

Update on reproductive biotechnologies in small ruminants and camelids

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Abstract

Recent advances in reproductive biotechnologies in small ruminants include improvement of methods for in vitro production of embryos and attempts at spermatogonial stem cell transplantation. In vitro production of embryos by IVM/IVF, intra-cytoplasmic sperm injection (ICSI), or nuclear transfer (NT) has been made possible by improvements in oocyte collection and maturation techniques, and early embryo culture systems. However, in vitro embryo production still is not very efficient due to several limiting factors affecting the outcome of each step of the process. This paper discusses factors affecting in vitro embryo production in small ruminants and camelids, as well as preliminary results with the technique of spermatogonial stem cell transplantation.

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Keywords: Sheep; Goat; Camelid; IVF; Nuclear transfer

1. Introduction

Several achievements have been made in recent years in reproductive biotechnologies in domestic animals. Small ruminants offer a good model for the development of these technologies and, therefore, it is not surprising that the first animal among the domestic species cloned by somatic cell nuclear transfer (NT) was a sheep. Camelids, on the other hand, are not as well studied, but could benefit from the introduction of reproductive

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biotechnologies, not only for traditional reasons (male and female infertility, genetic conservation), but also for the study of fertilization and early embryo development. Embryos of wild/endangered camelid species produced *in vitro* could also be transferred to domestic camelids.

In vitro production of embryos by IVM/IVF, cloning by NT, or intra-cytoplasmic sperm injection (ICSI) require basic manipulations such as oocyte collection and IVM, oocyte activation and IVC of zygotes or reconstructed embryos to a certain stage before they can be transferred into the uterus of a synchronous recipient. In males, the most exciting new development in reproductive biotechnology is spermatogonial stem cell transfer. The objective of this paper is to review these techniques in small ruminants and camelids, and factors affecting their success.

2. Oocyte collection and factors affecting yield and quality

Collection of good-quality oocytes is the first step for *in vitro* embryo production. In small ruminants the methods utilized are: (1) postmortem or postsurgical (ovariectomy) slicing of the ovaries or aspiration of ovarian follicles [1–5]; (2) aspiration of follicles after surgical exposure of the ovary by laparotomy or through laparoscopic folliculocentesis [1,2,6–8]; (3) transvaginal ultrasound-guided follicular aspiration [7]. Techniques used in South American camelids are generally similar to those used in small ruminants. Oocyte collection from live camels involves most commonly colpotomy and ultrasound-guided follicular aspiration.

2.1. Effect of collection method

2.1.1. Direct ovarian aspiration or dissection

Postmortem collection of oocytes is performed by follicular aspiration, using an 18 or 20 gauge hypodermic needle, or after mincing or slicing the ovary [3–5,9,10]. Oocyte yield is maximized, but the population of oocytes is heterogeneous and the technique is terminal for the donor. Recovery rate depends on the technique, follicular size, age, and reproductive status of the animal [11–14]. Ovarian mincing or slicing resulted in more oocytes recovered than follicular dissection or aspiration in prepubertal goats [14] and adult ewes [11–13]. In adult ewes, the average number of oocytes recovered per ovary ranged from 6 to 11 with the slicing method [12]. This technique resulted in the production of more debris, which interfered with the recovery of oocytes [11]. In the ewe, the recovery method does not seem to affect maturation or fertilization rates [12]. In goats, fewer oocytes recovered by mincing developed to the morula stage after fertilization when compared to oocytes recovered by aspiration [15]. In prepubertal goats, oocytes recovered from minced ovaries had lower normal fertilization rates than those from recovered by follicle dissection [14].

In camelids, both ovarian slicing and follicular aspiration have been used for oocyte collection (llamas [16]; camels [17–22]). In llamas, aspiration of follicles 1–12 mm resulted in a 62% recovery rate, with an average of six oocytes per female [23]. Mincing produced the highest yield of oocytes (27 per female), but maturation rate was reduced [16]. In dromedaries, aspiration with a needle attached to a syringe or to a vacuum pump resulted in oocyte recovery rates of 31–33%, whereas 94% of the oocytes were recovered

by follicle dissection [20]. Camel cumulus–oocyte complexes (COCs) are embedded in a thick sheet of granulosa cells and are difficult to retrieve without direct visualization, which may explain this difference in recovery rate [20]. Aspiration produced a recovery rate of 49%, with an average of seven oocytes per female [21]. Recovery rate from small follicles (1–4 mm) was low [17]. More oocytes are recovered during the breeding season [17,21] and in the absence of a corpus luteum [17].

2.1.2. Laparotomy and laparoscopic ovum pick-up

Collection of oocytes after exposure of the ovary by laparotomy is progressively being replaced by laparoscopic techniques in small ruminants. Laparoscopic folliculocentesis or laparoscopic ovum pick-up (LOPU) is relatively simple and efficient [6–8,24–31]. This procedure is quicker, less costly and can be repeated several times on the same animal without the complications that accompany laparotomy. The technique is performed under general anesthesia or heavy sedation after standard surgical preparation and can be completed in <20 min [7,25,29,32]. Recovery rates range from 75 to 85% in superstimulated (FSH, eCG) goats [6,7,25,32,33] and the average yield is 13.5 oocytes per animal [33,34]. The major problem with this technique is the increased loss of cellular layers, as the oocytes are aspirated from the follicle and pass through the tubing. Laparoscopic ovum pick-up has been used up to five times at weekly intervals in non-stimulated ewes, with a recovery rate of 68% resulting in an average of 4.6 (range 0.7–13) oocytes per ewe per session [29]. In camelids, laparotomy has been practiced primarily in alpacas and vicunas and allowed recovery of oocytes from up to 80% of aspirated follicles [35,36]. Laparoscopic ovum pick-up is more attractive and similar to that practiced in sheep [37].

2.1.3. Transvaginal ultrasound-guided aspiration

Transvaginal ultrasound-guided aspiration (TUGA) is a safe and practical alternative to surgical oocyte collection. A detailed description of a technique used in goats has been published [7]. This technique requires a lot of practice and is easier in smaller females. Oocyte recovery rates were similar to laparoscopic techniques; however, the number of follicles that can be visualized and aspirated was lower [7]. In camelids, TUGA has been used in llamas [38–40], alpacas [35], and camels (unpublished data). The technique is performed after standard MOET treatments with eCG or FSH, alone or in combination [41], with recovery rates ranging from 55 to 75% [38]. Gonadotropin stimulation did not improve recovery rates, but increased the number of oocytes obtained per female [35,38–40].

2.1.4. Cryopreserved oocytes and ovarian tissue

Oocytes can be frozen directly or recovered from frozen–thawed ovarian tissue sections [42]. Offspring have been obtained by IVM/IVF of vitrified oocytes from antral follicles in sheep [43]. Cryopreserved mature oocytes had premature release of cortical granules contents, hardening of the zona pellucida, and increased chromosomal abnormalities. These problems were not observed with cryopreserved, less-differentiated immature oocytes [44]. Isolated primordial follicles have been frozen successfully using 1 or 1.5 M DMSO [45].

The ovarian cortex contains a large supply of immature oocytes enclosed in preantral follicles. The cryopreservation of ovarian tissue and recovery of oocytes has been reported in sheep [43,45–47]. Oocytes obtained in this manner had only a slightly reduced ability to mature in vitro, compared to vitrified and non-vitrified oocytes [48]. A high rate of follicular degeneration was observed in goat [44] and sheep [47] ovarian tissue in the presence of cryoprotectant. In vitro culture of antral follicles obtained from frozen–thawed ovarian cortex resulted in low quality oocytes [49].

2.2. Gonadotropin stimulation before oocyte collection

Ovarian stimulation with gonadotropins increased the number of follicles available for aspiration and allows the use of juvenile or prepubertal animals as oocyte donors [33,50–53]. Several protocols of stimulation have been proposed [6–8,25,34,42,52,54–60]. Stimulation of female kids as young as 1.5–3 months resulted in the recovery of 15–35 oocytes per female, with an average of 22 oocytes suitable for IVM [61]. In one study, higher yield of oocytes was obtained from gonadotropin-primed prepubertal does than from adults, while in vitro development was similar [8]. In ewes, FSH treatment for 2 days resulted in better oocyte recovery and cleavage rates than treatment for 3 days [62]. A recent study on synchronized ewes showed that stimulation treatment with a constant FSH dose resulted in better oocytes and better embryonic tolerance to cryopreservation when compared to treatment with decreasing FSH doses [42]. In camelids, ovarian stimulation with eCG or FSH provided a uniform population of oocytes [35,40]. The best response to gonadotropins was obtained when treatment was initiated after elimination of the dominant follicle or synchronization of follicular waves [38,41]. These treatments also had the advantage of obtaining more expanded COCs [38]. In llamas, superstimulation with eCG was associated with a slightly higher proportion of expanded COCs and COCs in metaphase II compared to superstimulation with FSH [38]. Similar results were recently obtained in camels. Oocyte recovery method from non-stimulated ovaries does not affect nuclear maturation and rate of parthenogenetic activation/fragmentation [20]. Maturation rate of oocytes from non-stimulated animals were significantly lower than those from stimulated animals (63 versus 83%) [20].

2.3. Other factors affecting oocyte quality

Oocyte quality was influenced by the stage of follicular development [63] and the method of collection [15]. In addition, oocytes quality and the ability to develop to blastocyst was influenced by nutrition of the females during the stimulation period [64]. Treatment of ewes on the first and last day of superovulation with all-trans retinol, a regulator of cell growth, differentiation and development, increased embryonic viability and development in vivo [65].

3. Oocyte maturation

Embryo development is influenced by events occurring during oocyte maturation [66]. For successful IVM, oocytes must undergo nuclear and cytoplasmic maturation in vitro.

Sheep and goat oocytes are generally matured in buffered TCM-199 supplemented with pyruvate, heat-treated serum, and hormones (FSH, LH, estradiol) [3,25,48]. High maturation rates (70–90% reaching metaphase II) were achieved with pre-selected oocytes under specific conditions [1,5,67,68]. A maturation rate of 100% has been reported for goat oocytes aspirated from 2 to 6 mm follicles and incubated for 24 h in TCM-199 medium supplemented with 10% fetal bovine serum (FBS), 100 mg LH/mL, 0.5 mg FSH/mL, and 1 mg estradiol-17 β /mL at 38.5 °C in humid 5% CO₂ in air [1].

Camelid oocytes are matured in conditions similar to those described for small ruminants. The basic medium used was TCM-199 with 10% FBS, supplemented with 5 μ g/mL LH, 0.5 μ g/mL FSH, 1 μ g/mL estradiol-17 β , and 25 μ M pyruvate [16,21,23]. Camelid oocytes have a dark cytoplasm, due to presence of lipid particles, which makes evaluation very difficult [16,40,69]. A detailed description of maturation of camel oocytes was recently published [69]. The perivitelline space (PVS) increased as the maturation process progressed until 24 h, but no further increase occurred until 36 h of culture. A large number of microvilli were observed in the PVS in metaphase II oocytes. Cortical granules migrated towards the peripheral areas of the ooplasm and formed a lining in the subolemmal area [69].

3.1. Role of hormones

The addition of gonadotropins (FSH and LH) and estradiol-17 β to the maturation medium significantly improved maturation rates [3,9,10,15,25,42,51,70]. Gonadotropins are the primary regulators of in vitro nuclear maturation in mammalian oocytes. The beneficial effect of gonadotropins in the maturation medium was more pronounced for oocytes from juvenile or prepubertal females [51]. Estradiol may be involved in cytoplasmic maturation by stimulating DNA polymerase β and enhancing the synthesis of presumed male pronucleus growth factors. Blastocyst production was significantly increased for oocytes matured in the presence of estradiol-17 β [71].

3.2. Effect of serum

Maturation media are generally supplemented with 10–20% heat-treated serum (56 °C for 30 min). Serum provides nutrition to cells in the COCs and prevents zona hardening [72]. Sera utilized include estrous cow serum [48], FBS [3,25,42], and homologous or heterologous estrous serum [73]. Most studies in goats and sheep used FBS for oocytes IVM [74–76]. In goats, estrous goat serum (EGS) was used alone [15,56,70,77] or in combination with BSA [78]. Estrous sheep serum (ESS) has been used for ovine oocytes IVM [79]. The effect of type and concentration of serum on maturation rates has been investigated [2,76,80–82]. Although one study showed that the presence of EGS in the maturation medium was not essential [82], high maturation rates of caprine oocytes were obtained after 24–25 h of culture in maturation medium supplemented with 10% FBS (83%), 10% EGS (86%), or 10% ESS (94%), without addition of gonadotropins [81]. The addition of ESS, either alone or with hCG, stimulated maturation and subsequent embryo development in immature prepubertal sheep oocytes [80]. For oocytes from adult ewes, slightly higher maturation rates (82 versus 70%) were obtained with ESS compared to FBS [76].

3.3. *Effect of culture conditions*

Oocytes were matured by incubation in the desired medium at 38–39 °C in humidified atmosphere of 5% CO₂ in air (or 7% O₂ and 88% N₂) for 23–26 h for sheep [3,25,48] or 24–27 h for goats [1,73]. Incubation time necessary for maturation of goat oocytes seemed to be longer than that needed for sheep oocytes. A higher proportion of goat oocytes reach metaphase II after 27 h than after 24 h of culture [83]. Other studies suggested that culture for 32 h in TCM-199 with 20% EGS was the best alternative for IVM of goat oocytes [84,85].

In llamas, optimal maturation rate (62%) of oocytes recovered by slicing was achieved after incubation for 32–36 h [16,23]. Recent studies showed that an incubation time of 30 h resulted in higher maturation rates (80.6%) for oocytes aspirated after ovarian stimulation [38]. Camelids are induced ovulators and the induction of an LH surge is required for final oocytes maturation within follicles. Oocytes obtained 22 h after induction of an LH surge by administration of buserelin had a maturation rate of 62% in the absence of hormones in the medium [86]. In alpacas, oocyte maturation rates of 40–46% were obtained when compact COCs were collected 18–24 h after hCG administration and incubated for 26 h [35]. Similar results were obtained with vicuna oocytes incubated for 27 h [36].

In dromedary camels, TCM supplemented with estrous camel serum was superior to CRI or CMRL media for oocyte nuclear maturation [20]. Optimal dromedary oocytes maturation rate (51%) was observed after culture for 36 h in TCM supplemented with gonadotropins and bull serum [19]. A high nuclear maturation rate (85.4%) was obtained after 36 h of culture in CRI [17]. A 66% maturation rate was observed after 30 h of culture in Ham's F10 medium supplemented with 10% FCS and hCG; extending the culture beyond 36 h resulted in oocyte degeneration [69]. Ultrastructural studies of dromedary oocytes during nuclear maturation suggested that the optimal culture time is 30 h in TCM supplemented with estrous camel serum [69]. The camel oocyte was at the germinal vesicle stage in antral follicles, and therefore, the maturation of camel follicular oocytes was arrested in prophase I of the first meiotic division [22]. The time required for germinal vesicle breakdown ranged from <6 to 30 h [69]. Oocytes of non-pregnant females matured faster in vitro (32 h) than those of pregnant females (36 h) [19]. In the Bactrian camel, 46.7% of oocytes achieved meiotic maturation after 24–26 h of culture [87].

3.4. *Co-culture with follicular cells*

Follicular cells support oocyte maturation by providing nutrition and signals for the synthesis of specific structural and maturation proteins [72]. Incubation of goat oocytes over granulosa cell (GC) monolayer delayed maturation but significantly increased the maturation rate [88]. Higher fertilization and cleavage rates were achieved in oocytes matured over GC monolayer than those matured with GC co-culture [66,70]; this suggested that the GC monolayer improved cytoplasmic maturation [66]. Granulosa cells from small and large follicles were used for IVM and IVC, with approximately the same efficiency after conditioning with maturation medium and embryo development medium 18–24 h before culture [66].

3.5. Follicular fluid

Supplementation of IVM media with follicular fluid from non-atretic or gonadotropin-stimulated large follicles (>4 mm) had some beneficial effect in both sheep [89] and goats [26,90]. Addition of sheep or human follicular fluid to the maturation medium may enhance IVM/IVF of sheep follicular oocytes [89]. This beneficial effect on maturation may be due to growth factors, hormones and intra-ovarian peptides in more physiological proportions [26].

3.6. Effect of follicle size

The acquisition of meiotic competence in goat oocytes has been previously correlated with follicular size [91–93]. Goat oocytes from early antral follicles grew and acquired the ability to resume meiosis when cultured for 9 days on granulosa cell monolayers [74].

3.7. Effect of growth factors

Epidermal growth factor (EGF) influenced oocyte maturation and blastocyst production in various mammalian species and may be involved in the regulation of follicular growth and oocyte maturation in goats and sheep [94]. Oocytes matured in vitro in the presence of EGF had greater cumulus cell expansion and higher fertilization rates in sheep [95] and camels [21]. In camels, the addition of epidermal growth factor (EGF) and cysteamine to the maturation medium may have a beneficial effect on nuclear and cytoplasmic maturation [21]. In sheep, nerve growth factor (NGF) is produced in vitro by granulosa cells in response to gonadotropin stimulation and may be involved in the control of oocyte maturation [54]. The addition of NGF to the maturation medium resulted in resumption of meiosis in >70% of the oocytes [54]. Insulin-like growth factor-I (IGF-I) did not seem to affect oocyte IVM [96].

3.8. Effect of donor age

Developmental competence of oocytes from young animals has been controversial [53,58,59,97]. Lower developmental competence of oocytes from prepubertal females may be due to a deficiency in cytoplasmic maturation leading to reduced sperm penetration, lack of male pronucleus formation, failure to block polyspermy, cleavage failure, failure to reach or survive the transition from maternal to embryonic genomic expression, and developmental problems leading to pregnancy loss during the preimplantation and postimplantation stages [98,99]. Defects in the competence of lamb-derived embryos may account for the increased fetal loss during pregnancy and the occurrence of mummified fetuses delivered alongside normal healthy lambs [57,58].

In goats, oocytes recovered from prepubertal females had high rates of polyspermy [100], failure of sperm head decondensation and formation of male pronucleus [56], low rate of blastocyst production formation [97], and high percentage of haploid embryos [101]. The high rate of polyspermy may have been due to abnormal distribution of cortical

granules and a failure in the cortical reaction [99]. Oocytes with the same diameter derived from prepubertal and adult females had similar meiotic progression rates [53]. Prepubertal goat oocytes displayed the same maturation, but lower fertilization rate, compared to adult oocytes when cultured with adult goat granulosa cells [90,102]. Gonadotropin stimulation of prepubertal does resulted in high oocyte yield, with similar IVM and developmental rates than oocytes from adult does [8].

Supplementation of the medium with cystamine, a precursor for glutathione (an intracellular free thiol that improved development of the male pronucleus), improved normal fertilization and embryo development of ovine prepubertal oocytes [26,103–105]. However, addition of glutathione was not associated with a higher normal fertilization rate of prepubertal goat oocytes [106].

Low IVM rates were obtained with lamb oocytes after incubation for 24 h (60.0%) or 26 h (28.6%) when compared to adult oocytes incubated for 24 h (85.7%) [107]. Oocytes from prepubertal and adult ewes had similar cleavage rates but the proportion of embryos reaching the blastocyst stage was significantly lower for lamb-derived oocytes [107]. Oocytes did not reach full developmental competence until puberty and the lesser competence of prepubertal oocytes could have been due to morphological anomalies and alterations in physiological activity [108]. Gonadotropin treatment (400 IU eCG and 120 IU FSH) of neonatal and prepubertal (4–6 week old) females increased oocyte yield per female but had no effect on oocyte quality, in vitro embryo production, and lambing rates [52]. A single treatment with estrogen and progesterone prior to gonadotropin stimulation increased the yield and developmental capacity of oocytes from prepubertal sheep [59]. Maturation rates of oocytes from 30 to 40 days old juvenile lambs were lower than that of adult ewes oocytes in the absence of gonadotropins [51]. Fertilization rates were similar, but parthenogenetic activation and polyspermy were higher in juvenile oocytes. Blastocyst production was lower for juveniles but blastocyst viability was similar to those from obtained from oocytes of adult ewes [51].

3.9. *Effect of other substances and use of defined media*

The use of IVM media containing non-defined substances, particularly serum, has been suspected to be at the origin of the lack of repeatability of some IVM/IVF studies. Recent studies demonstrated that goat oocytes could be matured successfully under defined conditions (synthetic oviduct fluid (SOF), EGF, amino acids, gonadotropins, and BSA) [109]. A modified synthetic oviductal fluid maturation medium (mSOFmat) containing polyvinylalcohol (PVA) or hyaluronate with citrate as a macromolecular supplement resulted in better maturation and development rates for goat oocytes than TCM-199 supplemented with goat serum [110]. This medium contains cysteamine, a reducing agent shown to improve developmental competence of goat [104,111] and sheep [105] oocytes. In addition, the concentrations of pyruvate and lactate in the medium closely mimicked the concentrations normally present in follicular fluid [110]. Addition of MEM vitamins to SOF maturation medium was reported to be beneficial for subsequent blastocyst development and viability [5]. Maturation competence of goat oocytes was suppressed by streptomycin when compared to penicillin or gentamicin [112].

4. Sperm source and IVF

Most IVF studies use freshly ejaculated spermatozoa (goat [9,15,31,55,56,66,97,100,101,113–115]; sheep [3,25,72,100,116]). A few IVF trials have been carried out with frozen–thawed spermatozoa (goat [5,83,109]; sheep [42,95]) or epididymal spermatozoa (sheep [117]; goat [82,118]). Lambs have also been produced from embryos obtained by IVF with frozen–thawed spermatozoa that had been sexed by flow cytometry. Frozen spermatozoa can be used immediately after sexing or re-frozen for later use, with a high rate of correct predetermined sex (86.7%) [68]. The fertilization rate with sorted frozen–thawed spermatozoa was lower, but development capacity of fertilized oocytes was similar to fresh sorted spermatozoa [68].

Highly motile spermatozoa were selected by swim-up (goat [9,15,55,83,97,100,113–115]; sheep [42,95]), Percoll gradient centrifugation (goat [3,5,82,83,100,118]; sheep [25,116]) or sephadex filtration [83]. Greater yield of highly motile spermatozoa can be obtained by swim-up, when compared to Ficoll or Percoll density gradient centrifugation for fresh goat semen, but no differences were observed in terms of oocyte penetration and cleavage rate after IVF [100]. For frozen–thawed goat semen, Percoll treatment resulted in higher fertilization and development rates than swim-up or glass-wool separation [83]. There are indications that neither selection nor washing of spermatozoa were necessary when fresh, fertile semen was used for IVF [100].

Capacitation of sheep and goat sperm were obtained when heat-inactivated estrous sheep or goat serum was present in the medium, resulting in high cleavage rates [55,114,115]. Perhaps the addition of serum to the capacitation medium supported cholesterol efflux, thought to be a key event in capacitation [119]. Sperm capacitation was improved by addition of heparin (sheep [72]; goats [9,55,56,66,83,97,100,113]). Heparin improved fertilization, cleavage, and embryo yield when included in IVF media [120,121]. Addition of caffeine to heparin seemed to further improve cleavage rates [15]. In a recent study, addition of heparin was found helpful only when frozen–thawed semen was used; however, higher cleavage and blastocyst production rates were obtained after IVF with fresh sperm capacitated without heparin [55]. Although, the use of heparin in the fertilization medium has been reported to improve embryo yield, several studies have also reported reduced developmental competence and pregnancy rate, perhaps due to a high rate of polyspermy [26,31,55,122,123].

In goats, heparin was used in different IVF media (BO [109,114]; SOF [83]; TALP [31,55,56,100,113,120]) at concentrations ranging from 2 to 100 mg/mL. Incubation of sperm selected by swim-up in TALP–IVF with heparin (50 mg/mL) for 45 min resulted in excellent fertilization rates [55,100,113]. Similarly, high penetration and fertilization rates were obtained with modified defined medium (mDM) supplemented with heparin for capacitation and TALP supplemented with hypotaurine for fertilization. Addition of penicillamine and adrenaline to the fertilization medium reduced these rates [9].

Fertilization rates of capacitated semen were affected by individual males, in addition to conditions of capacitation and fertilization, and type of semen (fresh versus frozen and ejaculated versus epididymal) [116,124]. The incidence of polyspermy varied from 10 to 20%; however, after discarding polyspermic oocytes, an average of 60–70% normally-fertilized sheep and goat oocytes were routinely obtained *in vitro*.

In camelids, IVF studies are scarce. The first successful production of embryos by IVM/IVF in camelids was reported in llamas using epididymal sperm enriched by Percoll gradient in presence of heparin (2 or 5 $\mu\text{g/mL}$) [16]. Oocyte penetration and development to the pronucleus stage rates were 29.2 and 57.1%, respectively. Similarly treated llama epididymal sperm was also used to produce interspecies (*L. pacos* $\times$ *L. glama*) embryos [35]. In vitro production of camel embryos has been reported using Percoll-enriched fresh ejaculated [21] and epididymal semen [20]. Penetration (68%) and cleavage rates (X%) have been very promising with freshly ejaculated semen [21,125].

5. Intra-cytoplasmic sperm injection

There is little information on ICSI in small ruminants. This method has been used successfully to produce a lamb [126] and blastocysts in goats [127]. The acrosome reaction was generally not necessary for ICSI in sheep [126]. Blastocyst production after ICSI remains very low; in one study, 80% of the embryos produced by ICSI underwent development arrest on Day 4 (16-cell to morula stage) [128]. This could be due to a number of factors such as oocyte maturation, culture system, oocyte activation and abnormal fertilization [128,129]. In sheep, ICSI resulted in a higher rate of abnormal fertilization and lower total activation rate compared to IVF [126]. Inadequate oocyte activation after the injection could be responsible for lower sperm decondensation and pronuclear formation. Activation and fertilization rates were higher in the presence of calcium chloride in the culture medium at a concentration of 3.42 mM or higher [130,131]. In camelids, ICSI may be a valuable tool for production of embryos from infertile males and could have some value for the production and study of interspecies embryos within the camelidae family. The only report of ICSI-produced morulas was done in llamas [132].

6. Cloning

Cloning has generated tremendous interest in recent years [133–138]. The first reported birth of a clone obtained by NT was reported in sheep (Dolly) [139]. Dolly was the result of a batch of 277 embryos reconstructed by transfer of nuclei of cultured adult mammary epithelial cells, a success rate of 0.4% [139]. The major future use of NT in small ruminants is in the area of transgenic animals production/reproduction [6,33,135,140,141]. Transgenic lambs and goats have been produced by linking milk promoter genes to specific genes of interest (e.g. human coagulation factor IX in sheep [140] and human anti-thrombin III in goats [142]) and demonstrated the capacity of synthesizing the desired molecules in their milk. Gene modification can also serve other purposes such as prevention of rejection after xenotransplantation of organs [136] or “creation” of disease resistant animals (e.g. deletion of the PRion Protein responsible for scrapie in sheep [143]).

Nuclear transfer involves the transfer of nuclei from serum-starved fetal or adult cells into enucleated oocytes matured in vivo or in vitro. The type of donor cell used for nuclear transplantation seems to be the most significant source of variation in NT techniques [137,139,140,144–148]. In goats, births have been produced from embryos obtained by

transfer of either adult or fetal cell lines nuclei into enucleated ova and transfer of reconstituted embryos into recipients at 2–4-cell stage (1–2 days of culture) [27,28,142,149]. In general, 0.5–2% of reconstructed embryos develop to live offspring [142,144]. Fetal fibroblast quiescence is not required, but can improve the production of embryos and live offspring [138].

Adult cells used for cloning include mammary epithelial cells, granulosa cells (female), Sertoli cells (males), and skin cells (dermal fibroblasts) [148]. Embryos reconstructed by NT from cumulus and mural granulosa cells as karyoplast have resulted in 2–13% kidding rate [28]. Regardless of the cell type, the cell cycle stage of the donor cell at the time of NT to metaphase arrested or concurrently activated ooplasm can affect the efficiency. The quiescent (G0/G1) stage is preferred and readily available in culture of adult cells.

The poor results of cloning by NT are likely due to factors affecting nuclear reprogramming (i.e. cell-cycle stages, type, age and pretreatment of donor cell, cytoplasmic factors, and changes in chromatin and DNA structure driven by methylation and acetylation of donor cell DNA) [148]. The cytoplasm from the donor oocyte also has an involvement in the mechanisms of nuclear modifications through cyclins and kinases. Recently, it was shown that even ewe nutrition affects oocyte quality and development ability after NT [148].

In camelids, production of embryos by NT has been reported in llamas [150]. The couplets were obtained by transferring adult llama fibroblast nuclei from skin cell line biopsy into the perivitelline space of in vitro matured oocytes after removal of the metaphase plate and polar bodies. Fusion of the couplets in a microslide fusion chamber was successful in 62.5% of attempts ($n = 80$), followed by cleavage rates of 32 and 40% in CR1aa and G1.2 medium, respectively. Transfer of 8–32-cells embryos ($n = 10$; transferred surgically into the oviduct) and a morula (transferred non-surgically into the uterus) did not result in any pregnancies 14 days later [150].

7. Early embryo development competence

Early goat embryos cultured in vitro fail to develop past the 8- to 16-cell stages in traditional culture media. This block occurred around the time of activation of the embryonic genome [151]. Presumptive zygotes have been successfully cultured to the blastocyst stage with granulosa cells [9,56,152], G1.2 and G2.2 medium [8], oviductal cells [10,55,82,113], cumulus cell [10], and SOF medium [152]. Co-culture with oviductal cells resulted in the best development rates for oocytes from prepubertal goats [10,97]. The stage of the cycle when oviductal cells were harvested did not seem to affect development [97]. In vitro embryonic development following IVF ranged from 50 to 70% [55,56,83,100]; however, the proportion of embryos developing to blastocyst stage was variable and ranged from 8 to 40% [55,82,113,114,153], often not exceeding 10% [8,56,127].

More than 50% of goat embryos produced in vitro may show chromosomal abnormalities [101]. Modifications of IVM conditions by addition of follicular fluid from goats treated with gonadotropin or cysteamine to the culture medium improved the efficacy of in vitro embryo production [26]. Higher development rates to morula and blastocysts stages was observed in culture with bovine or caprine oviductal cells than with

cumulus cells [10]. Oviductal cell co-culture had a marked effect on cleavage and development of goat IVF embryos. Buffalo oviductal cells were used for goat embryo development [154]. The effect of age of the donor on development capacity of IVF oocytes has been a subject of debate. Some studies reported a lower developmental competence of prepubertal oocytes in sheep [51,57,58,155,156], whereas others did not find any difference between adult and prepubertal oocytes in sheep [98] and goats [8,56,97]. Age of the donor (prepubertal or adult) of oviductal cells for in vitro culture did not affect the development of in vitro produced embryos, neither in vitro nor in vivo, after transfer of early embryos into rabbit oviducts [97].

Epidermal growth factor (EGF) enhanced in vitro blastocyst production in several species. The use of EGF in culture media for oocytes collected from FSH-treated ewes increased in vitro blastocyst production [95]. However, in a recent study in sheep, fertilization and morula production rates were similar for COCs cultured with or without EGF [96]. In vitro culture of sheep embryos in SOF resulted in greater cleavage and blastocyst production rates than culture in TCM-199 [3,4]. Embryos produced by IVM/IVF with sexed semen were cultured in Sidney IVF medium to the blastocyst stage [68]. Sheep embryos were also successfully cultured to blastocyst stage in SOF with or without human serum. Fetuses produced with embryos co-cultured with granulosa cells or in SOF supplemented with human serum were heavier at birth and had significantly larger livers and hearts [152]. In camels, we have recently shown that embryos obtained by IVM/IVF and cultured until hatching in semi-defined medium (mKSOMaa) had better in vivo development ability than those cultured with oviductal cells [125].

8. Effect of in vitro embryo production on pregnancy and offspring health

Pregnancy rates after transfer of in vitro produced sheep embryos to recipients ranged from 41 to 66% for fresh embryos [50,59,157–160] and from 33 to 50% for vitrified embryos [50,160–162]. Overall, pregnancy rates ranged from low (<20%) to acceptable (66%). Live birth rates obtained after transfer of embryos produced by NT were low. Low rates of cleavage and development to blastocyst combined with increased pregnancy wastage were the main causes of the low efficiency of NT [113]. High pregnancy losses (30%) have been reported with sheep embryos cultured in SOF [4]. To improve pregnancy rates in small ruminants, multiple embryos are generally transferred to the same recipient [58,59,153]. However, the optimal number of embryos per recipient is not well defined and may be breed-dependant.

Pregnancy and fetal abnormalities are reasons for great concern with embryos produced in vitro. Gestation length and birth weight of lambs produced by IVM/IVF were increased, suggesting that IVM/IVF affected subsequent embryonic and fetal development. Other problems encountered with pregnancies from embryos produced in vitro included increased rates of dystocia and perinatal death, increased respiratory problems, suckling difficulties, and compromised immune systems [163–165]. Transfer of ovine embryos produced in SOF supplemented with 20% human serum resulted in prolonged gestation and birth weights that were higher than normal [152]. These anomalies were apparently not observed after culture in serum-free medium [123].

Large offspring syndrome (LOS) and several other developmental abnormalities were reported in lambs produced in vitro [152,165–169]. Transfer of sheep embryos 2 days after IVF did not decrease the incidence of LOS or congenital abnormalities compared to transfer of 6 days old embryos [166]. Although serum used in embryo culture may interfere with embryonic and fetal development, it is not the only factor responsible for LOS [166], since early development in vivo cannot rescue embryos produced in vitro from deleterious effects during oocyte maturation and fertilization [170]. The mechanisms involved in aberrant development of embryos produced in vitro are speculative. Candidate perturbing agents include ammonia, progesterone, and growth factors [79,165]. The long-term effects of these abnormalities of development have not been investigated. Lambs born oversized tended to have the same weight as contemporary lambs by 1 year of age [79,171].

Sheep studies in NT have been plagued by high rates of defective placentation (hydrops amnii), fetal abnormalities, abortion, and lamb mortality [64,134,172]. Large offspring syndrome is also a characteristic of pregnancies from cloned embryos resulting in increased rates of organomegaly and dystocia [165,173,174]. This syndrome may be due to fetal and placental growth deregulation even in early gestation [175,176] and has been associated with use of serum and complex culture media [168,171]. These abnormalities were probably due to abnormal epigenetic organization with incomplete re-programming of imprinted gene methylation pattern in the donated somatic cell nucleus and/or to inappropriate allocation of cells during early lineage specification [177]. These problems seemed less common in goats. It is possible that culture conditions of the karyoplast donor cells as well as the reconstructed embryos are responsible for most of the wastage. Number of culture passages may be responsible for telomere shorting.

9. Spermatogonial stem cell transplantation

Spermatogonial stem cell transplantation is a technique in which spermatogonial stem cells from the testis of a donor male are transferred into the seminiferous tubules of a recipient male [178,179]. This technique provides a unique approach for the study of spermatogenesis, manipulation of the male germ line, and reproduction of transgenic domestic animals. In goats, the technique resulted in donor-derived spermatogenesis within recipient testes and generation of offspring with the donor genotype following natural breeding [180]. In goats, isolated donor cells were infused directly into the rete testis of immature recipients using an ultrasound-guided technique. Histologically, tissue disruption was limited to the injection site [179]. No detectable local or systemic signs of immunologic reactions to the transplantations have been observed, suggesting that in some species the recipient animals do not have to be immunosuppressed or genetically matched to the donors to receive germ cell transplantation [179]. In adult recipient, germ cell depletion by induction of testicular degeneration may facilitate intratesticular infusion [178]. Recent observations in rams show that germ cell depletion of the recipient testis may be obtained by immunization against LHRH or a combination of immunization and irradiation, without affecting Sertoli cell function [178].

10. Conclusion

In vitro production of embryos by IVM/IVF, NT, and ICSI is not yet efficient in small ruminants and camelids. However, progress achieved in recent years in developing new oocyte harvesting techniques and improving early embryo culture has shown promise to increase pregnancy and parturition rates. The understanding of the control of early embryo development at the molecular level may lead to the discovery of factors affecting fetal and placental development and causes of fetal and gestational abnormalities seen with embryos produced in vitro. Studies on in vitro embryo production in camelids are lagging behind other traditional domestic species, but there has been increased research activity in this field recently. The first births of camels produced by IVM/IVF were born in our laboratory February 2005. The possibility of transferring spermatogonial stem cell from one male to the testis of a recipient male opens new exciting prospect for the multiplication of superior males. There is no doubt that these techniques will improve our understanding of gamete physiology, but their application in animal production will depend on efficiency and acceptance by consumers.

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