Theriogenology 81 (2014) 1149–1162

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Review

In vitro production of small ruminant embryos: Late improvements and further research

Joanna Maria Gonçalves de Souza-Fabjan [a,b,*], Barbara Panneau [a], Nicolas Duffard [a,c], Yann Locatelli [a,c], José Ricardo de Figueiredo [d], Vicente José de Figueirêdo Freitas [b], Pascal Mermillod [a]

[a] *INRA, Physiologie de la Reproduction et des Comportements, Nouzilly, France*
[b] *Faculty of Veterinary, Laboratory of Physiology and Control of Reproduction (LFCR), State University of Ceará, Fortaleza, Ceara, Brazil*
[c] *Museum National d'Histoire Naturelle, Réserve de la Haute Touche, Obterre, France*
[d] *Faculty of Veterinary, Laboratory of Manipulation of Oocyte and Preantral Follicles (LAMOFOPA), State University of Ceará, Fortaleza, Ceara, Brazil*

ARTICLE INFO

Article history:
Received 5 October 2013
Received in revised form 28 January 2014
Accepted 1 February 2014

Keywords:
Sheep
Goat
Oocyte
In vitro embryo production
Embryo

ABSTRACT

Beyond the potential use of *in vitro* production of embryos (IVP) in breeding schemes, embryos are also required for the establishment of new biotechnologies such as cloning and transgenesis. Additionally, the knowledge of oocyte and embryo physiology acquired through IVP techniques may stimulate the further development of other techniques such as marker assisted and genomic selection of preimplantation embryos, and also benefit assisted procreation in human beings. Efficient *in vitro* embryo production is currently a major objective for livestock industries, including small ruminants. The heterogeneity of oocytes collected from growing follicles by laparoscopic ovum pick up or in ovaries of slaughtered females, remains an enormous challenge for IVM success, and still limits the rate of embryo development. In addition, the lower quality of the IVP embryos, compared with their *in vivo*–derived counterparts, translates into poor cryosurvival, which restricts the wider use of this promising technology. Therefore, many studies have been reported in an attempt to determine the most suitable conditions for IVM, IVF, and *in vitro* development to maximize embryo production rate and quality. This review aims to present the current panorama of IVP production in small ruminants, describing important steps for its success, reporting the recent advances and also the main obstacles identified for its improvement and dissemination.

1. Introduction

Throughout the world, the importance of small ruminants as providers of essential food, meat and dairy products, has been well reported. Beyond genetic selection for productive traits, reproductive efficiency is one of the most important factors to improve goat and sheep production. After Artificial Insemination (AI) and Multiple Ovulation and Embryo Transfer (MOET) schemes, *in vitro* production of embryos (IVP) represents the third generation of techniques aimed at better control of animal reproduction [1]. This technique involves four major steps: oocyte collection, oocyte IVM, IVF, and *in vitro* development (IVD) of the resulting embryos up to the blastocyst stage, at which these embryos can be efficiently cryopreserved or transferred into the uterus of synchronized recipient females. These different steps are now well established in domestic ruminant species (cattle, sheep, and goat). *In vitro* embryo

* Corresponding author. Tel.: +33 608 279 759; fax: +33 2 47 42 77 43.
E-mail address: joannavet@gmail.com (J.M.G. de Souza-Fabjan).

0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.02.001

production is also under progress in nonruminant species like horse [2] and pig [3], although far less efficient than in ruminants. Despite heavy research efforts during the past 30 years, the variability of the number and quality of the oocytes collected and the low viability of frozen-thawed IVP embryos still limit the large-scale use of this technology [4].

In small ruminants, *in vivo* embryo recovery requires surgical procedures that impair repeated embryo production from individual donors. Therefore, IVP has some advantages such as reliability [5], reproducibility [6], the possibility to collect oocytes from females hormonally stimulated or not [7], the use of prepubertal donors [8], senile [9], pregnant females, or even in postmortem cases. Moreover, oocyte and embryo techniques are necessary to develop other biotechnologies such as cloning and transgenesis [10], and it has been proposed as a valuable strategy for the conservation of endangered species [11]. Furthermore, gametes and embryo technologies give access to fundamental research on cell cycle and gene expression regulations, and it may help the identification of new targets for marker-assisted genomic selection of precise reproductive characters.

Studies concerning IVF in mammals were initiated as early as 1878 by Schenk [12], and the first domestic animal born after IVF was a rabbit, in 1959 [13]. Some years later, Hanada [14] reported the first goat birth after IVF using IVM oocytes and only in 1993, a study was published reporting for the first time a development to term after transfer of an embryo produced totally *in vitro* in this species [15]. The technique is extremely versatile, and thus has been intensely studied in recent years. However, despite considerable efforts aiming to improve IVP steps, success rates are still far from *in vivo*–derived embryos [16,17]. This review aims to present the current panorama of embryo IVP in small ruminants, describing important steps for its success, reporting the recent advances and also the main obstacles identified for its improvement and dissemination, and clues for further progress of this technology.

2. Oocytes: Source and recovery

The first step of IVP is the recovery of developmentally competent oocytes. Oocyte developmental competence may be defined as its ability to resume and achieve meiosis, be fertilized, to develop into an embryo, and give rise to normal and fertile offspring after normal gestation. Oocyte developmental competence thus reflects the oocyte's intrinsic quality. In mammals, developmental competence is acquired progressively by the oocyte during folliculogenesis, and increases with the size of follicle to be ready to support embryo development at the time of ovulation [18].

Immature oocytes may be recovered from slaughterhouse ovaries or from live animals. Slaughterhouse ovaries provide a cheap and abundant source of oocytes that can be recovered by follicle aspiration, slicing, or follicle dissection. These large number of oocytes from unknown females are helpful for research and improvement of IVP conditions. This strategy may be of interest for females of high genetic value that had to be unexpectedly culled [19], which obviously consists in the single use of a particular animal but can provide a last offspring from theses valuable females. However, the use of IVP for genetic improvement or diffusion requires repeated oocyte recovery from live females with high genetic value to maximize the number of embryos that can be produced and optimize genetic gain. It is possible to obtain immature oocytes by follicular aspiration performed by abdominal laparotomy [20], but this method presents the disadvantage of generating adhesions, avoiding repetitions of the procedure. In cattle, the transvaginal ultrasound-guided aspiration technique based on ovarian mobilization through rectum wall is currently the most used technique to successfully obtain oocytes [21]. However, in small ruminants this method is not adapted as ovaries are difficult to grasp through the rectum. Graff, et al. [22] obtained lower numbers of oocytes from goats submitted to transvaginal ultrasound-guided aspiration as compared with those submitted to laparoscopy (4.3 vs. 11.5 oocytes collected per female). Laparoscopic ovum pick-up procedure appeared less stressful, less invasive, has a shorter duration (each session takes between 10 and 20 minutes in does and ewes) and can be repeated at short intervals without affecting oocyte developmental competence [5–7,23,24].

In goat, meiotic, cleavage, and development (blastocyst) rates were positively correlated with the size of follicles after IVM, IVF, and IVD [25,26]. Consequently, antral follicles from 3 to 5 mm may be preferentially aspirated for subsequent IVP [25]. At the step of recovery of immature oocytes, the integrity of cumulus-oocyte complexes (COC), defined by density of cumulus cells and homogeneity of oocyte cytoplasm are the main morphologic criteria, as cumulus cells play important role during the IVM process [27,28]. To respect the integrity of COC structure and optimize their recovery rate, the material and aspiration conditions used during LOPU are of critical importance [29] (Fig. 1). Depending on laboratories, the needle diameter used varies from 16 to 21 ga, and the vacuum connected to the needle is regulated from 25 to 70 mm Hg for both goats and sheep. Under these conditions, oocyte recovery rates range from 40% to 90%, and the number of harvested structures may reach frequently around 12 to 13 oocytes per female in different laboratories [1,5,7,30].

In sheep, Rodriguez, et al. [29] compared different aspiration devices and flow rates for aspiration. With the increase of aspiration flow rate, the proportion of good quality oocytes decreased dramatically (69.5%–28.3% with aspiration flows of 10 and 50 mL/min respectively; $P < 0.05$). Thin and intermediate tubings were more effective as laminar flow during aspiration of COC within tubulure to prevent damages on COC. Using slaughterhouse ovaries, follicle size did not affect recovery rate, but proportion of good quality oocytes was higher for large (78%) and medium (64%) follicles ($P < 0.05$). They observed that 18-ga needle promoted a significant better oocyte recovery rate than 20-ga needle, whereas no influence was noted in oocyte quality after aspiration [29].

The aspiration device is also important to respect female tract integrity. It has been shown that repeated ovum pick-up does not affect fertility of donor sheep, even when repeated up to 20 times [6]. It is noteworthy that after repeated LOPU in sheep no complication such as adhesions

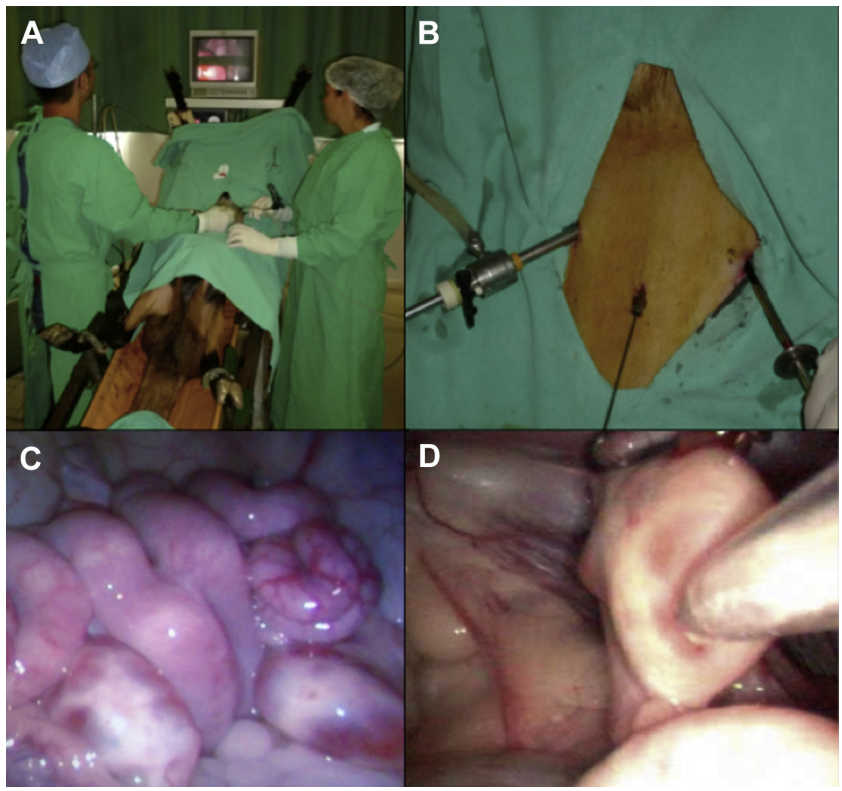

Fig. 1. Different stages of LOPU in small ruminants. Overview of operators, animal, and equipment (A), detail of puncture points in the abdomen (B); initial view of the abdominal cavity and reproductive tract (C) and puncture in a follicle with specific needle. LOPU, laparoscopic ovum pick-up.

and fibroses occurred and normal histology of ovaries were observed [24], confirming that laparoscopy is a minimally invasive procedure. Besides that, the repetition of ovum collections did not cause painful discomfort to the animals, which can be detrimental to animal welfare [24].

2.1. Influence of ovarian status and stimulation

In small ruminants, emergence of large antral follicle occurs in different waves during reproductive cycle, two to four in goats [31] and two to three waves in sheep [32]. Selection of ovulatory follicle(s) may occur during the last wave, whereas other follicles become atretic [31]. Competent oocytes may be recovered from these follicles for IVP before atresia occurs. As oocyte developmental competence increases with follicular size, a strategy for improving number of embryos per females relies on decreasing selection pressure during folliculogenesis for maximizing number of large antral follicles.

Therefore, smaller follicles present on the ovary at the end of progestagen treatment may be stimulated by administration of purified exogenous gonadotropin such as FSH or eCG, increasing follicle survival [24,33]. This strategy must be used at precise moments during the emergence of follicular waves, when follicle survival depends on FSH. Two main strategies were assessed in small ruminants. The first one concerned the administration of gonadotropin before follicular aspiration by LOPU at random time of the reproductive cycle [6]. However, this appeared unsatisfying regarding prevention of atresia [34], as this event may have already occurred for a follicular population. The second and most used nowadays represents the association of progestagen treatment to gonadotropin (Fig. 2). Its steady and continuous release is ensured by the administration of intravaginal sponges impregnated with progestagen and/or progesterone (fluorogestone, medroxiprogesterone acetate or controlled internal drug release) which inhibit the endogenous secretion of LH, allowing regression of dominant follicle(s) by atresia. Different attempts of hormonal stimulation have been evaluated to improve the quantity and quality of oocytes from does submitted to LOPU [35].

Gonzalez-Bulnes, et al. [36] demonstrated that in comparison with ewes that received just PGF2α for estrus synchronization, ovulatory follicles from progestagen-treated ewes showed deficiencies in different phases. First, in the secretion of estradiol during the preovulatory phase; and second, in the ability to ovulate an oocyte able to be fertilized and develop into a viable embryo; and third, in the secretion of progesterone by the subsequent CL. Likewise, Berlinguer, et al. [37] demonstrated that ewes treated with FSH after estrus synchronization without progestagen (just PGF2α) produced a higher oocyte recovery rate, and oocytes with higher competence to cleave after IVF and to develop to the blastocyst stage. Although it is important to associate estrus synchronization to stimulatory treatments, more studies should be performed to verify the real benefits of progestagen in these procedures.

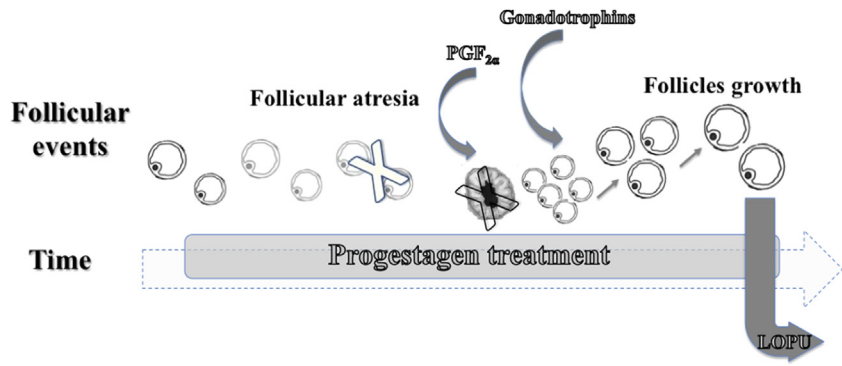

Fig. 2. Diagrammatic representation of protocols used to synchronize estrus and to stimulate healthy estrogenic follicle growth. Prostaglandin (PGF2α) is administered normally just before the end of progestagen treatment. At progestagen removal, laparoscopic ovum pick-up (LOPU) is performed in goats or sheep. Adapted from Reby, et al. [141].

It is well described that FSH treatment before LOPU increases the number of ovarian follicles and oocytes collected from each female [6,7,38]. Morton, et al. [7] reported similar IVD rates in ewes submitted to treatment with or without FSH before LOPU; although, these results were obtained in relation to the cleaved oocytes. In fact, although not statistically different, when we take into account ewes that received FSH (∼59%) they had a lower cleavage rate than those not treated (∼79%) [7]. Similarly in another study, a lower cleavage rate was obtained in FSH-stimulated does rather than in the control group, but this difference was balanced by the numerically higher developmental rates to the blastocyst stage in the FSH-treated group [38]. In our laboratory, we observed that does receiving high dose of ovine FSH (1 IU) had greater proportion of zygotes reaching the blastocyst stage than goats treated with half dose [39]. These data suggest that FSH may enhance the amount of oocytes recovered per doe, however, some reports indicate an adversely effect in further cleavage rate although it does not affect development rates. Perhaps, either the positive or negative effect of FSH may be in relation to its plan of administration. Protocols use decreasing doses of FSH or a constant dose every 12 or 24 hours [35]. In sheep, Baldassarre, et al. [23] observed no difference in the IVD of oocytes collected from females treated with different FSH administration regimes. Conversely, the use of a stimulation protocol for goats with a higher number of FSH injections (five vs. three) resulted in G1/G2 COCs with higher epidermal growth factor receptor expression in cumulus cells, which is correlated with an elevated meiotic competence after IVM [40]. Abdullah, et al. [41] reported an increase in IVP efficiency when ovarian stimulation started 72 hours before LOPU when compared with 36 or 60 hours. Furthermore, an increase in blastocyst rate after IVP and cryotolerance to vitrification were reported when FSH was administered in constant versus decreasing doses before LOPU [42]. Therefore, purity and dose of gonadotropin, the stimulation regimen used and timing of administration are of particular importance, affecting not only follicles and/or oocytes yield but also oocyte intrinsic quality.

The use of GnRH antagonist before the stimulatory treatment has been proposed to synchronize emergence of follicular waves regarding ovarian stimulation and thus maximizing the responses to exogenous FSH by reducing the effect of dominant follicles in MOET programs [43]. When applied to small ruminants, the antagonist treatment overcomes the problem of nonresponding females. However, terminal follicular growth was impaired in goats, and the treatment beneficial effect on the ovulation rate was negated by an increase in the proportion of unfertilized ova and degenerated embryos. Using such treatment, it is likely that high response observed (≥28 CL per goat) may also have impaired female's physiology, especially regarding maternal environment or oocyte developmental competence. When this strategy was applied for LOPU IVP, it enhanced follicular stimulation but also affected the oocyte competence to develop after IVF and IVD [44] indicating that oocyte itself was affected by the treatment.

2.2. *Influence of age and season*

Some studies focused on the effect of donor age on oocyte yield and developmental competence in small ruminants. The use of prepubertal animals is a real possibility for embryo IVP, allowing to reduce the interval between generations and to accelerate the processes of genetic improvement in selection schemes [45]. The effect of the goat donors age on oocyte quality, and the effect of follicle and oocyte size were reviewed [17]. Generally, prepubertal animals are good responders to hormonal stimulation with exogenous gonadotropin. It has been shown in various ruminant species that oocytes collected from prepubertal animals are less competent for development than those collected from adult females (cattle, [46]; sheep, [20]; and goat, [20,47]). Moreover, ultrastructural and functional deficiencies were reported in prepubertal goat oocytes [17] and lower embryo cryotolerance [47]. On the other hand, it has been recently shown that the developmental competence of oocytes obtained from large follicles (≥3 mm) were equally competent between nontreated prepubertal and adult goats [48]. These results may suggest that oocytes of good quality may be recovered from prepubertal animals in a physiological hormonal environment and that ovarian stimulation in prepubertal animals may require further adjustment. With a decrease of ovarian reserve and impaired hormonal environment, aged females may be

associated with poor reproductive performance. In goat, LOPU or IVP has shown to be an efficient strategy for the reproductive rescue of aged goats of high genetic value [9]. Berlinguer, et al. [49] reported that reproductive aging in sheep is associated with impaired follicle functionality and an increase in the proportion of oocytes showing morphologic abnormalities. Total oocyte yield was not affected by age, but the amount of oocyte suitable for IVP was decreased (7%). Despite these observations, the developmental competence of oocytes after IVP and embryo cryotolerance was not affected by aging process.

In addition to the age of the females, their physiological status (follicular or luteal phase, season, and nutrition) could also influence the quality of LOPU oocytes. In goats, the number of aspirated follicles was similar, whereas the proportion of good quality COC (grade I/II) was enhanced in the breeding season (unpublished data). However, interestingly, the cleavage rate of LOPU oocytes was higher in the anestrous season and blastocyst development rate was also higher in the same period when using slaughterhouse oocytes (unpublished data). In sheep, the season influenced the number and competence of recovered oocytes, but cleavage rate tended to be higher in the anestrous season [50]. Conversely, it was recently reported in sheep that the cleavage rate was not affected, whereas the blastocyst rate was higher during the breeding season in comparison with the anestrous season [51]. The oocyte developmental competence in buffalo was also shown to be affected by different seasons because a higher cleavage and embryo yields were recorded in autumn compared with spring [52]. A subcutaneous implant of melatonin improved sheep oocyte developmental competence during the anestrous season [50]. It was demonstrated earlier that LOPU could be performed at different seasons with little or no important change in overall response [53]. It is likely that the progestagen exposure and ovarian stimulation may be sufficient to overcome a possible seasonal effect in small ruminants.

3. *In vitro* maturation

As earlier mentioned, during folliculogenesis, the oocyte undergoes cytoplasmic changes (ultrastructure, organites, RNA, and protein storage) leading to the acquisition of meiotic competence and finally to developmental competence [54]. During this step, the oocyte is maintained at meiotic prophase stage (germinal vesicle) by follicular environment, impairing chromosomes condensation, and thus allowing the continuation of transcription activities, important for its final developmental competence [55]. As the follicular inhibition is stopped by LH surge (ovulation) or by removing oocyte from its follicle (oocyte collection), maturation occurs. Oocyte maturation includes meiotic resumption and progression to the fertilizable stage of metaphase II after emission of the first polar body (transition from oocyte I to II), and related events in oocyte cytoplasm and surrounding cumulus cells [55]. Therefore, the result of IVM depends on the intrinsic quality of immature oocytes, but also the maturation conditions can widely modulate the final competence of IVM oocytes [56]. It is clear that the whole process of oocyte differentiation and maturation is coordinated by surrounding somatic cells (granulosa, cumulus) through a constant dialogue mediated by follicular fluid (FF) and through the network of gap junctions maintained between these compartments [18,57]. It was well demonstrated that IVM oocytes are compromised in their developmental capacity compared with those matured *in vivo* [4,58,59]. The lower potential of IVM oocytes is probably related to the heterogeneity of the oocytes obtained, in terms of differentiation status, and to inappropriate conditions used in IVM. This results in a relatively low rate of oocytes finally reaching the blastocyst stage, which is one of the main limitations of IVP from immature oocytes in mammals. Although many studies aim at searching for molecular biomarkers of oocyte quality in follicular cells [60] or in oocyte itself [61], the morphologic evaluation of COC (cumulus cells number and appearance, homogeneity of oocyte cytoplasm) remains the only noninvasive way to select homogenous COC population after collection [62,63]. Nonetheless, the assessment of the COC morphology before IVM has proved to be a poor predictor of oocyte developmental competence [40], resulting in a great variability in the production of embryos. Brilliant cresyl blue (BCB) staining may represent a good complement to simple morphologic evaluation of COC. The BCB staining reflects activity of glucose-6-phosphate dehydrogenase, an enzyme synthesized in growing oocytes but with decreased activity in oocytes that have finished their growth phase [64]. This staining allows for the selection of two populations of COC with different competences, BCB^+ oocytes being more competent than BCB^-. It has been shown that BCB staining also reflects mitochondrial activity [61] and apoptosis regulator genes expression in oocytes [64]. The BCB^+ oocytes that develop to the blastocyst stage have a higher number of cells compared with the BCB^-oocytes. However, the results presented in the available literature on the utility of the test are not always conclusive [61,64].

In vitro maturation is commonly performed using tissue culture media enriched with amino acids and glucose, supplemented with hormones and heat-inactivated serum (Table 1). In general, the most commonly used system for small ruminants is tissue culture medium (TCM) 199 medium supplemented with FSH (porcine or ovine origin), LH, estradiol, and 10% fetal calf serum (FCS) [1,65]. Our group used FF from nonatretic and large follicles as an IVM medium supplement (10%) for some years with good results [1,4]. However, all complex supplements such as FCS, estrous goat or sheep serum or FF lead to a lack of reproducibility, because there is high biochemical variability among sources or among batches from the same source [17] and exposure to sanitary risks. For these reasons, there has been a trend to use more defined maturation media. Our laboratory, to make IVM simpler, more safe, and repeatable, proposed a maturation medium using just defined compounds—TCM 199 supplemented with 10 ng/mL epidermal growth factor (EGF) and 100 μM cysteamine—and obtained good results in embryo development of adult goat oocytes [16,66]. Indeed, the EGF family of growth factors has been pointed out as a possible intrafollicular mediator of the LH preovulatory surge in rodent species [67] and the addition of EGF to the IVM

Table 1
Recent results of laparoscopic ovum pick-up (LOPU) or slaughterhouse oocytes recovered and submitted to IVM, IVF, and *in vitro* development (IVD) in goats and sheep.

Species	Oocyte origin	Oocyte recovery rate (COC, n)	IVM		IVF		IVD	MII	Cleavage	Blastocyst rate		Embryo	Authors (y)
			Media[a]	Duration (h)	Media[a]	Duration (h)	media[a]	rate[b]	rate[c]	From cleaved	From oocytes	destination	
Goat	LOPU	88 (NI, ~160)	10% EGS	26	20% EGS	18–22	—	NI	51	NI	7	Cell staining	Koeman, et al. (2003) [111]
Goat	LOPU	81 (658)	10% FCS	24–26	10% ESS	NI	—	NI	82	50	NI	Embryo transfer	Cox and Alfaro (2007) [30]
Goat	LOPU	NI (347)	10% EGS	26	10% EGS	24	—	85	55	34	18	Vitrification	Leoni, et al. (2009) [47]
Goat	Slaughterhouse	NI (1231)	—	22–24	10% FCS	18–20	—	NI	53–61	24–25	12–16	Cell staining	Bormann, et al. (2003) [72]
Goat	Slaughterhouse	NI (422)	—	24	10% ESS	18	10% FCS	NI	83	23	28	Embryo transfer	Rodriguez-Dorta, et al. (2007) [16]
Goat	Slaughterhouse	NI (490)	5% to 15% FF	27	—	6	—	54–68	NI	NI	0–10	NI	Masudul Hoque, et al. (2012) [112]
Goat	Slaughterhouse	NI (1447)	—	22	10% ESS	20	10% FCS	NI	64–72	59–75	38–54	NI	Souza, et al. (2013) [66]
Sheep	LOPU	80 (415)	10% FCS	24	20% ESS	20	5% FCS	NI	72–76	39–42	28–32	Embryo transfer	Ptak, et al. (1999) [20]
Sheep	LOPU	83 (142)	10% FCS	24	2% ESS	20	—	74	27	37	10	NI	Berlinguer, et al. (2007) [37]
Sheep	LOPU	85 (512)	10% FCS	24–26	10% ESS	NI	—	NI	93	62	NI	Embryo transfer	Cox and Alfaro (2007) [30]
Sheep	Slaughterhouse	NI (292)	10% FCS	24	20% ESS	18	—	75	73	26	19	NI	Wang, et al. (2012) [86]
Sheep	Slaughterhouse	NI (482)	10% FCS	24	20% ESS	22	10% FCS	NI	79–82	39–40	32	Vitrification	Shirazi, et al. (2012) [113]
Sheep	Slaughterhouse	NI (5035)	—	24	20% ESS	20	—	NI	79–84	38–52	30–42	Embryo transfer	Mara, et al. (2013) [51]

These values obtained above are related only to adult females.
Abbreviations: COC, cumulus-oocyte complex; EGS, estrus goat serum; ESS, estrus sheep serum; FCS, fetal calf serum; FF, follicular fluid; IVD, *in vitro* development; LOPU, laparoscopic ovum pick-up; MII, metaphase II; NI, not informed.
[a] Only biological fluids supplements are indicated; see cited articles for more details.
[b] MII rate represents the rate of oocytes reaching nuclear maturation.
[c] Cleavage rate represents the number of cleaved oocytes in relation to the number of oocytes entering to maturation.

medium has been shown to improve matured oocyte quality in several species [62,68]. Cysteamine is a stable permeating precursor of glutathione (GSH) metabolism (Fig. 3) which increases the reduced GSH content of matured oocytes, improving their post-IVF viability by facilitating sperm nucleus decondensation and pronuclei formation [69]. The effects of cysteamine, cystine, and cumulus cells on the GSH synthesis were intensively evaluated, and the highest blastocyst rate was achieved only at 100 μM cysteamine or 200 μM cystine, with blastocyst rates decreasing at higher concentrations. When both were added, the blastocyst rate was further increased for goat COC. Interestingly, the authors reported that the improvement of denuded oocytes required for the supplementation of both cystine and cysteamine [28]. Also, significant amounts of cysteamine have been detected in the FF of the cow, sow, goat, and dog [70]. These simplified maturation conditions (TCM 199, EGF, cysteamine) provide good and reliable results in terms of cleavage and development rate [66,69] for slaughterhouse-derived COC. However, recent experiments performed in our laboratory observed that LOPU-derived COC may have different requirements during IVM and more complex media would be necessary to achieve high IVP rates (article accepted for publication).

The alteration of basic maturation conditions can significantly affect oocyte competence as reflected by the morula and blastocyst yield after IVF [71]. The use of vitamins in IVM also promoted beneficial effects in overall blastocyst development, cleavage rate, and in the mean number of blastocyst cells [72]. It was shown [71] that cleavage rate and developmental capacity of cleaved ovine oocytes matured in undefined medium (FCS) were higher than those matured in semidefined (BSA) or defined medium (polyvinyl alcohol). This result indicates that serum may have an additional effect beyond EGF, insulin-like growth factor 1 (IGF-I), and cysteamine leading to a better completion of cytoplasmic maturation in oocytes. Serum is a highly complex combination of components, including proteins, fatty acids, vitamins, trace elements, hormones, and growth factors [73]. The same group demonstrated later that the maturation media containing FCS supplemented with human menopausal serum, estrous sheep serum, or estrous goat serum supported better rates of IVM, IVF, and embryo development than bovine or ovine FF [74]. Also in the presence of FCS, the supplementation with GH or FSH reached similar blastocyst rates, but a lower rate was obtained when both hormones were concomitantly used [75]. Different interactions may exist among the substances, and more studies are needed to establish which molecules or combinations to optimize this step [76]. Regarding IVM physical conditions, the literature does not vary considerably, and IVM is usually performed incubating COC in large groups (50–70) in four-well plates with 500 μL of medium, under 5% CO_2 in air at 38 °C to 39 °C with maximum humidity for 22 to 27 hours [16,30,66].

Another way to improve the whole success of IVP would be to increase the intrinsic quality of the oocytes before IVM by allowing them to complete *in vitro* the late differentiation process that occurs into the growing follicle *in vivo* [56]. This prematuration step would require mimicking *in vitro* the meiotic inhibition signal provided by

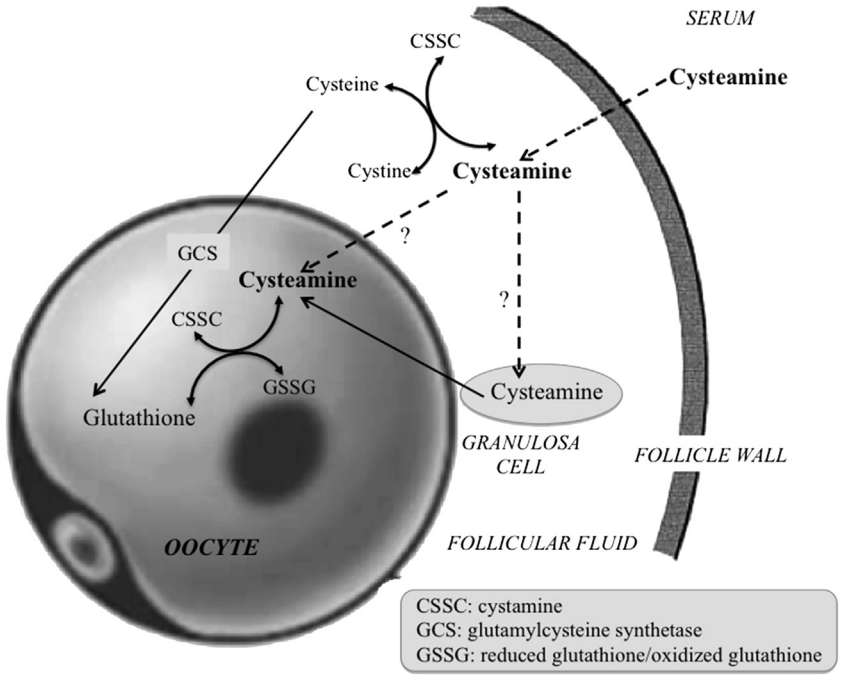

Fig. 3. Metabolism of cysteamine in the oocyte and its environment. Cysteine readily autoxidizes to cystine in the extracellular fluid; once it enters the cell, cystine is rapidly reduced to cysteine. Inside the cell, the majority of cysteine is incorporated into glutathione. Adapted from Guérin, et al. [142].

the follicle, to allow the oocyte to continue his transcription and translation activities at the germinal vesicle stage. The key element of meiotic resumption control cascade in the mammalian oocyte is the M-phase-promoting factor (MPF) kinase, which can induce chromosome condensation, germinal vesicle breakdown, and spindle formation by phosphorylating specific target proteins [55]. Several chemical methods aimed at inhibiting MPF activation [77] or MPF kinase activity [78] have been proposed. These methods provided an efficient meiotic block *in vitro* but did not allow positive progression of treated oocytes toward improved competence, because several MPF-independent pathways related to maturation were still activated [79]. More recently, some interesting results have been obtained in cattle by targeting upstream of MPF activation, such as phosphodiesterase 3, the enzyme which regulates cAMP level in cumulus cells, and in turn MPF activation in the oocyte [80]. This more physiological inhibition process is able to increase final oocyte intrinsic quality before gonadotropin addition allowing meiotic resumption. In addition, the same group showed that oocyte secreted factors could be involved in an autocrine–paracrine manner in the regulation of oocyte quality [81]. Such oocyte-secreted factors have been shown to increase the developmental competence of prepubertal goat oocytes [82]. These interesting findings may open the way for new improvement of IVM success in small ruminants.

4. *In vitro* fertilization

The IVF technique should be able to produce embryos using both female and male gametes. Studies of sperm capacitation for IVF purposes have provided evidence that male variability, the origin of sperm (ejaculated or cauda epididymal, fresh or frozen-thawed), and the presence of seminal plasma play crucial roles in embryo IVP in cattle [58]. Either fresh or frozen-thawed semen can be used to fertilize matured oocytes. In any case, it is essential to assess live or dead sperm cells. IVF rates after Percoll or swim-up separation of motile spermatozoa did not differ significantly, regarding the rates of single pronucleus formation, normal fertilization with two pronuclei, and polyspermic fertilization in goats [59].

It has been suggested that procedures used in bovine IVP can be applied in small ruminants after minimal modifications, mainly during sperm capacitation and IVF, such as the reduction in the centrifugation speed and the addition of sheep serum in IVF medium for spermatozoa capacitation [30]. Capacitation is a crucial process that mammalian sperms require to achieve fertilizing ability, and the importance of capacitating agents for the success of IVF has been already described in many species [59]. Heparin has been shown to increase fertilization rate in cattle [83], and is thus widely used for spermatozoa capacitation in this species. Similarly, there are some reports confirming the role of heparin in goats and sheep [30,38]. However, some studies indicate an adverse effect on fertilization [84] or reduced embryo development and quality [85]. Interestingly, an alternative effect of heparin occurred when using goat fresh or frozen-thawed sperm, probably because of a capacitating action of sperm freezing [38]. We have recently demonstrated that greater numbers of blastocysts were obtained from COC fertilized in the presence of 5 μg/mL heparin than those without heparin, suggesting that the addition of heparin to the fertilization medium improves sperm capacitation of frozen-thawed

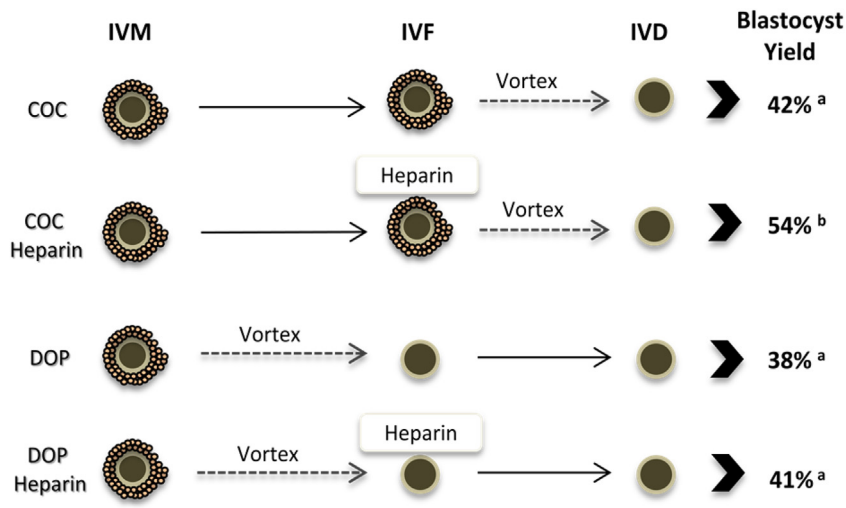

Fig. 4. Effect of culturing goat oocytes during IVF as intact cumulus-oocyte complexes (COCs) or denuding them before IVF (DOP), and the absence (−) or presence (+) of heparin in the IVF medium on the blastocyst production at 8 days postinsemination of goat embryos [66]. IVD, *in vitro* development.

goat sperm [66] (Fig. 4). The treatment of sperm with ionomycin has also been shown to increase the fertilization rate in goats [97].

Regarding IVF conditions for small ruminants, sperm concentrations vary from one to 3.5 × 10^6 cells/mL (depending on the male and IVF system) with sperm and oocytes coincubated for 16 to 20 hours at 38 °C to 39 °C in humidified atmosphere of 5% CO_2 in air [5,30,38]. In our laboratory, we routinely use a pool of two or three straws from different males [15], because this may increase the repeatability of IVF results (unpublished observations). In prepubertal goat oocytes, it was possible to achieve elevated sperm penetration with 4 × 10^6 cells/mL without increasing polyspermy [87], which is a major concern when using a high sperm concentration. In sheep and goats, polyspermy is the main abnormality detected after IVF, affecting almost 20% of the inseminated oocytes, much higher than what is observed *in vivo* [88]. Some recent sheep IVF data show that sex-sorted spermatozoa elicit equal or greater cleavage and blastocyst rates than nonsorted spermatozoa [89]. Despite its interesting applicability, there are no reports related to the use of sexed spermatozoa in goat IVF.

A high variation is observed between bucks or rams and also between ejaculates from the same animal in terms of fertilizing ability *in vitro* and embryo development [90]. Currently, in most IVP laboratories, male selection is based on their capacity to fertilize oocytes in previously established IVF conditions. This practice could make it easier to solve the problem and maintain IVP. However, genetic IVP requires the use of a specific male, which is generally of unknown *in vitro* fertility. To make this technique more applicable in the field, these variations should be better understood and IVF conditions optimized for each specific male of interest. This optimization should be based on the number of spermatozoa used and/or heparin concentration. A capacitation treatment before insemination could be tried for lower fertility males.

The appropriate time of cumulus cell removal from oocytes during IVP also remained controversial. Evaluating recent studies, some reports describe that oocytes are denuded immediately after IVM [7,16,50,63], just after IVF [30,48] or even partially denuded after IVM with cumulus-free presumptive zygotes denuded after IVF [74]. Without direct comparison of these two procedures, it is difficult to precisely analyze their respective interests. In our laboratory, cumulus oophorus was usually removed after maturation, before IVF [4]. Nonetheless, we recently demonstrated the importance of cumulus cells during the IVF process [66] and currently, goat COCs are denuded just after IVF (Fig. 4). This parameter may also be important while setting up conditions adapted to specific males, with the presence of cumulus cells being able to regulate the sperm capacitation process.

5. *In vitro* development and embryo quality

After IVF, the presumptive zygotes are removed from the fertilization medium, and placed in an embryo culture medium that allows the development up to a stage that is compatible with its transfer to the recipient uterus. It has been clearly demonstrated in cattle that although the rate of success of IVP in terms of blastocysts yield relies on the oocyte's intrinsic quality and maturation conditions, the quality of the resulting blastocysts (cryosurvival, viability) relies on the conditions encountered during earlier steps of development [91]. It was demonstrated that bovine *in vitro*–derived embryos show certain differences when compared with embryos produced *in vivo* in relation to their morphology, timing of development, resistance to low temperature [92], embryo metabolism [93], and especially gene expression [94]. One of the most dramatic effect of *in vitro* environment is the modification of embryo lipid metabolism leading to increased storage of triglycerides and decreased phospholipid production [95], translating into altered membrane fluidity, and in turn, lower cryoresistance. *In vitro* conditions may also induce sex-ratio deviation, because of metabolic advantage of male embryos in *in vitro* environment [96].

Although different culture media have been successfully used for small ruminant embryo development such as TCM 199 [97,98], Menezo B2 [38,63], potassium simplex optimized medium [99], Sydney IVF blastocyst medium [7,8], the most widely used medium is the synthetic oviduct fluid (SOF). First developed for sheep embryos [100], the SOF medium has then been used and adapted for other species like cattle [101], pig [102], and goat [1]. Sequential media (G1.2/G2.2, CR 1 aa), more adapted to changing embryo requirements have also been successfully used for small ruminants [28,103]. To ensure embryo confinement, which facilitates the action of autocrine factors known to stimulate early development, embryos are usually cultured in mineral oil overlaid droplets of medium (1 μL per embryo) [17]. This confinement improves the development to the blastocyst stage [104]. This is particularly important when working with LOPU, because a high variation in female response can be observed, and in any case, the number of embryos per female remains low.

Embryo development media are usually supplemented with various protein sources (BSA, FCS, growth factors), although these supplements may be associated to "large offspring syndrome" in several species, including small ruminants [105]. Indeed, serum can induce morphologic and physiological differences in embryos [74]. Therefore, a chemically defined medium for culturing IVP embryos may allow to overcome some inconvenient of the use of complex biological additives such as serum (sanitary risks, batch effect, large offspring syndrome, and embryo metabolic defects), but results still consist in lower development. Consequently, while setting up IVP conditions, it is important to evaluate the quality of the embryos produced in addition to the final rate of development. It is also important to keep in mind that early embryos, as any eukaryotic cells, have active securities to recognize genome abnormalities and consequently block the cell cycle progression, and drive the affected cell to apoptosis [106]. Therefore, selecting fast-growing embryos or accelerating development kinetics by modifying culture conditions may favor embryos, which lost their protections against genetic abnormalities and finally lead to the transfer of genetically defected embryos.

The best way to assess embryo quality or viability is to check their capacity of establishing pregnancy after transfer to recipients and consequently give birth to normal offspring. However, as embryo transfers are costly in domestic species, various methods have been proposed to approximate embryo quality in laboratory experiments. Some reliable indicators of embryo viability are the evaluation of the level of expression of specific gene sets, embryo metabolism, kinetic of development, and the resistance to cryopreservation [18]. The lower quality of IVP embryos is probably because of inappropriate culture conditions compared with natural maternal environment. Early development taking place in the oviduct, many studies have been aimed at understanding the physiology of this organ [107], and to mimic *in vitro* its beneficial effect on embryo quality. Indeed, developing IVP embryos in oviducts of a transient recipient allows reaching the same level of quality than *in vivo*–derived embryos [45]. This recipient female could be from another species; ewes or rabbit does being the most frequently used, although mouse oviducts could also provide valuable results [108]. However, because this methodology is laborious and requires the presence of transient recipients and two surgeries, efforts have been done to reproduce this maternal environment in culture.

The first successes of ruminant embryo development up to the blastocyst stage *in vitro* have been obtained by using coculture systems involving oviduct epithelial cells [109]. Further evolution of this technique involved the use of new media especially designed to support early embryo development based on the composition of oviduct fluid, such as SOF medium [110]. Rodríguez-Dorta, et al. [16] used the SOF medium on goat oviduct epithelial cells (GOEC) monolayers for coculture of goat IVP embryos to evaluate the effect of cells on embryo survival after vitrification. Although the rate of zygotes reaching the blastocyst stage was significantly higher in SOF (28%) as compared with GOEC (20%), after direct transfer of vitrified-thawed embryos, kidding rate and embryo survival rate were significantly higher in GOEC coculture (Table 2).

The oviducts used to generate GOEC primary cultures come usually from slaughtered animals, and because of heterogenous cell origin, they can introduce some variability. Therefore, further research using more reliable GOEC coculture or purified GOEC factors is still required in view to enhance the success rate of IVP in goats. Goat IVM/IVF oocytes were successfully cocultured with GOEC that were simultaneously collected at the same moment as immature oocytes [38]. However, in cases when LOPU is applied, there is no opportunity to collect oviduct epithelial cells. An interesting possibility would be to use oviducts from other species (cattle), easier to obtain and providing high number of cells. Indeed, the positive effect of OEC is not species specific [114], and we obtained similar results with BOEC (bovine cells) than those described for GOEC (goat cells), in terms of development rate and quality (article in preparation). Surprisingly, porcine cells were even more effective in supporting bovine embryos development than with bovine-derived cells [115]. The mechanisms of the beneficial effects of coculture are not well understood. Oviduct hosts the first events of reproduction, such as oocyte maturation, selection, transport, and storage of sperm, fertilization of the ovum, early embryonic development [116]. These activities may rely on the production of embryotrophic factors by oviduct cells, the depletion of potentially harmful substances, the modification of the medium components to better fit embryo requirements or even the modification of physico-chemical environment parameters such as pH or oxygen concentration [117]. In addition, the oviduct may act on early reproductive processes through a combination of these mechanisms.

Regarding embryo quality, as described previously, COC morphology evaluation before IVM is not sufficient as a good predictor of oocyte intrinsic meiotic competence [40]. Han, et al. [26] showed that developmental competence of goat oocytes with the same COC morphology, follicle size, and grade of cumulus expansion may be different. This strongly indicates that the developmental potential of an oocyte is determined by multifactorial interactions. Researchers have begun to study the relationship between oocyte quality and their competence to develop up to blastocyst after IVF. Epidermal growth factor receptor may

Table 2
Success rate after direct transfer of goat IVP embryos cultured in different development systems.

Treatment	Recipients (n)	Embryos (n)	Pregnant goats at		Kidding	Kids born
			Day 34, n (%)	Day 90, n (%)	n (%)	n (%)
SOF fresh	13	26	12[a] (92)	12[a] (92)	12[a] (92)	16[a] (62)
SOF vitrified	29	58	6[b] (21)	4[b] (14)	4[b] (14)	5[b] (9)
GOEC vitrified	18	36	13[a] (72)	10[c] (56)	10[c] (56)	12[c] (33)

[a,b,c] Values with different superscripts in the same column are significantly different ($P < 0.05$).
Abbreviations: GOEC, goat oviduct epithelial cells; SOF, synthetic oviduct fluid. Adapted from Rodriguez-Dorta, et al. [20].

indeed be a good candidate marker for indirect prediction of goat oocyte quality [40]. Nonetheless, fewer and less conclusive results have been obtained about the relationship between oocyte and blastocyst quality. It was suggested that the conditions of embryo culture have a crucial role in determining blastocyst quality [48,118].

The investigation of molecular markers using highly sensitive techniques, such as microarray and real-time polymerase chain reaction, is a promising approach for studies in COC gene expression for a better inference of oocyte competence to development [57,119]. Successful embryonic development is dependent on a rigorous, time- and site-specific gene expression program of appropriate genes. Identification of these differentially expressed genes and the analysis of their pattern of expression are powerful tools to gain information about functions relevant to processes such as the oocyte competence. These biochemical markers for embryo development would be able to improve pregnancy by optimizing oocyte and embryo selection. The differentially expressed genes may be important markers of the oocyte's ability to reach the blastocyst stage, and allow direct assessment of the fertility potential of an individual oocyte without compromising its integrity [57]. A first class of genes is required for normal folliculogenesis and fertilization. Other genes are maternal effect genes, i.e., they are dispensable until fertilization but essential for proper embryo development. Besides those, there is some functional importance of other oocyte-enriched genes that remains to be assessed [120]. Moreover, some important research could be done to evaluate the genic expression profile of LOPU or slaughterhouse-derived oocytes.

6. Related technologies

6.1. Cloning and transgenesis

Some biotechnologies associated with genetic engineering, such as transgenesis and cloning, are strongly related to the IVP methods. Additionally, small ruminants are presented as an excellent model for these techniques. Tremendous interest in cloning has been generated in the recent years. It is the asexual production of genetically identical animals that can be obtained by nuclear transfer (NT). The NT involves the transfer of nuclei from serum-starved fetal or adult cells into enucleated oocytes matured *in vivo* or *in vitro*. Fetal fibroblast cell or a variety of adult cells can be used. Since the birth of "Dolly" [121], a ewe derived from the transfer of an adult somatic cell to an enucleated oocyte, research on cloning of somatic cells has gained force. In goats, several reports have been published about the birth of clones, most of these reports pertaining to the production of genetically modified animals [122,123].

A transgenic animal may be defined as one containing recombinant DNA molecules in its genome that were introduced by genetic engineering manipulation. Transgenic small ruminants are mainly produced by two techniques: NT [122,123] and pronuclear microinjection of DNA [124]. Recently, however, Pereyra-Bonnet, et al. [125] obtained transgenic sheep embryos using the sperm-mediated gene transfer technique. After the birth of first transgenic mammal [126], transgenic sheep were obtained few years after that [127]. On the other hand, goat is particularly an efficient system of producing recombinant proteins as they produce considerable amounts of milk and incur lower investment and maintenance costs than cows. Currently, the human antithrombin III produced by transgenic goats is the unique recombinant protein from animal bioreactor that was approved for clinical use in Europe [128], and in the United States [129]. Other examples of human recombinant proteins expressed in the milk of transgenic goats are alpha-1-antitrypsin, blood clotting factor IX, granulocyte colony-stimulating factor, growth hormone, prolactin, and tissue plasminogen activator [130]. Thus, the transgenic technology was validated using a goat model as a viable alternative method for the production of recombinant pharmaceutical proteins.

6.2. Manipulation of preantral follicle-enclosed oocytes in goats and sheep

Preantral follicles (PFs) form a far larger oocyte reservoir (90% of all ovarian follicles) compared with the number of antral follicles. However, the majority (99.9%) of preantral and antral follicles become atretic during their growth and maturation. Therefore, recovery of PFs before atresia followed by IVC might increase the availability of fertilizable oocytes, enhance the knowledge of the mechanisms involved in ovarian folliculogenesis, complement other reproductive technologies such as IVP, nuclear transfer, transgenic animal production, embryonic stem cell development, and aid in studies on reproductive toxicology.

Taking into consideration that farm or endangered animals are normally found far away from the laboratory, one extremely important step is the preservation of ovarian tissue during transportation. This step aims to ensure good quality oocytes enclosed in PF for further cryopreservation and/or IVD. Experiments performed by our group demonstrated that the best temperature for the transportation of caprine or ovine PFs enclosed in ovarian tissue is 4 °C independent of the media used. Methods for the isolation of

caprine PFs include mechanical and enzymatic procedures. Although mechanical isolation of PFs is mainly performed using 25-ga needles [131] or a tissue chopper [132], enzymatic isolation is performed by proteolytic digestion, incubating the ovarian tissue with nonspecific enzymes such as collagenase [129] or trypsin [133].

Preantral follicles, isolated or enclosed in the ovarian tissue, can be cryopreserved by applying conventional slow freezing or vitrification methods. Both methods are efficient and possess advantages and disadvantages. Recently, we have shown that ovarian tissue–enclosed PFs from sheep and goats respond differently to the same cryopreservation protocol [134]. Furthermore, caprine PFs present some peculiarities that might influence cryopreservation, such as the absence of concentration of cytoplasmic components in the juxtanuclear region as observed in other species [135]. Recovery of gonadal function and harvesting of mature viable oocytes after autotransplantation of frozen-thawed caprine ovarian tissue are the best results reported so far [136]. In addition, using this procedure, live offspring were produced in sheep [137].

Caprine PFs are usually cultured either in ovarian cortical slices or after isolation. Although IVD of PFs enclosed in cortical slices is practical, non-timeconsuming, maintains three-dimensional follicle architecture, and preserves interactions between follicles and surrounding stroma cells, the cortical tissue may act as a barrier to IVD medium perfusion, resulting in the loss of PFs to apoptosis. In addition, the growth of primordial follicles only until the secondary stage is physically possible as the cortex pieces are unable to support antral follicle development [138]. Conversely, IVD of isolated PFs allows monitoring of individual follicles throughout the growing period, but is time consuming, may be affected by the isolation procedure, demands more sophisticated IVD systems, and is often applied to secondary and not to primordial and primary follicles. A major achievement was the IVD of isolated secondary PFs up to antral stages and subsequent production of mature oocytes and embryos in goats [139] and sheep [140].

7. Conclusions

In vitro embryo production has a great potential for efficient propagation of valuable females after obtaining oocytes generated by LOPU or slaughterhouse ovaries. However, oocyte and embryo physiology need to be better understood to improve the technique and to produce a large number of good quality embryos of high genetic merit for production traits or for biodiversity preservation.

Three different research lines appear promising in this view:

- Based on the comparison of requirements between LOPU and slaughterhouse oocytes and on new meiotic inhibition strategies, it would be possible to set up innovative maturation treatments beneficial to the oocyte's intrinsic quality.
- The fertilization step of IVP should be adapted to be able to use any male for IVP. A systematic check of the most appropriate IVF parameters with slaughterhouse oocytes should be designed and used for any new male.
- A deeper study of oviduct embryo interaction and dialogue will allow identifying the embryotrophic factors produced by the oviduct epithelium. These factors could then be used as supplement of semidefined culture media.

Finally, *in vitro* technologies ignite new hopes for increased accuracy of selection scheme and improved efficiency of genetic gain.

Acknowledgments

J.M.G. Souza-Fabjan was supported by CAPES and Ceará State University. The authors wish to thank CAPES-COFECUB bilateral framework for financial support of collaboration between the Ceará State University and INRA on goat IVP (Grant 728/11, 2011–2013). This work was supported by a grant from Région Center, France (PIVER program, #200800030493, 2008–2011). We thank Michael J. Bertoldo for proof reading the manuscript.

References

[1] Cognié Y, Poulin N, Locatelli Y, Mermillod P. State-of-the-art production, conservation and transfer of *in-vitro*-produced embryos in small ruminants. Reprod Fertil Dev 2004;16:437–45.
[2] Hinrichs K. *In vitro* production of equine embryos: state of the art. Reprod Domest Anim 2010;45:3–8.
[3] Gil MA, Cuello C, Parrilla I, Vazquez JM, Roca J, Martinez EA. Advances in swine *in vitro* embryo production technologies. Reprod Domest Anim 2010;45:40–8.
[4] Cognié Y, Baril G, Poulin N, Mermillod P. Current status of embryo technologies in sheep and goat. Theriogenology 2003;59:171–88.
[5] Baldassarre H, Karatzas CN. Advanced assisted reproduction technologies (ART) in goats. Anim Reprod Sci 2004;82-83:255–66.
[6] Stangl M, Kuhholzer B, Besenfelder U, Brem G. Repeated endoscopic ovum pick-up in sheep. Theriogenology 1999;52:709–16.
[7] Morton KM, de Graaf SP, Campbell A, Tomkins LM, Maxwell WM, Evans G. Repeat ovum pick-up and *in vitro* embryo production from adult ewes with and without FSH treatment. Reprod Domest Anim 2005;40:422–8.
[8] Morton KM. Developmental capabilities of embryos produced *in vitro* from prepubertal lamb oocytes. Reprod Domest Anim 2008;43:137–43.
[9] Baldassarre H, Rao KM, Neveu N, Brochu E, Begin I, Behboodi E, et al. Laparoscopic ovum pick-up followed by *in vitro* embryo production for the reproductive rescue of aged goats of high genetic value. Reprod Fertil Dev 2007;19:612–6.
[10] Baldassarre H, Wang B, Keefer CL, Lazaris A, Karatzas CN. State of the art in the production of transgenic goats. Reprod Fertil Dev 2004;16:465–70.
[11] Souza JMG, Batista RITP, Melo LM, Freitas VJF. Reproductive biotechnologies applied to the conservation of endangered ruminant - past, present and future. Rev Port Ciênc Vet 2011;110:31–8.
[12] Schenk SL. Das Säugetierei Künstlich befruchtet ausserhalb des Muttertieres Mittheilungen aus dem Embryolischen Institute der Kaiserlich-Königlichen Universität in Wien 1878;1:107.
[13] Chang MC. Fertilization of rabbit ova *in vitro*. Nature 1959;184:466–7.
[14] Hanada A. *In vitro* fertilization in goats. Jpn J Anim Reprod 1985;31:21–6.
[15] Crozet N, De Smedt V, Ahmed-Ali M, Sevellec C. Normal development following *in vitro* oocyte maturation and fertilization in the goat. Theriogenology 1993;39:1.
[16] Rodriguez-Dorta N, Cognié Y, Gonzalez F, Poulin N, Guignot F, Touzé JL, et al. Effect of coculture with oviduct epithelial cells on viability after transfer of vitrified *in vitro* produced goat embryos. Theriogenology 2007;68:908–13.
[17] Paramio MT. *In vivo* and *in vitro* embryo production in goats. Small Rumin Res 2010;89:144–8.
[18] Mermillod P, Dalbies-Tran R, Uzbekova S, Thelie A, Traverso JM, Perreau C, et al. Factors affecting oocyte quality: who is driving the follicle? Reprod Domest Anim 2008;43:393–400.

[19] Tibary A, Anouassi A, Khatir H. Update on reproductive biotechnologies in small ruminants and camelids. Theriogenology 2005;64:618–38.
[20] Ptak G, Loi P, Dattena M, Tischner M, Cappai P. Offspring from one-month-old lambs: studies on the developmental capability of prepubertal oocytes. Biol Reprod 1999;61:1568–74.
[21] Viana JH, Almeida Camargo LS, Moraes Ferreira A, Sa WF, Carvalho Fernandes CA, Pinho Marques Junior A. Short intervals between ultrasonographically guided follicle aspiration improve oocyte quality but do not prevent establishment of dominant follicles in the Gir breed (*Bos indicus*) of cattle. Anim Reprod Sci 2004;84:1–12.
[22] Graff KJ, Meintjes M, Dyer VW, Paul JB, Denniston RS, Ziomek C, et al. Transvaginal ultrasound-guided oocyte retrieval following FSH stimulation of domestic goats. Theriogenology 1999;51:1099–119.
[23] Baldassarre H, Furnus CC, De Matos DG, Pessi H. *In vitro* production of sheep embryos using laparoscopic folliculocentesis: alternative gonadotrophin treatments for stimulation of oocyte donors. Theriogenology 1996;45:707–17.
[24] Teixeira PP, Padilha LC, Oliveira ME, Motheo TF, da Silva AS, Barros FF, et al. Laparoscopic ovum collection in sheep: gross and microscopic evaluation of the ovary and influence on oocyte production. Anim Reprod Sci 2011;127:169–75.
[25] Crozet N, Ahmed-Ali M, Dubos MP. Developmental competence of goat oocytes from follicles of different size categories following maturation, fertilization and culture *in vitro*. J Reprod Fertil 1995; 103:293–8.
[26] Han ZB, Lan GC, Wu YG, Han D, Feng WG, Wang JZ, et al. Interactive effects of granulosa cell apoptosis, follicle size, cumulus-oocyte complex morphology, and cumulus expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system. Reproduction 2006;132:749–58.
[27] Gandolfi F, Brevini TAL. *In vitro* maturation of farm animal oocytes: a useful tool for investigating the mechanisms leading to full-term development. Reprod Fertil Dev 2010;22:495–507.
[28] Zhou P, Wu YG, Li Q, Lan GC, Wang G, Gao D, et al. The interactions between cysteamine, cystine and cumulus cells increase the intracellular glutathione level and developmental capacity of goat cumulus-denuded oocytes. Reproduction 2008;135:605–11.
[29] Rodriguez C, Anel L, Alvarez M, Anel E, Boixo JC, Chamorro CA, et al. Ovum pick-up in sheep: a comparison between different aspiration devices for optimal oocyte retrieval. Reprod Domest Anim 2006;41:106–13.
[30] Cox JF, Alfaro V. *In vitro* fertilization and development of OPU derived goat and sheep oocytes. Reprod Domest Anim 2007;42: 83–7.
[31] De Castro T, Rubianes E, Menchaca A, Rivero A. Ovarian dynamics, serum estradiol and progesterone concentrations during the interovulatory interval in goats. Theriogenology 1999;52:399–411.
[32] Evans AC, Duffy P, Hynes N, Boland MP. Waves of follicle development during the estrous cycle in sheep. Theriogenology 2000; 53:699–715.
[33] Gibbons A, Pereyra Bonnet F, Cueto M, Catala M, Salamone D, González-Bulnes A. Procedure for maximizing oocyte harvest for *in vitro* embryo production in small ruminants. Reprod Domest Anim 2007;42:423–6.
[34] Jablonka-Shariff A, Reynolds LP, Redmer DA. Effects of gonadotropin treatment and withdrawal on follicular growth, cell proliferation, and atresia in ewes. Biol Reprod 1996;55:693–702.
[35] Gibbons A, Pereyra Bonnet F, Cueto MI, Salamone D, Catala M. Recovery of sheep and goat oocytes by laparoscopy. Acta Sci Vet 2008;36:223–30.
[36] Gonzalez-Bulnes A, Veiga-Lopez A, Garcia P, Garcia-Garcia RM, Ariznavarreta C, Sanchez MA, et al. Effects of progestagens and prostaglandin analogues on ovarian function and embryo viability in sheep. Theriogenology 2005;63:2523–34.
[37] Berlinguer F, Gonzalez-Bulnes A, Succu S, Leoni G, Mossa F, Bebbere D, et al. Effects of progestagens on follicular growth and oocyte developmental competence in FSH-treated ewes. Domest Anim Endocrinol 2007;32:303–14.
[38] Katska-Ksiazkiewicz L, Rynska B, Gajda B, Smorag Z. Effect of donor stimulation, frozen semen and heparin treatment on the efficiency of *in vitro* embryo production in goats. Theriogenology 2004;62: 576–86.
[39] Locatelli Y, Poulin N, Baril G, Touzé JL, Bouttier A, Cognié Y, et al. FSH treatment before laparoscopic ovum pick-up in goat influences quantity and quality of recovered oocytes. Reprod Fertil Dev 2004;16:513.
[40] Almeida KC, Pereira AF, Alcantara Neto AS, Avelar SR, Bertolini LR, Bertolini M, et al. Real-time qRT-PCR analysis of EGF receptor in cumulus-oocyte complexes recovered by laparoscopy in hormonally treated goats. Zygote 2011;19:127–36.
[41] Abdullah RB, Liow SL, Rahman ANMA, Chan WK, Wan-Khadijah WE, Ng SC. Prolonging the interval from ovarian hyperstimulation to laparoscopic ovum pick-up improves oocyte yield, quality, and developmental competence in goats. Theriogenology 2008;70:765–71.
[42] Berlinguer F, Leoni G, Bogliolo L, Pintus PP, Rosati I, Ledda S, et al. FSH different regimes affect the developmental capacity and cryotolerance of embryos derived from oocytes collected by ovum pick-up in donor sheep. Theriogenology 2004;61:1477–86.
[43] Cognié Y. State of the art in sheep-goat embryo transfer. Theriogenology 1999;51:105–16.
[44] Berlinguer F, Gonzalez-Bulnes A, Succu S, Leoni GG, Veiga-Lopez A, Mossa F, et al. GnRH antagonist enhance follicular growth in FSH-treated sheep but affect developmental competence of oocytes collected by ovum pick-up. Theriogenology 2006;65:1099–109.
[45] Galli C, Crotti G, Notari C, Turini P, Duchi R, Lazzari G. Embryo production by ovum pick up from live donors. Theriogenology 2001;55:1341–57.
[46] Revel F, Mermillod P, Peynot N, Renard JP, Heyman Y. Low developmental capacity of *in vitro* matured and fertilized oocytes from calves compared with that of cows. J Reprod Fertil 1995;103:115–20.
[47] Leoni GG, Succu S, Satta V, Paolo M, Bogliolo L, Bebbere D, et al. *In vitro* production and cryotolerance of prepubertal and adult goat blastocysts obtained from oocytes collected by laparoscopic oocyte-pick-up (LOPU) after FSH treatment. Reprod Fertil Dev 2009;21:901–8.
[48] Romaguera R, Moll X, Morato R, Roura M, Palomo MJ, Catala MG, et al. Prepubertal goat oocytes from large follicles result in similar blastocyst production and embryo ploidy than those from adult goats. Theriogenology 2011;76:1–11.
[49] Berlinguer F, Gonzalez-Bulnes A, Spezzigu A, Contreras-Solis I, Succu S, McNeilly AS, et al. Effect of aging on follicular function may be relieved by exogenous gonadotropin treatment in a sheep model. Reproduction 2012;144:245–55.
[50] Vazquez MI, Abecia JA, Forcada F, Casao A. Effects of exogenous melatonin on *in vivo* embryo viability and oocyte competence of undernourished ewes after weaning during the seasonal anestrus. Theriogenology 2010;74:618–26.
[51] Mara L, Sanna D, Casu S, Dattena M, Mayorga-Muñoz IM. Blastocyst rate of *in vitro* embryo production in sheep is affected by season. Zygote 2013;5:1–6.
[52] Di Francesco S, Boccia L, Campanile G, Di Palo R, Vecchio D, Neglia G, et al. The effect of season on oocyte quality and developmental competence in Italian Mediterranean buffaloes (*Bubalus bubalis*). Anim Reprod Sci 2011;123:48–53.
[53] Pierson J, Wang B, Neveu N, Sneek L, Cote F, Karatzas CN, et al. Effects of repetition, interval between treatments and season on the results from laparoscopic ovum pick-up in goats. Reprod Fertil Dev 2004;16:795–9.
[54] Sirard MA, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. Theriogenology 2006;65:126–36.
[55] Mermillod P, Marchal R. Mammalian oocyte maturation. M S-Medecine Sci 1999;15:148–56.
[56] Mermillod P, Marchal R. *In-vitro* maturation of oocytes from domestic species. Hum Reprod 1999;14:384–5.
[57] Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S, et al. Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate *in vitro*. Biol Reprod 2008;79:209–22.
[58] Smorag Z, Katska-Ksiazkiewicz L, Skrzyszowska M, Jura J, Gajda B, Bochenek M. Animal reproduction biotechnology in Poland. Int J Dev Biol 2008;52:151–5.
[59] Khatun M, Bhuiyan MM, Ahmed JU, Haque A, Rahman MB, Shamsuddin M. *In vitro* maturation and fertilization of prepubertal and pubertal black Bengal goat oocytes. J Vet Sci 2011;12:75–82.
[60] Salhab M, Papillier P, Perreau C, Guyader-Joly C, Dupont J, Mermillod P, et al. Thymosins beta-4 and beta-10 are expressed in bovine ovarian follicles and upregulated in cumulus cells during meiotic maturation. Reprod Fertil Dev 2010;22:1206–21.
[61] Catala MG, Izquierdo D, Uzbekova S, Morato R, Roura M, Romaguera R, et al. Brilliant Cresyl Blue stain selects largest oocytes with highest mitochondrial activity, maturation-promoting factor activity and embryo developmental competence in prepubertal sheep. Reproduction 2011;142:517–27.
[62] Guler A, Poulin N, Mermillod P, Terqui M, Cognié Y. Effect of growth factors, EGF and IGF-I, and estradiol on *in vitro* maturation of sheep oocytes. Theriogenology 2000;54:209–18.

[63] Katska-Ksiazkiewicz L, Opiela J, Rynska B. Effects of oocyte quality, semen donor and embryo co-culture system on the efficiency of blastocyst production in goats. Theriogenology 2007;68:736–44.
[64] Opiela J, Katska-Ksiazkiewicz L. The utility of Brilliant Cresyl Blue (BCB) staining of mammalian oocytes used for *in vitro* embryo production (IVP). Reprod Biol 2013;13:177–83.
[65] Cocero M, Alabart J, Hammami S, Marti J, Lahoz B, Sanchez P, et al. The efficiency of *in vitro* ovine embryo production using an undefined or a defined maturation medium is determined by the source of the oocyte. Reprod Domest Anim 2011;46:463–70.
[66] Souza JMG, Duffard N, Bertoldo MJ, Locatelli Y, Corbin E, Fatet A, et al. Influence of heparin or the presence of cumulus cells during fertilization on the *in vitro* production of goat embryos. Anim Reprod Sci 2013;138:82–9.
[67] Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 2004;303:682–4.
[68] Lonergan P, Carolan C, Van Langendonckt A, Donnay I, Khatir H, Mermillod P. Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro*. Biol Reprod 1996;54:1420–9.
[69] de Matos DG, Gasparrini B, Pasqualini SR, Thompson JG. Effect of glutathione synthesis stimulation during *in vitro* maturation of ovine oocytes on embryo development and intracellular peroxide content. Theriogenology 2002;57:1443–51.
[70] Guyader-Joly C, Guerin P, Renard JP, Guillaud J, Ponchon S, Menezo Y. Precursors of taurine in female genital tract: effects on developmental capacity of bovine embryo produced *in vitro*. Amino Acids 1998;15:27–42.
[71] Shabankareh HK, Zandi M. Developmental potential of sheep oocytes cultured in different maturation media: effects of epidermal growth factor, insulin-like growth factor I, and cysteamine. Fertil Steril 2010;94:335–40.
[72] Bormann CL, Moige Ongeri E, Krisher RL. The effect of vitamins during maturation of caprine oocytes on subsequent developmental potential *in vitro*. Theriogenology 2003;59:1373–80.
[73] Harper KM, Brackett BG. Bovine blastocyst development after follicle-stimulating hormone and platelet-derived growth factor treatment for oocyte maturation *in vitro*. Zygote 1993;1:27–34.
[74] Karami Shabankareh HK, Sarsaifi K, Mehrannia T. *In vitro* maturation of ovine oocytes using different maturation media: effect of human menopausal serum. J Assist Reprod Genet 2011;28:531–7.
[75] Shirazi A, Shams-Esfandabadi N, Ahmadi E, Heidari B. Effects of growth hormone on nuclear maturation of ovine oocytes and subsequent embryo development. Reprod Domest Anim 2010;45:530–6.
[76] Fonseca JF, Souza JMG, Camargo LSA. Produção de oócitos e embriões de pequenos ruminantes: passado, presente e futuro. Acta Sci Vet 2010;38:337–69.
[77] Sirard MA, First NL. *In vitro* inhibition of oocyte nuclear maturation in the bovine. Biol Reprod 1988;39:229–34.
[78] Mermillod P, Tomanek M, Marchal R, Meijer L. High developmental competence of cattle oocytes maintained at the germinal vesicle stage for 24 hours in culture by specific inhibition of MPF kinase activity. Mol Reprod Dev 2000;55:89–95.
[79] Vigneron C, Perreau C, Dupont J, Uzbekova S, Prigent C, Mermillod P. Several signaling pathways are involved in the control of cattle oocyte maturation. Mol Reprod Dev 2004;69:466–74.
[80] Gilchrist RB, Thompson JG. Oocyte maturation: emerging concepts and technologies to improve developmental potential *in vitro*. Theriogenology 2007;67:6–15.
[81] Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update 2008;14:159–77.
[82] Romaguera R, Morato R, Jimenez-Macedo AR, Catala M, Roura M, Paramio MT, et al. Oocyte secreted factors improve embryo developmental competence of COCs from small follicles in prepubertal goats. Theriogenology 2010;74:1050–9.
[83] Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988;38:1171–80.
[84] Malik RK, Lohan IS, Dhanda OP, Tuli RK. Test for the acrosomal reaction of goat spermatozoa treated with heparin. Small Rumin Res 1997;26:163–6.
[85] Cognié Y, Poulin N, Pignon P, Sulon J, Beckers JF, Guerin Y. Does heparin affect developmental ability of IVP goat oocytes? In: AETE (Ed.), 11th AETE, Hannover, Germany, p. 146, 1995.
[86] Wang B, Baldassarre H, Tao T, Gauthier M, Neveu N, Zhou JF, et al. Transgenic goats produced by DNA pronuclear microinjection of *in vitro* derived zygotes. Mol Reprod Dev 2002;63:437–43.
[87] Palomo MJ, Mogas T, Izquierdo D, Paramio MT. The influence of sperm concentration, length of the gamete co-culture and the evolution of different sperm parameters on the *in vitro* fertilization of prepubertal goat oocytes. Zygote 2010;18:345–55.
[88] Coy P, Aviles M. What controls polyspermy in mammals, the oviduct or the oocyte? Biol Rev Camb Philos Soc 2010;85:593–605.
[89] de Graaf SP, Beilby KH, Underwood SL, Evans G, Maxwell WM. Sperm sexing in sheep and cattle: the exception and the rule. Theriogenology 2009;71:89–97.
[90] Larsson B, Rodriguez-Martinez H. Can we use in vitro fertilization tests to predict semen fertility? Anim Rep Sci 2000;60-61:327–36.
[91] Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 2002;61:234–48.
[92] Massip A, Mermillod P, Dinnyes A. Morphology and biochemistry of *in-vitro* produced bovine embryos: implications for their cryopreservation. Hum Reprod 1995;10:3004–11.
[93] Donnay I, Partridge RJ, Leese HJ. Can embryo metabolism be used for selecting bovine embryos before transfer? Reprod Nutr Dev 1999;39:523–33.
[94] Rizos D, Clemente M, Bermejo-Alvarez P, de La Fuente J, Lonergan P, Gutierrez-Adan A. Consequences of *in vitro* culture conditions on embryo development and quality. Reprod Domest Anim 2008;43:44–50.
[95] Abd El Razek IM, Charpigny G, Delalleau N, Marquant-Leguienne B, Mermillod P, Guyader-Joly C, et al. Lipid composition of *in vivo* and *in vitro* produced bovine embryos and of *in vitro* embryos showing different osmotic behavior. Theriogenology 2000;53:46.
[96] Gutierrez-Adan A, Lonergan P, Rizos D, Ward FA, Boland MP, Pintado B, et al. Effect of the *in vitro* culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. Theriogenology 2001;55:1117–26.
[97] Pradeep MA, Jagadeesh J, De AK, Kaushik JK, Malakar D, Kumar S, et al. Purification, sequence characterization and effect of goat oviduct-specific glycoprotein on in vitro embryo development. Theriogenology 2011;75:1005–15.
[98] Yadav EN, Kharche SD, Goel AK, Jindal SK, Sinha NK, Johri DK. Effect of serum source on in vitro maturation and fertilization of pre pubertal goat oocytes. Indian J Anim Sci 2008;78:146–9.
[99] Chiamenti A, Aguiar Filho CR, Freitas Neto LM, Chaves RM, Palua-Lopes FF, Lima PR, et al. Effects of retinoids on the in vitro development of Capra hircus embryos to blastocysts in two different culture systems. Reprod Dom Anim 2010;45:68–72.
[100] Tervit HR, Whittingham DG, Rowson LE. Successful culture *in vitro* of sheep and cattle ova. J Reprod Fertil 1972;30:493–7.
[101] Takahashi Y, First NL. *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 1992;37:963–78.
[102] Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Mermillod P. Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology 2001;56:17–29.
[103] Garcia-Garcia RM, Ward F, Fair S, O'Meara CM, Wade M, Duffy P, et al. Development and quality of sheep embryos cultured in commercial G1.3/G2.3 sequential media. Anim Reprod Sci 2007;98:233–40.
[104] Ferry L, Mermillod P, Massip A, Dessy F. Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. Theriogenology 1994;42:445–53.
[105] Sinclair KD, McEvoy TG, Maxfield EK, Maltin CA, Young LE, Wilmut I, et al. Aberrant fetal growth and development after *in vitro* culture of sheep zygotes. J Reprod Fertil 1999;116:177–86.
[106] Mu XF, Jin XL, Farnham MM, Li Y, O'Neill C. DNA damage-sensing kinases mediate the mouse 2-cell embryo's response to genotoxic stress. Biol Reprod 2011;85:524–35.
[107] Leese HJ, Hugentobler SA, Gray SM, Morris DG, Sturmey RG, Whitear SL, et al. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. Reprod Fertil Dev 2008;20:1–8.
[108] Rizos D, Ramirez MA, Pintado B, Lonergan P, Gutierrez-Adan A. Culture of bovine embryos in intermediate host oviducts with emphasis on the isolated mouse oviduct. Theriogenology 2010;73:777–85.
[109] Gandolfi F, Moor RM. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J Reprod Fertil 1987;81:23–8.
[110] Fukui Y, McGowan LT, James RW, Pugh PA, Tervit HR. Factors affecting the in-vitro development to blastocysts of bovine oocytes matured and fertilized *in vitro*. J Reprod Fertil 1991;92:125–31.

[111] Koeman J, Keefer CL, Baldassarre H, Downey BR. Developmental competence of prepubertal and adult goat oocytes cultured in semi-defined media following laparoscopic recovery. Theriogenology 2003;60:879–89.
[112] Masudul Hoque SA, Yahia Khandoker MAM, Kabiraj SK, Asad LY, Fakruzzaman M, Tareq KMA. Effect of goat follicular fluid on *in vitro* production of embryos in black Bengal goats. Iranian J Appl Anim Sci 2012;2:287–94.
[113] Shirazi A, Ardali MA, Ahmadi E, Nazari H, Mamuee M, Heidari B. The effect of macromolecule source and type of media during *in vitro* maturation of sheep oocytes on subsequent embryo development. J Reprod Infertil 2012;13:13–9.
[114] Yadav PS, Saini A, Kumar A, Jain GC. Effect of oviductal cell coculture on cleavage and development of goat IVF embryos. Anim Reprod Sci 1998;51:301–6.
[115] Pavasuthipaisit K, Lhuangmahamongkol S, Tocharus C, Kitiyanant Y, Prempree P. Porcine oviductal cells support *in vitro* bovine embryo development. Theriogenology 1994;41:1127–38.
[116] Buhi WC, Alvarez IM, Kouba AJ. Oviductal regulation of fertilization and early embryonic development. J Reprod Fertil 1997;52:285–300.
[117] Goncalves RF, Staros AL, Killian GJ. Oviductal fluid proteins associated with the bovine zona pellucida and the effect on *in vitro* sperm-egg binding, fertilization and embryo development. Reprod Domest Anim 2008;43:720–9.
[118] Rizos D, Ward F, Boland MP, Lonergan P. Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. Theriogenology 2001;56:1–16.
[119] Wrenzycki C, Herrmann D, Niemann H. Messenger RNA in oocytes and embryos in relation to embryo viability. Theriogenology 2007;68:77–83.
[120] Thelie A, Papillier P, Pennetier S, Perreau C, Traverso JM, Uzbekova S, et al. Differential regulation of abundance and deadenylation of maternal transcripts during bovine oocyte maturation *in vitro* and *in vivo*. BMC dev biol 2007;7:125–38.
[121] Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. Nature 1997;385:810–3.
[122] Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, et al. Production of goats by somatic cell nuclear transfer. Nat Biotechnol 1999;17:456–61.
[123] Keefer CL, Keyston R, Lazaris A, Bhatia B, Begin I, Bilodeau AS, et al. Production of cloned goats after nuclear transfer using adult somatic cells. Biol Reprod 2002;66:199–203.
[124] Freitas VJF, Serova IA, Andreeva LE, Dvoryanchikov GA, Lopes ESJ, Teixeira DIA, et al. Production of transgenic goat (Capra hircus) with human granulocyte colony stimulating factor (hG-CSF) gene in Brazil. An Acad Bras Cienc 2007;79:585–92.
[125] Pereyra-Bonnet F, Gibbons A, Cueto M, Sipowicz P, Fernández-Martín R, Salamone D. Efficiency of sperm-mediated gene transfer in the ovine by laparoscopic insemination, *in vitro* fertilization and ICSI. J Reprod Dev 2011;57:188–96.
[126] Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH. Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Nat Acad Sci 1980;77:7380–4.
[127] Hammer RE, Pursel VG, Rexroad CEJ, Wall RJ, Bolt DJ, Ebert KM, et al. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 1985;315:680–3.
[128] Schmidt C. Belated approval of first recombinant protein from animal. Nat Biotechnol 2006;24:877.
[129] Kling J. First US approval for a transgenic animal drug. Nat Biotechnol 2009;27:302–4.
[130] Moura RR, Melo LM, Freitas VJF. Production of recombinant proteins in milk of transgenic and non-transgenic goats. Braz Arch Biol Technol 2011;54:927–38.
[131] Demeestere I, Delbaere A, Gervy C, Van den Bergh M, Deyreker F, Englert Y. Effect of preantral follicle isolation technique on *in-vitro* follicular growth, oocyte maturation and embryo development in mice. Hum Reprod 2002;17:2152–9.
[132] Lucci CM, Amorim CA, Bao SN, Figueiredo JR, Rodrigues AP, Silva JR, et al. Effect of the interval of serial sections of ovarian in the tissue chopper on the number of isolated caprine preantral follicles. Anim Reprod Sci 1999;56:39–49.
[133] Itoh T, Hoshi H. Efficient isolation and long-term viability of bovine small preantral follicles *in vitro*. In vitro Cell Dev Biol Anim 2000;36:235–40.
[134] Faustino LR, Santos RR, Silva CM, Pinto LC, Celestino JJ, Campello CC, et al. Goat and sheep ovarian tissue cryopreservation: effects on the morphology and development of primordial follicles and density of stromal cell. Anim Reprod Sci 2010;122:90–7.
[135] Lucci CM, Silva RV, Carvalho CA, Figueiredo JR, Bao SN. Light microscopical and ultrastructural characterization of goat preantral follicles. Small Rumin Res 2001;41:61–9.
[136] Santos RR, Knijn HM, Vos PL, Oei CH, Van Loon T, Colenbrander B, et al. Complete follicular development and recovery of ovarian function of frozen-thawed, autotransplanted caprine ovarian cortex. Fertil Steril 2009;91:1455–8.
[137] Salle B, Demirci B, Franck M, Rudigoz RC, Guerin JF, Lornage J. Normal pregnancies and live births after autograft of frozen-thawed hemi-ovaries into ewes. Fertil Steril 2002;77:403–8.
[138] Martins FS, Celestino JJ, Saraiva MV, Matos MH, Bruno JB, Rocha-Junior CM, et al. Growth and differentiation factor-9 stimulates activation of goat primordial follicles *in vitro* and their progression to secondary follicles. Reprod Fertil Dev 2008;20:916–24.
[139] Magalhães DM, Duarte ABG, Araújo VR, Brito IR, Soares TG, Lima IM, et al. *In vitro* production of a caprine embryo from a preantral follicle cultured in media supplemented with growth hormone. Theriogenology 2011;75:182–8.
[140] Arunakumari G, Shanmugasundaram N, Rao VH. Development of morulae from the oocytes of cultured sheep preantral follicles. Theriogenology 2010;74:884–94.
[141] Reby D, Charlton BD, Locatelli Y, McComb K. Oestrous red deer hinds prefer male roars with higher fundamental frequencies. Proceeding R Soc B 2010;277:2747–53.
[142] Guerin P, El Mouatassim S, Menezo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod update 2001;7:175–89.