 24 hours.
3- The fertilized oocytes then placed in culture medium. After a few days the normally developing embryos, which reached the blastocyst stage are transferred in to recipients or frozen for later transfer (Wang et al., 1998; Stenbak et al., 2001 and Grazul-Bilska et al., 2003).

In vitro maturation, fertilization and culture (IVMFC) have become valuable techniques for production of farm animals embryo for use in embryo transfer, nuclear transfer studies and for production of transgenic animals (Chauhan et al., 1998). The IVMFC methodology is being routinely used for production in farm animal embryos on an industrial scale (Gordon, 1991 and Lu and Polge, 1992). The potential application of in vitro embryo production technologies partially depends on the development of reliable and efficient techniques for oocytes recovery from genetically valuable animals. The oocytes collection from living small ruminant has been limited (Galli et al., 2001). Most work reported in sheep has been accomplished with slaughterhouse-derived oocytes (Naitana et al., 1992; Gordon, 1997; Cognie, 1999 and Guler et al., 2000).

However, Snyder and Dukelow (1974) pioneered laparoscopic oocyte collection in sheep and their technique was further improved in recent years (Tervit et al., 1992; Baldassarre et al., 1994; Earl and Kotaras,

1997 and Stangle et al., 1999). Laparoscopic oocyte collection is an effective and minimally invasive technique, which offers the possibility of repeated ovum pick-up and allows for repeated production of embryos from a single donor ewe (Stangel et al., 1999). This technique does not cause permanent damage to the donor ewe's reproductive health. In addition, the donor ewe can be in almost any physiological status and still be suitable for oocyte recovery (Gordon, 1997). For small ruminant, laparoscopic oocyte collection is the technique of choice for its simplicity, minimal invasiveness, repeatability and efficiency. Oocytes collected can be subsequently utilized for the in vitro production (IVP) of embryos (Earl and Kotaras, 1997). In addition, the rates of development are similar or even greater when the oocytes are recovered from live donors compared with oocytes collected from ovaries of slaughtered animals (Galli et al., 2001).

In vitro production of embryos is used in farm animals to increase the number of progenies from selected mature or juvenile females and salvage oocytes from valuable dead or dying animals. One very important issue to be solved in in vitro production embryos remains the survival rate of frozen sheep embryos. Although cryopreservation of bovine embryos has made great progress in recent years, little achievement was obtained on ovine embryo freezing especially in vitro produced embryos (Zhu et al., 2001).

The adoption of advanced reproductive technologies for embryo production and transfer in ruminants may result in the occurrence of large offspring syndrome (LOS) also termed as fetal over size syndrome (Walker et al., 1996; Thompson, 1997; Young et al., 1998 and Farin et al., 2001). This syndrome has been associated with increased dystocia and abnormal growth and development at fetal, neonatal and later stages in life. A number of treatments have been shown to cause this perturbation including in vitro culture and transfer of embryos to recipients (Walker et al., 1996; Thompson et al., 1995; Young et al., 1998 and Farin et al., 2001). This emphasizes need to optimize culture conditions to stimulate natural uterine environment.

b- Cloning

In nature, clones or genetically identical animals produced when early embryos splitting into halves, creating identical twins. This has been stimulated in the laboratory by splitting embryos in half with micro tools. Large commercial applications of bisection procedures, 422 embryos were spilt and produced 441 pregnancies after transfer as single embryo. However, transfer of 515 intact embryos from the same donor produced 291 pregnancies (Leibo and Rall, 1987). Thus conception rate in the two recipients groups are similar, being 52.4% after splitting compared with 56.5% in intact embryos.

The splitting procedure has found commercial application, but there are three limitations. Firstly, repeated splitting is not possible (Woolliams and Wilmut, 1989). Secondly, normal development does not occur if the embryo is split into more than four portions (Rossant, 1976). Thirdly, as splitting reduces the ability of the embryos to survive freezing and thawing, then splitting and transfer must be carried out on the same time (Woolliams and Wilmut, 1989).

Recently, mammals have been cloned through nuclear transfers. During this technique, a nucleus is transferred from one cell to another enucleated cell (that has had its nucleus removed). Nuclear transfer technologies use embryonic, fetal and adult somatic cells from a range of mammalian species (Wilmut et al., 1997). Nuclear transfer has been used for embryo cloning in sheep and other species (Campbell et al., 1996, 2001; Kuhholzer and prather, 2000; Westhusin et al., 2001 and Campbell, 2002,a,b). The first cloned sheep named Dolly, has been produced through nuclear transfer using a mammary gland cell from mature ewe (4 years old) as a cell "donating" genetic materials (karyoplast) to the enucleated oocyte (cytoplast). In addition to producing Dolly from an adult mammary cell, the same researchers team produced lambs by using fetal sheep cell as karyoplast (Wilmut et al., 1997). Research efforts have been intensified toward improving the efficiency of reprogramming cells such as adult mammary gland cells and fibroblast for cloning by nuclear transfer.

Cultured embryonic cells when transferred into enucleated oocytes have resulted live calves and lambs (Wilmut et al., 1997 and Wells et al., 1997). However a high incidence of abortion around day 40 has

been seen with the cell-cloned embryos, accompanied by poorly developed placentomes (Stice et al., 1996). There are many factors known to influence the efficiency of embryo cloning by nuclear transfer; (a) the stage of development and cell cycle of donor cells, (b) the choice of recipient cell, (c) the activation methods for oocyte, (d) the cell cycle coordination between nuclear donor and recipient cytoplast and (e) the method for fusion between nuclear donor and recipient cytoplast (Wolf, et al., 1998).

Finally, bringing nuclear transfer to routine practice requires greater knowledge and understanding the basic biological processes underlying epigenetic controls of nuclear activities (Renard et al., 2002).

The advancements in nuclear transfer technology can now be applied for improvement and increase of farm animals rescue of endangered species and assisted reproduction (Tsunoda and Kato, 2002).

c- Gene transfer

Gene transfer has been used to produce both random and targeted insertion of direct DNA fragments into animal's genome. The most common procedure for gene incorporation in farm animals depends upon direct injection of few hundred copies of a gene directely into a nucleus of an early embryo (Ward and Brown, 1998).

Although there have been recent advances in nuclear transfer technology in livestock and laboratory species (Wilmut et al., 1997 ; Schnieke et al., 1997; Wakayama et al., 1998; Eyestone and Campbell, 1999; Kuhholzer and Prather, 2000; Wheeler and Walters, 2001; Escriba et al., 2002; Niemann et al., 2003; Wang and Zhou, 2003 and Mullins et al., 2004), much still needs to be learned regarding the biology and application of these methods to production of transgenic animals. This technology is very inefficient at present (Wilmut et al., 1997; Campbell, 1999; Niemann and Hues, 2000 and Paterson et al., 2003) and needs improvement before it can be widely used for livestock systems. The economic significance of transgenic animals to animal agriculture in the future can not be estimated with any confidence. However, the livestock and dairy industries generated over 68 billion dollars of on-farm receipts in 1992 (Anonymous, 1994). Even small effects on efficiency would repay research costs several times over.

The benefits and applications of gene transfer in farm animals are; (i) improvement of product quality and quantity; (ii) produce disease resistance animals and (iii) the production of valuable proteins in the mammary gland and other organs (Wolf, et al., 2000). The use of gene transfer alone or in combination with nuclear transfer is very useful for obtaining transgenic animals.

d- Imprinting

Imprinting mean reconstructed oocyte containing two haploid sets of maternal genome and/or paternal genome, derived from the pronuclei stages (Kono et al., 2004).

References

Andersen, V. K.; J. Aamdal and J. A. Fougner (1973). Intrauterine and deep cervical insemination with frozen semen in sheep. Zuchthygiene (Berl.) 8,113-121.

Anonymous (1994). Agricultural Outlook 204. Economic Research Service, U.S. Dept. Agriculture. January-February.

Assey, R.J.; P. Hyttel; T. Greve and B. Purwantara (1994). Oocyte morphology in dominant and subordinate follicles. Mol. Reprod. Dev., 37, 335-344.

Baldassare, H.; B. wang; N. Kafidi; C. Keefer; A. Lazaris and C.N. Karatzas (2002). Advances in the production and propagation of transgenic goat using laparoscopic ovum pic-up and in vitro embryo production technologies. Theriogenology, 57, 275-284.

Baldassare, H.; D.G. de Matos; C.C. Furnus; T.E. Castro and E.I.C. Fischer (1994). Technique for efficient recovery of sheep oocytes by laparoscopic folliculocentesis. Animal. Reprod. Sci., 35:145-150.

Barry, D.M.; C.H. Van Niekerk; J. Rust and T. Van der Walt (1990). Cervical embryo collection in sheep after ripening of cervix with prostaglandin E_2 and estradiol. Theriogenology, 33,190.

Bindon, B.M. and L.R. piper (1986). Induction of superovulation in sheep and cattle by injections of PMSG and ovine anti-PMSG immune serum. Theriogenology, 8, 171.

Blond, M.P.; T.F. Crosby and I. Gordon (1983). Facctors influencing the superovulation response in sheep. Theriogenology, 19, 114.

Bunch, T. D. and H. S. Ellsworth (1981). Gross anatomy of the ovine cervix. Int. Goat Res., 1,282-285.

Campbell, K.H. (2002,a). A background to nuclear transfer and its application in agriculture and human therapeutic medicine. J. Anat., 200, 267-275.

Campbell, K.H. (2002,b).Transgenic sheep from culture cells. Methods Mol. Biol., 180, 289-301.

Campbell, K.H. 1999. Nuclear transfer in farm animal species. Semin. Cell. Dev. Biol., 10, 245-252.

Campbell, K.H.; J. McWhir and W.A. Richie (1996). Sheep cloned by nuclear transfer from a cultured cell line. Nature, 380, 64-66.

Campbell, K.H.; R. Alberio; J.H. Lee and W.A. Ritchie (2001). Nuclear transfer in practice.Cloning Stem Cell, 3, 201-208.

Carlson, C.D. (2000). The effect of melatonin dosage and progesterone on reproduction in anestrous ewes. *http://www.csuchico.edu/agr/tempDocs:/ccarlson.html*.

Cast (1999). Animal Agriculture and Global Good Supply.Ames. IA: Councel and technology. Task Force Report., No. 135.

Chauhan, M.S.; S.K. Singla; P. Palta; R.S. Manik and O.S.Tomer (1998). Development of in vitro produced buffalo (Bubalus bubalis) embryos in relation to time. AJAS., 11(4) 398-403.

Cognie, Y. (1999). State of the art in sheep and goat embryo-transfer. Theriogenology. 51, 105-116.

Cognie, Y.; G. Baril; N. Poulin and P. Mermillod (2003). Current status of embryo technologies in sheep and goat. Theriogenology, 59, 171-188.

Dattena, M.; V. Isachenko; J.L. Alabart; J. Folch and C. Cappai (2001). Comparison between two embryo transfer methods of vitrified sheep blastocystes. Proc.17thMtg Assoc Eur Trans Emb (AETE) 1, 14 (abstract).

Dobrinsky, J.R. (2002). Advancements in cryopreservation of domestic animals embryos. Theriogenology, 57, 285-302.

Dobrinsky, J.R. (2001). Cryopreservation of pig embryos: adaptation of virtification technology for embryo transfer. Reprod. Suppl., 58, 181-184.

Dun, R. B. (1955). The cervix of the ewe. Its importance in artificial insemination in sheep. Aust. Vet. J., 31,101-103.

Earl, C.R.and P.J. Kotaras (1997). Oocyte retrieval and in vitro embryo production in sheep and cattle: current status and future perspectives. Aust. J. Agric. Res., 48: 125-135.

East, N.E. and J.D. Rowe (1989). Subcutaneous progestin implants versus intravaginal sponge for dairy goat oestrus synchronization during the transitional period. Theriogenology, 32, 921-928.

EL-Gayar, M. and W. Holtz (2001). Technical note: vitrification of goat embryos by the open pulled-straw method. J. Anim. Sci., 97, 2436-2438.

Eppleston, J., and W. M. C. Maxwell (1993). Recent attempts to improve the fertility of frozen ram semen inseminated ito the cervix. Wool Technol. Sheep Breed., 41, 291-302.

Escriba, M.J.; D. Vaalbuena; J. Remohi; A. Pellicer and C. Simon (2002). New techniques on embryo manipulation. J. Reprod. Immunol., 55, 149-161.

Evans, G. and D.T. Armstrong (1984). Reduction in fertilization rate in vitro of oocyte from immature rats induced to superovulation. J. Reprod. Fertil., 70, 131-135.

Eyestone, W.H. and K.H. Campbell (1999). Nuclear transfer from somatic cells: applications in farm animals species. J. Reprod. Fertil. (Suppl.) 54; 277-93.

Farin, P.W.; A.E. Croiser and C.E. Farin (2001). Infulence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology, 55, 151-170.

Flores-Foxworth, G.; B.M. McBride; D.C. Kraemer and L.C. Nuti (1992). A comparison between laparoscopic and transcervical embryo collection and transfer in goats. Theriogenology, 37,213.

Flores-Foxworth, G.; S.A. Coonrod; J.F. Moreno; S.R.Byrd; ED.C. Kraemer and M. Westhu (1995). Interspecific transfer of IVM IVF-derived Red sheep (*Ovis orientalis gmelint*) embryos to domestic sheep (*Ovis arêtes*). Theriogenology, 44, 681-690.

Fukui, Y. and E.M. Roberts (1977). Fertility of ewes treated with prostaglandin $F_{2\alpha}$ and artificially inseminated at predetermined intervals. Aust. J. Agric. Res., 28, 891-897.

Galli, C.; G. Crotti; C. Notari; P. Turini; R. Duchi and G.Lazzari (2001). Embryo production by ovum pick up from live donors. Theriogenology, 55, 1341-1357.

Gordon, I. (1997). *Controlled reproduction in sheep and goats*. CAB International. New York.

Gordon,I. (1991). Potential application of cattle in vitro fertilization in commercial practice and research. Embryo Transfer Newsletter., 9, 4-9.

Greve, T.; H. Callesen; P. Hyttel; R. Hoier and R. Assey (1995). The effects of exogenous gonadotropins on oocyte and embryo quality in cattle. Theriogenology, 43, 41-50.

Grazul-Bilska, A.T.; J.T. Choi; R.M. Weigl; J.D. Kirsch; K.C. Kraft; L.P.Reyno and D.A. Redmer (2003). Effects of epidermal growth factor on early embryonic development after in vitro fertilization of oocytes collected from ewes treated with follicle stimulating hormone. Theriogenology, 59, 1453-1461.

Guler, A.; N. Poulin; P. Mermillod; M. Terqui; Y. Cognie (2000). Effects of growth factors, EGF and IGF-1, and estradiol on in vitro maturation of sheep oocytes. Theriogenology, 54,209-218.

Halbert, G. W., H. Dobson, J. S. Walton, and B. C. Buckrell (1990). The structure of the cervical canal of the ewe. Theriogenology, 33, 977-992.

Haresign, W. (1992). Manipulation of reproduction in sheep. Reprod. Fertil. (Suppl), 45, 127-139.

Hawk, H.W. (1983). Sperm survival and transport in the female reproductive tract. J. Dairy Sci., 66, 2645-2660.

Hazlerigg D.G. (2001). What is the role of melatonin in the anterior pituitary? Endocrinology, 170, 493-501.

Ishwar, A.K. and M.A. Memon (1996). Embryo transfer in sheep and goats: a review. Small Ruminant Res. 19, 35-43.

Ishwar, A.K. and J.N. Pandey (1990). Oestrus synchronization and fertility behavior in Black Bengal goats following either progesterone or prostaglandin treatment. Theriogenology, 34, 1015-1024.

Jablonka-Shariff, A.; P.M. Fricke; A.T. Grazul-Bilska; L.P.Reynolds and D.A. Redmer (1994). Size, number, cellular proliferation

and atresia of gonadotropin induced follicles in ewes. Biol. Reprod., 51, 531-540.

Kono, T.; Y. Obata; Q. Wu; K. Niwa; Y. Ono; Y. Yanamoto; E.S. Park; J.S. Seo and H. Ogawa (2004). Birth of parthenogenetic mice that can develop to adulthood. Nature, 22(428) 860-864.

Knights, M.; T.D. Maze; P.J. Bridges; P.E. Lewis and E.K. Inskeep (2001). Short-term treatment with a controlled internal drug releasing (CIDR) device and FSH to induce fertile estrus and increase prolificacy in anestrous ewes. Theriogenology, 55, 1181-1191.

Kuhholzer, B. and R.S. Prather (2000). Advances in livestock nuclear transfer. Proc. Soc. Ex. Biol.Med., 224, 240-245.

Leyva, V.; B.C. Buckrell and J.S. Walton (1998). Follicular activity and ovulation regulated by exogenous progestagen and PMSG in anestrous ewes. Theriogenology, 50, 377-393.

Leibo, S.P. and W.F. Rall (1987). Increase production of pregnancies by bisecting of bovine embryos. Theriogenology, 27, 245 (Abst.).

Loi, P.; G. Patk; M. Dattena; S. Ledda; S. Naitana and P. Cappai (1998). Embryo transfer and related technologies in sheep reproduction. Reprod. Nutr. Dev., 38,615-628.

Lu, K.H. and C. Polge (1992). A summary of two years research in large scale in vitro bovine embryo production. Proceeding of 12th Int. Cong. Anim. Reprod., 3, 1315-1317.

McKelvey, W.A.C.; J.J. Robinson; R.P. Aitken and I.S. Robertson (1986). Repeated recover embryos from ewes by laparoscopy. Theriogenology, 25, 855-865.

Meinecke-Tillman, S. (1993). Induction of superovulation in Merinolandshaf ewes after single or multiple FSH injections. Reprod. Domest. Anim., 28, 433-442.

Moore, N.W. and J. Eppleston (1979). Embryo transfer in Angora goat. Aust. J. Agric. Res., 30, 973-981.

Mullins, L.J.; I. Wilmut and J.J. Mullins (2004). Nuclear transfer in rodents. J. Physiol., 554, 4-12.

Naitana, S.; P.Loi; S. Leda and P. Cappai (1992). Embryo transfer and new technology in sheep production. In: A. Lauria and F.

Gandolfi (editors) *Embryonic development and manipulation in animal production: Trends in research and applications.* Portland Press, London 1992; pp.183-194.

Nellenshulte, E and H. Nieman (1992). Collection and transfer of ovine embryos by laparoscopy. Anim. Reprod. Sci., 27, 293-304.

Niemann, H.; D.Rath and C. Wrenzycki (2003). Advances in biotechnology: new tools in future pig production for agriculture and biomedicine. Reprod. Domest. Anim., 38:82-89.

Niemann, H. and W.A Hues (2000). Transgenic livestock: premises and promises. Anim. Reprod. Sci., 60-61, 277-293.

Nuti, L.C.; B.S. Minhas; W.C. Baker; J.S. Capehart and P. Marrack (1987). Superovulation and recovery of zygotes from Nubian and Alpine dairy goats. Theriogenology, 28, 481-488.

Paterson, L.; P. DeSousa; W. Ritchie; T. King and I. Wilmut (2003). Application of reproductive biotechnology in animals: implications and potentials. Applications of reproductive cloning. Anim. Reprod. Sci., 79, 137-43.

Pugh, P.A.; Y. Fukui; H.R. Tervit and J.G. Thompson (1991). Development ability of in vitro matured sheep oocytes collecting during the nonbreeding season and fertilized in vitro with frozen ram semen. Theriogenology, 36, 771-778.

Rajkumar R.R.; C.M. Argo and RG.. Rodway (1989). Fertility of ewes given either melatonin or progestogen sponges. Vet Rec., 124, 215-217.

Renard, J.P.; Q. Zhou; D. LeBourhis; P. Chavatte-Palmer; I. Hur; Y. Heyman and X. Vignon (2002). Nuclear transfer technology: between successes and doubts. Theriogenology, 57(1) 203-222.

Reynolds, L.P.; J.D. Kirsch; K.C. Kraft; D.L.Knutson; W.J. McClaflin and D.A. Redmer (1998). Time-course of the uterine response to estradiol-17 β in ovariectomized ewes: Uterine growth and microvascular development. Biol. Reprod., 59, 606-612

Robinson, T.J. (1965). Use of progestagen-impregnated sponges inserted intravaginally or subcunaneously for control of estrous cycle in sheep. Nature, 206, 39-43.

Rossant, J. (1976). Post-implantation development of blastomeres isolated from 4- and 8-cell mouse eggs. J. Embry. Exper. Morph., 36, 283-290.

Ryan, J.P.; J.R. Hunton and W.M. Maxwell (1991). Increased production of sheep embryos following superovulation of Merino ewe with a combination of pregnant mare serum gonadotropin and follicle stimulating hormone. Reprod. Fertil.Dev., 3, 551-560.

Safranski, T.; W.R. Lamberson and D.H. Keisler (1992). Use of melengestrol acetate and gonadotropins to induce fertile estrus in seasonally anestrous ewes. J. Anim. Sci., 70, 2935-2941.

Sayre, B. L., and G. S. Lewis (1997). Fertility and ovum fertilization rate after laparoscopic or transcervical intrauterine artificial insemination of oxytocin-treated ewes. Theriogenology, 48, 267-275.

Schnieke, A.E.; A.J. Kind; W.A. Ritchie; K. Mycock; A.R. Scott; M. Ritchie; I. Wilmut; A. Colman; K.H.S. Campbell (1997). Human Factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science, 278, 2130-2133.

Senn, B.J. and M.E. Richardson (1992). Seasonal effects on caprine response to synchronization of estrus and superovulatory treatment. Theriogenology, 371, 579-685.

Shelton, M. (1995). Harnessing the biological potential of sheep in providing protein for growing world population. J. Anim. Sci., 73, 243.

Shelton, J.N. and N.W.Moore (1966). Survival of fertilized eggs transferred to ewes after progesterone treatment. J. Reprod. Fertil., 11, 149.

Snyder, D.A.and R. Dukelow (1974). Laparoscopic studies of ovulation, pregnancy and follicle aspiration in sheep. Theriogenology, 2;143-148.

Stangl, M.; B. Kuhholzer; U. Besenfelder and G. Brem (1999). Repeated endoscopic ovum pick-up in sheep. Theriogenology, 52, 709-716.

Stefani, J.S.; M.D.C. Pahla; L. Christmann; J.M. Rosa; M.C. Silveira and J.L. Rodrigues (1990) Laparoscpic versus surgical transfer of ovine embryos. Theriogenology, 33, 330.

Stenbak, T.K.; A.T. Grazul-Bilska; H.R. Berginski; C. Navanukraw; M.J. Toutges; J.D. Kirsch; K.C. Kraft ; J.J. Bilski; L.P. Reynolds and D.A. Redmer (2003). Evaluation of ovulation rate in ewes synchronized with Syncro-Mate-B (SMB) and treated with follicle stimulating hormone (FSH). Small Ruminant Res., 48, 1-8.

Stenbak, T.K.; D.A. Redmer; H.R. Berginiski; E.S. Erickson; C. Navanukraw; M.J. Toutges; J.J. Bilski; J.D. Kirsch; K.C. Kraft; L.P. Reynolds and A.T. Grazul-Bilska (2001). Effects of follicle stimulating hormone (FSH) on follicular development, oocyte retrieval and in vitro fertilization (IVF) in ewes during breeding season and seasonal anestrus. Theriogenology, 56, 51-64.

Stice, S.L.; N.S. Strelchenko; C.L. Keefer and L. Mattheus (1996). Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. Biol. Reprod., 54, 100-110.

Szell, A.Z. and D.P. Windsor (1994). Survival of vitrified sheep embryos in vitro and in vivo. Theriogenology, 42, 227-238.

Tervit, H.R.; D.G. Whittingham and L.E.A. Rowson (1992). Successful culture in vitro of sheep and cattle ova. J. Reprod. Fertil., 30, 493-497

Thompson, J.G. (1997). Comparison between in vitro-drived and in vitro produced pre-elongation embryos from domestic ruminants. Reprod. Fertil. Dev. 9, 341-354.

Thompson, J.G.; D.K. Gander; P.A. Pugh; W.H. McMillan and H.R. Tervit (1995). Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biol. Reprod., 53, 1385-1391.

Trounson, A.O. (1976). Effect of prostaglandin analogue cloprostenol on oestrous, ovulation and embryonic viability in sheep. J. Agric. Sci., 86, 609.

Trounson, A.O. and N.W. Moore (1974). Fertilization in the ewe following multiple ovulation and uterine insemination. Aust. J. Biol. Sci., 27, 301.

Tsunoda, Y. and Y. Kato (2002). Recent progress and problems in animal cloning. Differentiation, 64 (4-5) 158-161.

Wakayama, T.; A.C.F. Perry; M. Zuccotti; K.R. Johnson; R. Yanagimachi (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature, 394, 369.

Walker, S.K.; D.H. Smith and R.F. Seamark (1986). Timing of multiple ovulation in the ewe after treatment with FSH or PMSG with or without GnRH. J. Reprod. Fertil., 77, 135-142.

Walker, S.K.; K.M. Hartwich and R.F. Seamark (1996).The production of unusually large offspring following embryo manipulation: Concepts and challenges. Theriogenology, 45, 111-120.

Waller, S.L.; R.E. Hundgens; M.A. Diekman and G.E. Moss (1988). Effect of melatonin on induction of estrous cycles in anestrous ewes. J. Anim. Sci., 66, 459-463.

Wang, B. and J. Zhou (2003). Specific genetic modifications of domestic animals by gene targeting and animal cloning. Reprod. Biol. Endocrinol., 1, 103.

Wang, S.; Y. Liu; G.R.Holyoak; R.C. Evans and T.D. Bunch (1998). A protocol for in vitro maturation and fertilization of sheep oocytes. Small Ruminant Res., 29, 83-88.

Ward, K.A. and B.W. Brown (1998). The production of transgenic domestic livestock: Successes, failures and the need for nuclear transfer. Reprod. Fertil. Dev., 10 (7-8) 659-665.

Wells, D.N.; P.M. Misica; T.A. Day and H.R. Tervit (1997). Production of cloned lambs from an established embryonic cell line: A comparison between in vivo- and in vitro-matured cytoplasts. Biol. Reprod., 57, 385-393.

Westhusin, M.E.; C.r. Long; T.Shin; J.R. Hill; C.R. Loony; J.H.Pryor and J.A.Piedrahita (2001). Cloning to produce desired genotype.Theriogenology, 55, 35-49.

Wheaton, J.E.; H.A. Pohl and H.F. Windels (1990). Effects of melatonin and progesterone administered to ewes in spring and summer. J. Anim. Sci., 68, 923-930

Wheeler, M.B. and E.M.Walters (2001). Transgenic technology and applications in swine. Theriogenology, 56, 1345-1370.

Willadsen, S.M.; C. Polge; R.E.A. Rowson and R. M. Moor (1976). Deep freezing of sheep embryos. J. Reprod. Fertil., 46, 151-154.

Wilmut, A.E.; J. Schnieeke; A.J. Whir;.S. Kind and K. H. Campbell (1997).Viable offspring derived fron fetal and adult mammalian cells. Nature, 385, 810-813.

Wolf, E.; V. Zakhartechenko and G. Brem (1998). Nuclear transfer in mammals: Recent developments and future perspective. J. Biotech., 65 (2-3) 99-110.

Wolf, E.; W. Schernthaner; V. Zakhartchenko; K. Prelle; M. Stojkovic and G. Brem (2000). Transgenic technology in farm animals' progress and perspectives. Exp. Physiol., 85(5) 615-625.

Woolliams, J.A. and I. Wilmut (1989). Embryo manipulation in cattle breeding and production. Ainm. Prod., 48, 3-30.

Wulster-Radcliffe, M.C., Wang, S., Lewis, G.S. (2004). Transcervical artificial insemination in sheep: Effects of a new transcervical artificial insemination instrument and traversing the cervix on pregnancy and lambing rates. Theriogenology, 62,990-1002.

Young, L.E.; K.D. Sinclair and I. Wilmut (1998). Large offspring syndrome in cattle and sheep. Rev. Reprod., 3, 155-163.

Zhu, S.E.; S.M. Zeng; W.L. Yu; S.J. Li; Z.C. Zhang and Y.F. Chen (2001). Vitrification of in vivo and in vitro produced ovine blastocysts. Anim. Biotechnol., 12, 1993-2003.