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

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Abstract. This review presents the latest advances in and main obstacles to the application of *in vitro* embryo production (IVEP) systems in small ruminants. This biotechnology is an extremely important tool for genetic improvement for livestock and is essential for the establishment of other biotechnologies, such as cloning and transgenesis. At present, the IVEP market is almost non-existent for small ruminants, in contrast with the trends observed in cattle. This is probably related to the lower added value of small ruminants, lower commercial demand and fewer qualified professionals interested in this area. Moreover, there are fewer research groups working on small ruminant IVEP than those working with cattle and pigs. The heterogeneity of oocytes collected from growing follicles in live females or from ovaries collected from abattoirs remains a challenge for IVEP dissemination in goats and sheep. Of note, although the logistics of oocyte collection from live small ruminant females are more complex than in the bovine, in general the IVEP outcomes, in terms of blastocyst production, are similar. We anticipate that after appropriate training and repeatable results, the commercial demand for small ruminant *in vitro*-produced embryos may increase.

Keywords: goat, IVF, IVM, reproductive biotechniques, sheep.

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Introduction

Small ruminant production is an important source of milk, meat, wool or fibres and skin, especially in developing countries. Overall, livestock populations continue to expand worldwide, with the number of small ruminants globally increasing over the past 30 years (from 1980 to 2018), from 0.6 to 1.0 billion goats and from 1.1 to 1.2 billion sheep, totalling 2.2 billion animals (FAOSTAT, http://www.fao.org/faostat/en/#home, accessed 18 April 2020). The Consultative Group on International Agricultural Research estimates that the small ruminant population will expand to nearly 2.7 billion in the next 30 years (Nierenberg and Reynolds 2013). However, although small ruminants represent a relatively high number of animals, they have not been genetically improved to the same degree as, for example, as cattle (for a review, see Paramio *et al.* 2020). In actual fact, assisted reproductive technologies (ARTs) are excellent tools to circumvent this bottleneck for genetic breeding programs in small ruminants.

In vitro embryo production (IVEP) and multiple ovulation and embryo transfer (MOET) are techniques that have substantially contributed to the genetic improvement of livestock. In contrast with bovine species, where the recent main trends in the embryo market are related to a decrease in MOET and an increase in *in vitro*-produced (IVP) embryos, MOET remains the primary ART used for small ruminant embryos (Fonseca *et al.* 2019*a*; Viana 2019). Data from the International Embryo Transfer Society (IETS) newsletters summarising the global trends in sheep and goat MOET and IVEP-derived embryos are

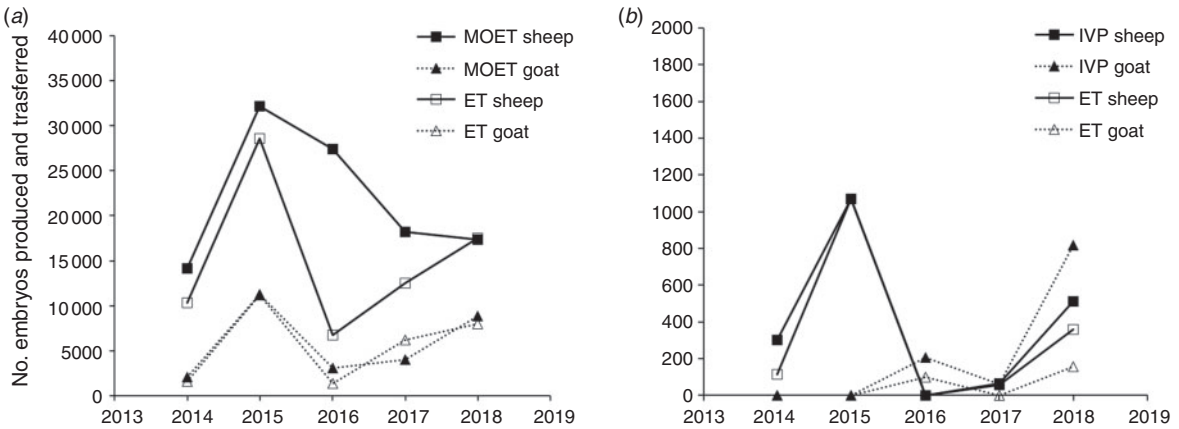

Fig. 1. Data for global (*a*) *in vivo* (multiple ovulation and embryo transfer (MOET)) and (*b*) *in vitro* production (IVP) of embryos and embryo transfer (ET) in sheep and goats. Data were sourced from International Embryo Transfer Society newsletters published between 2015 and 2019 and referring to the years between 2014 and 2018 (http://www.iets.org/comm_data.asp, accessed 21 April 2020).

presented in Fig. 1. Even though these data are underestimated because many countries do not communicate their commercial activities to IETS, at a commercial level small ruminant embryo production is notably irregular. However, it is known that the production of sheep embryos has been considerably greater than that of goat embryos. Because of the limited demand, there are lower numbers of technicians and researchers involved in the small ruminant ART industry (Fonseca *et al.* 2019*a*), making ART progress slower in small ruminants than in bovine (Candappa and Bartlewski 2011).

Although non-surgical embryo recovery may be used efficiently in small ruminants (Fonseca *et al.* 2019*a*), *in vivo* embryo collection worldwide is generally performed by laparotomy. This surgical technique requires prior fasting of the animals and the use of anaesthetic drugs that could pose risks to the animal. Moreover, there is the possibility of adhesions, post-surgical sequelae and stress, which could impair repeated embryo production. Recently, we demonstrated in sheep that transcervical embryo collection is as effective and less stressful regarding animal welfare than laparotomy (Santos *et al.* 2020). The MOET procedure does have some limitations, including high variability in the ovulatory response to hormone treatment (Souza-Fabjan *et al.* 2017), fertilisation failures (Gibbons *et al.* 2007) and premature luteal regression (Souza-Fabjan *et al.* 2017). Therefore, the advantages of IVEP include the possibility of collecting oocytes from females regardless of hormone stimulation and the use of prepubertal, senile, pregnant or even dead females. It is widely accepted that IVEP techniques are essential to any laboratory interested in preserving endangered breeds (Souza-Fabjan *et al.* 2013), as well as cloning and transgenesis (Pereira *et al.* 2013). Finally, understanding early embryo development will provide valuable information that can be used, for example, in developing therapies for human reproductive failure (Zhu *et al.* 2018).

The IVEP technique involves four steps: the collection of cumulus–oocyte complexes (COC), IVM, IVF and *in vitro* development (IVD) of the embryos up to the blastocyst stage. Despite considerable efforts in the field by scientists worldwide to enhance blastocyst rates, the most critical problems related to IVEP remain unresolved. The inconsistency of results obtained among laboratories, the high variability in the number and quality of oocytes recovered and the low embryo viability after cryopreservation still restrict the dissemination of IVEP (for reviews, see Souza-Fabjan *et al.* 2014*c*; Paramio *et al.* 2020). Furthermore, the success rates of IVEP are still far below those for MOET, with IVEP overall resulting in approximately 75–90% of immature oocytes undergoing meiotic maturation, 50–80% cleaving and only 25–50% of oocytes from adult females reaching the blastocyst stage in both goats (Table 1) and sheep (Table 2). Thus, the aim of the present review was to summarise the recent advances in and main obstacles to small ruminant IVEP, including intrinsic and extrinsic factors affecting oocyte quality at the time of collection, IVM, IVF and IVD procedures, and embryo cryopreservation.

Stages of IVEP and factors affecting their success

Collection of COCs

Source of oocytes

Immature oocytes may be collected either from abattoir-derived ovaries or from live animals. The abattoir-derived ovaries is a cheap source of oocytes, which are usually recovered by follicle aspiration or ovary slicing. Moreover, these ovaries enable IVEP to be established in the laboratory to test the roles of different molecules or protocols, for example (for a review, see Souza-Fabjan *et al.* 2014*c*). However, the use of IVEP as a tool for genetic improvement obviously requires oocyte recovery from live select females (or after unexpected donor culling). Laparoscopic ovum pick up (LOPU) is the minimally invasive technique used to recover oocytes from live ewes (Baldassarre 2012) and does (Souza-Fabjan *et al.* 2014*b*).

Oocyte quality is one of main factors affecting IVEP outcomes. Oocyte quality is affected by both intrinsic and extrinsic factors, such as individual donor responses to hormone treatments, body condition score, breed, age, season and technician experience, among others. The effects of hormone protocols for

Table 1. Recent results for oocytes obtained from adult goats by laparoscopic ovum pick-up (LOPU), abattoir or ovariectomy and subjected to IVM, IVF and *in vitro* development (IVD)

Note, the MII rate represents the proportion of oocytes reaching nuclear maturation, and the cleavage rate represents the number of cleaved oocytes relative to the number of oocytes undergoing maturation. COC, cumulus–oocyte complexes; NA, not available; semi-defined medium, medium supplemented with bovine serum albumin or another semi-defined component; undefined medium, medium supplemented with serum

COC origin	IVM medium	IVF medium	IVD medium	MII rate (%)	Cleavage rate (%)	% Blastocysts		References
						From cleaved	From COCs	
LOPU	Defined	Undefined	Semi-defined	53–72	39	71	28	Souza-Fabjan *et al.* (2014*a*)
	Undefined	Defined	Undefined	NA	53	39	21	Romaguera *et al.* (2011)
		Undefined	Semi-defined	85	70	34	33	Leoni *et al.* (2009)
			Undefined	NA	59	87	51	Souza-Fabjan *et al.* (2013)
Abattoir	Defined	Defined	–	61	–	–	–	Purohit *et al.* (2012)
			Undefined	NA	34–72	32–77	11–59	Souza *et al.* (2013)
		Undefined	Semi-defined	40–85	68–77	60–70	46–47	Souza-Fabjan *et al.* (2014*a*)
			Undefined	NA	70–73	47–62	34–45	Fieni *et al.* (2012)
			Undefined + coculture	NA	83	20–28	16–23	Rodríguez-Dorta *et al.* (2007)
	Semi-defined	–	–	20–70	–	–	–	Cadenas *et al.* (2018), Correia *et al.* (2019)
		Defined	NA + coculture	NA	24–30	9–11	5–8	Conceição *et al.* (2015)
			Semi-defined + coculture	NA	27–36	14–24	6–8	Conceição *et al.* (2016)
		Undefined	Semi-defined	62–90	66	65–68	43–45	Souza-Fabjan *et al.* (2014*a*)
			Undefined	41–68	56–68	24–38	13–26	Veshkini *et al.* (2015)
	Undefined	–	–	13–89	–	–	–	Rao *et al.* (2012), Zhang *et al.* (2013, 2015), Abazari-Kia *et al.* (2014), Fernandes *et al.* (2014), Mukherjee *et al.* (2014), Pu *et al.* (2014), Krishan *et al.* (2016), Zhou *et al.* (2016), Gomes *et al.* (2018), Saeedabadi *et al.* (2018), Silva *et al.* (2018)
		NI	Semi-defined + coculture	NA	10	20–40	2–4	Chiamenti *et al.* (2010)
			Undefined	41–85	31–85	3–60	6–49	Han *et al.* (2008), De *et al.* (2011), An *et al.* (2018)
		Defined	Undefined	NA	18–53	11–39	3–21	Pradeep *et al.* (2011), Romaguera *et al.* (2011)
		Semi-defined	–	60–66	–	–	–	Khatun *et al.* (2011)
		Undefined	NA	86	31	10	3	Kouamo and Kharche 2015)
			Semi-defined	92	36–72	17–63	6–45	Souza-Fabjan *et al.* (2014*a*), Agarwal *et al.* (2017)
			Undefined	86–95	63–72	65–90	41–66	Wang *et al.* (2017)
Ovariectomy	Undefined	Undefined	Semi-defined	NA	63–82	26–38	16–31	Berlinguer *et al.* (2009)

Table 2. Recent results for oocytes obtained from adult sheep by laparoscopic ovum pick-up (LOPU), from an abattoir or after ovariectomy and subjected to IVM, IVF and *in vitro* development (IVD)

Note, the MII rate represents the proportion of oocytes reaching nuclear maturation, and the cleavage rate represents the number of cleaved oocytes relative to the number of oocytes undergoing maturation. COC, cumulus–oocyte complexes; NA, not available; semi-defined medium, medium supplemented with bovine serum albumin or another semi-defined component; undefined medium, medium supplemented with serum

COC origin	IVM medium	IVF medium	IVD medium	MII rate (%)	Cleavage rate (%)	% Blastocysts		References
						From cleaved	From COCs	
LOPU	Defined	Undefined	Undefined	NA	54–57	33–58	19–31	Cocero *et al.* (2011)
	Undefined	–	–	33	–	–	–	Padilha *et al.* (2014)
		Semi-defined	Undefined	NA	3–54	–	–	Tsiligianni *et al.* (2009)
		Undefined	Semi-defined	92	70–83	92–95	28–39	Fang *et al.* (2016), Menchaca *et al.* (2018)
			Undefined	NA	71–88	26–57	22–41	Cocero *et al.* (2011), Lahoz *et al.* (2013)
Abattoir	Defined	–	–	4–59	–	–	–	Crocomo *et al.* (2015*b*), Byri *et al.* (2017)
		Undefined	Semi-defined	NA	55–85	21–41	13–35	Shabankareh and Zandi 2010), Zhao *et al.* (2012)
			Undefined	NA	74	40	30	Cocero *et al.* (2011)
	Semi-defined	–	–	7–93	–	–	–	Cao *et al.* (2009), Sreenivas *et al.* (2012), Buell *et al.* (2015), Byri *et al.* (2017), Wang *et al.* (2018)
		Semi-defined	–	21–84	–	–	–	Yasmin *et al.* (2015)
			Semi-defined	NA	40–76	23–60	16–45	Shabankareh *et al.* (2012)
			Undefined	11–70	62	0–6	0–3	Fernández-Reyez *et al.* (2012)
		Undefined	Semi-defined	64–68	59–85	16–64	11–52	Shabankareh and Zandi (2010), Kafilzadeh *et al.* (2012), Shirazi *et al.* (2012), Wang *et al.* (2013), Mara *et al.* (2014), Quan *et al.* (2017)
		Undefined	Undefined	87	40–51	7–96	3–43	Romão *et al.* (2013, 2015)
	Undefined	–	–	1–97	–	–	–	Ebrahimi *et al.* (2010*a*, 2010*b*), Kyasari *et al.* (2012), Sreenivas *et al.* (2012), Palmerini *et al.* (2014), Abazari-Kia *et al.* (2015), Crocomo *et al.* (2015*a*, 2015*b*, 2016*b*), Gharibzadeh *et al.* (2015), Veshkini *et al.* (2015), Valiollahpoor Amiri *et al.* (2016), Azari-Dolatabad *et al.* (2016), Barakat *et al.* (2016), Nadri *et al.* (2016), Wei *et al.* (2016*a*), Byri *et al.* (2017), Tian *et al.* (2017), Zhang *et al.* (2018*a*), Al-Mutary *et al.* (2019), Javvaji *et al.* (2019)
		NA	NA	NA	77	39	30	Hosseini *et al.* (2015)
			Semi-defined	NA	49–70	4–29	2– 20	Natarajan *et al.* (2010*a*, 2010*b*, 2010*c*)
			Undefined	41–95	10–85	0–70	0–55	Maalouf *et al.* (2009), Hosseini *et al.* (2011), Gharibi *et al.* (2013), Tripathi *et al.* (2016), Nandi *et al.* (2018), Ahmadi *et al.* (2019*a*)
		Defined	Semi-defined	NA	20–90	0–65	0–45	Shabankareh and Akhondi (2012), Sanaei *et al.* (2018*a*, 2018*b*)
		Semi-defined	NA	20–77	32–98	0–63	0–45	Beilby *et al.* (2011), Davachi *et al.* (2016), Martino *et al.* (2017), Goodarzi *et al.* (2018), Rouhollahi Varnosfaderani *et al.* (2020)
			NA + coculture	NA	81–91	30–50	27–45	Dashti *et al.* (2016)
			Semi-defined	52–93	29–96	8–77	4–61	Shabankareh *et al.* (2011), Heidari *et al.* (2013), Davachi *et al.* (2014), Aghaz *et al.* (2015), Moradi *et al.* (2015), Shirazi *et al.* (2015), Davachi *et al.* (2017), Zabihi *et al.* (2019)
			Semi-defined + coculture	NA	89–92	25–43	23–39	Heidari *et al.* (2013)
			Undefined	52–88	20–91	8–51	2–45	Aghaz *et al.* (2016), Eshtiyaghi *et al.* (2016), Mishra *et al.* (2016*a*, 2016*b*, 2018), Hajarian *et al.* (2017), Pezhman *et al.* (2017)
			Undefined + coculture	NA	82–88	13–31	11–26	Heidari *et al.* (2013)

Undefined	Semi-defined	24–97	7–96	5–76	1–62	Bai *et al.* (2008), Bebbere *et al.* (2010), Modina *et al.* (2010), Shabankareh and Zandi (2010), Vázquez *et al.* (2010*a*, 2010*b*), Shirazi *et al.* (2011), Succu *et al.* (2011, 2018), Berlinguer *et al.* (2012*a*, 2012*b*), Moawad *et al.* (2012, 2013, 2018), Mohammadi-Sangcheshmeh *et al.* (2012, 2014), Wang *et al.* (2012), Shirazi and Motaghi (2013), Vázquez *et al.* (2013), Leoni *et al.* (2015), Crispo *et al.* (2016), Crocomo *et al.* (2016*a*), Ledda *et al.* (2016), Naderi *et al.* (2016*a*), Quan *et al.* (2016, 2017), Amir *et al.* (2018), Barrera *et al.* (2018), Masala *et al.* (2018), Zhang *et al.* (2018*b*, 2019), Tian *et al.* (2019), Sánchez-Ajofrín *et al.* (2020), dos Santos-Neto *et al.* (2020)
	Undefined	30–96	26–89	12–59	4–50	Wan *et al.* (2009), Cocero *et al.* (2011, 2019), Reader *et al.* (2015), Amini *et al.* (2016), Golchin *et al.* (2016), Naderi *et al.* (2016*b*), Zacchini *et al.* (2017), Ahmadi *et al.* (2019*b*)
	Semi-defined	NA	50–85	5–36	2–31	Grazul-Bilska *et al.* (2012)
Ovariectomy	Undefined	NA				

ovarian follicle stimulation and overall IVEP conditions are discussed in detail later in the review.

Age of donor

Oocyte quality is strongly affected by the age of the female donor. The developmental competence of oocytes from juvenile or prepubertal females is lower than that of oocytes from their adult counterparts in both goats (24 vs 34%; Leoni *et al.* 2009) and sheep (19.9 vs 51.3%; Leoni *et al.* 2015; Table S1). This lower competence is related to the small follicles from which the oocytes develop, mostly follicles <3 mm in diameter (for a review, see Paramio and Izquierdo 2014). Comparing prepubertal and adult goat oocytes, Romaguera *et al.* (2011) observed similar blastocyst development in the two groups when oocytes were obtained from follicles >3 mm in diameter (18% vs 20% respectively). In adult goats, Crozet *et al.* (1995) also observed a direct and positive relationship between follicle size and oocyte competence to develop up to the blastocyst stage. In mice, blastocyst development was similar between adult and prepubertal females when animals were previously treated with equine chorionic gonadotrophin (eCG) to stimulate follicle growth (Jiao *et al.* 2013). Jiao *et al.* (2013) concluded that the development of oocytes from small follicles from juvenile females is impaired because of the high susceptibility of these oocytes to the harmful effects of reactive oxygen species (ROS). In addition, ultrastructural and functional deficiencies have been reported for oocytes from prepubertal goat ovaries, such as altered distribution of cortical granules and mitochondria, disorganisation of microtubules and microfilaments, as well as changes in total RNA content, p34 (cdc2) and cyclin B1 expression and maturation-promoting factor (MPF) activity (for a review, see Paramio and Izquierdo 2014).

The interest in using prepubertal oocytes is because these oocytes could be a promising tool for animal husbandry as a result of a reduction in the generation interval and increased genetic gain in breeding programs. This technology is called juvenile *in vitro* embryo technology (JIVET). In sheep, using embryos produced from 4-week-old females reduced the generation interval to 6 months, compared with 12 months using MOET (Morton 2008). Thus, in conclusion of JIVET in merino breeding programs improves genetic gain by 21% in meet and 33% in wool productions (Granleese *et al.* 2015).

Season of collection

In seasonal species, reproductive seasonality has a considerable effect on ART efficiency (for a review, see Mastromonaco and Gonzalez-Grajales 2020). In sheep, more oocytes of higher quality are retrieved during the breeding season, positively affecting blastocyst development (Davachi *et al.* 2014). However, although some studies have reported that season affects oocyte cleavage rate (Stenbak *et al.* 2001; Davachi *et al.* 2014), others have not (Mara *et al.* 2014). Still, even when cleavage was unaffected, greater blastocyst yield was achieved for oocytes collected during the breeding season (Mara *et al.* 2014). In hormone-stimulated adult goats, season did not affect the number of follicles aspirated and oocytes recovered (Pierson *et al.* 2004). We also assessed the role that the season playson

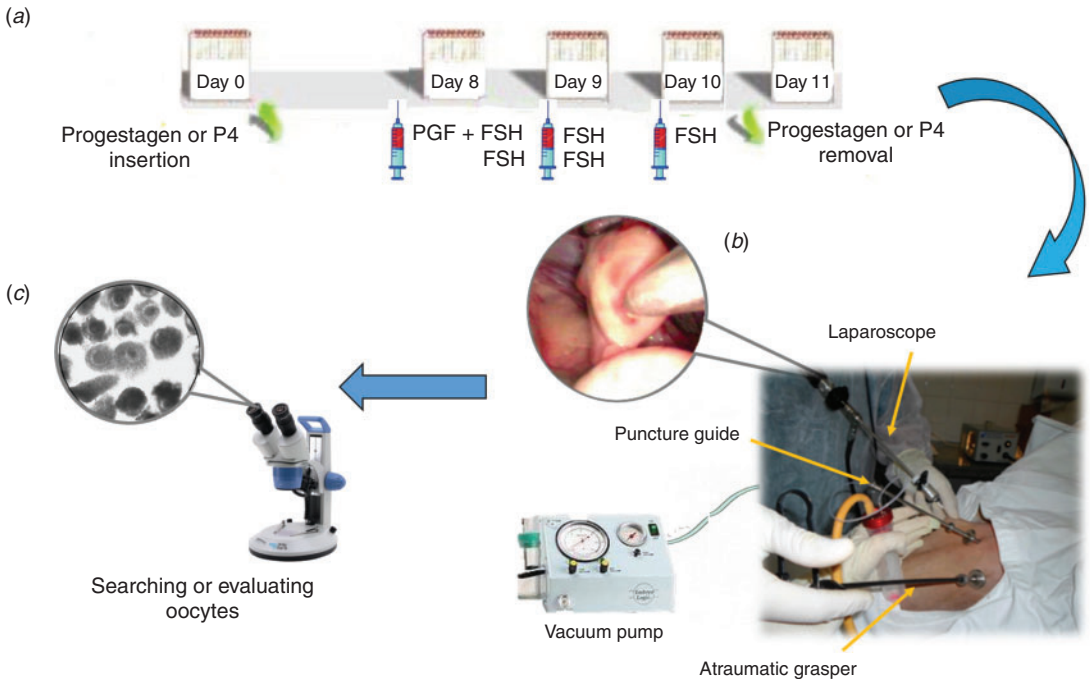

Fig. 2. Schematic representation of the (*a*) traditional hormone treatment administered to donors, (*b*) oocyte recovery and (*c*) oocyte evaluation. (*a*) Hormone treatment for ovarian stimulation consists of 11 days of progestogen priming associated with i.m. injections of decreasing doses of prostaglandin $F_{2\alpha}$ (PGF) and FSH from Day 8 to Day 10. P4, progesterone. (*b*) Laparoscopic ovum pickup (LOPU) using a 22-G needle connected to a puncture guide and vacuum pump. (*c*) Searching and evaluation of oocytes under a stereomicroscope (magnification ×70–80).

COC developmental competence in adult goats and found that both cleavage and blastocyst rates were higher in autumn (breeding season) than in the spring (anoestrous season), with no difference in the number of blastomeres (embryo quality; J. M. G. Souza-Fabjan, L. F. L. Correia, R. I. T. P. Batista, V. J. F. Freitas, P. Mermillod, unpubl. data). Surprisingly, in prepubertal goats, cleavage and blastocyst rates were greater during the anoestrous season (Catalá *et al.* 2018). The reason for the differences between prepubertal and adult goats require further investigation, but it should be considered that the reproductive parameters of prepubertal females are not affected by variations in the photoperiod. These data indicate that season may be expected to affect oocyte quality throughout the year (for a review, see Zhu *et al.* 2018). In sheep, a melatonin implant enhanced COC developmental competence during the anoestrous season (Vázquez *et al.* 2010*a*), indicating that some technical approaches could be used to overcome the effect of season when live females are used.

Hormone treatment of donors before collection

Hormonal ovarian stimulation is normally used in live ewes and does because it increases the number of follicles available and, consequently, the number of oocytes recovered by LOPU. Numerous ovarian hormone stimulation protocols have been used in small ruminants. Studies in cows have shown that global gene expression in cumulus cells (CC) is affected when using stimulatory treatment (Dias *et al.* 2013). However, little is known about the molecular status of oocytes growing under different stimulatory treatments in small ruminants. Overall, hormone stimulation combines the use of progesterone (P4; or its analogue)-releasing devices for 10–14 days to synchronise oestrus, prostaglandin $F_{2\alpha}$ (or its analogue) to promote luteolysis and gonadotrophin(s) for follicle growth (for a review, see Souza-Fabjan *et al.* 2014*c*). Among the gonadotrophins, FSH can be used in different concentrations, from either porcine or ovine origin and administered as either once or in multiple (three to six) doses. A traditional protocol for ovarian stimulation is shown in Fig. 2*a*. Normally, when single doses of FSH are used in association with eCG, this is known as the 'one-shot' protocol. This treatment protocol is frequently used in small ruminants because of its practical management (Pierson *et al.* 2004; Teixeira *et al.* 2011; Baldassarre 2012; Sanchez *et al.* 2014).

Recently, we used the 'Day 0 protocol', initially developed for MOET (Menchaca *et al.* 2007), for the first time in sheep for follicle stimulation and oocyte collection (Bragança *et al.* 2018). We compared the effects of stimulation protocols (starting on Day 0) containing 80 or 120 mg FSH alone in a three-dose regimen or in association with 300 IU eCG (one-shot regimen) on follicle population and the molecular status of immature COCs. We concluded that 80 mg FSH was sufficient to stimulate the growth of multiple follicles, generating COCs of good morphological quality (Bragança *et al.* 2018). Similarly, the multiple-dose regimen (80 mg FSH) appeared to be more appropriate for producing better-quality oocytes, because the expression of quality markers (i.e. maternal antigen that embryo requires (*MATER*), zygote arrest 1 (*ZAR1*), bone morphogenetic

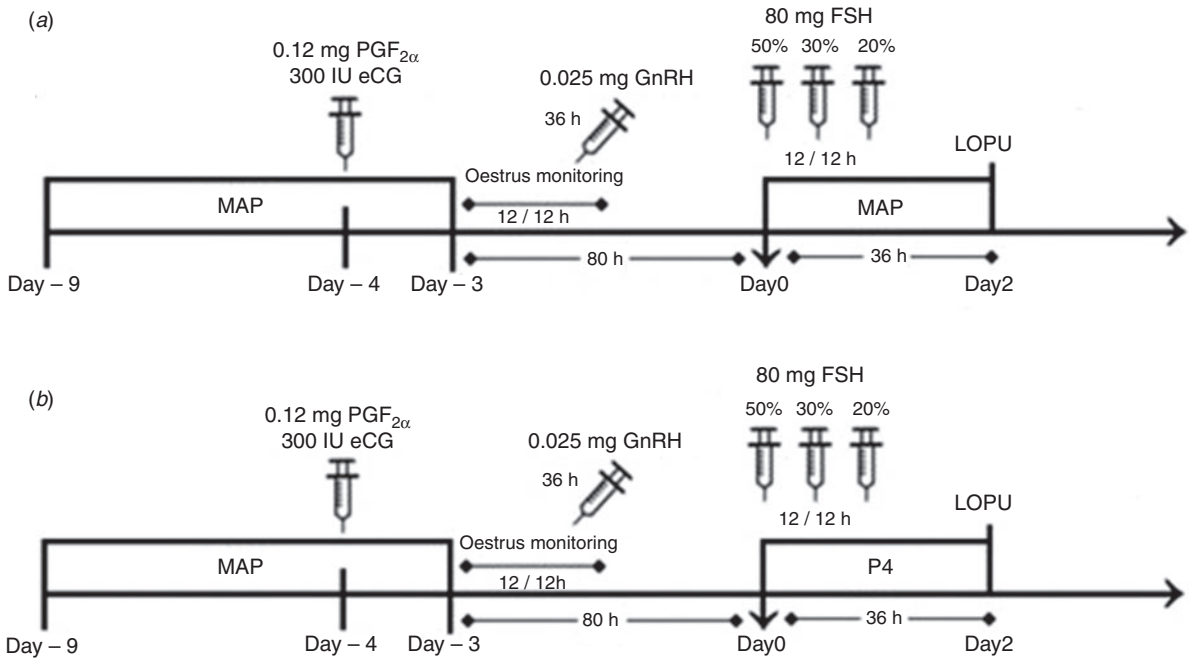

Fig. 3. Schematic diagram of the best protocols for hormone stimulation in Santa Inês ewes determined in two studies (Bragança *et al.* 2018, 2021). In both protocols, the same oestrus synchronisation treatment is used to reach ovulation on Day 0, consisting of insertion of a medroxyprogesterone acetate (MAP) sponge on Day –9 and injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and equine chorionic gonadotrophin (eCG) on Day –4. (*a*) In the first study (Bragança *et al.* 2018), a new progestogen (MAP) sponge is inserted Day 0 and ovarian stimulation is performed using a total of 80 mg FSH, administered in three decreasing doses every 12 h. (*b*) The current protocol used differs only in the insertion of a natural progesterone (P4)-releasing intravaginal device on Day 0 instead of the MAP sponge (Bragança *et al.* 2021). GnRH, gonadotrophin-releasing hormone; LOPU, laparoscopic ovum pick-up.

protein 15 (*BMP15*) and growth differentiation factor 9 (*GDF9*)) was upregulated compared with the non-stimulated control, as was the expression of genes related to the steroidogenic pathway (*FSHr*, oestrogen receptor α (*ERα*)), in the multiple-dose versus one-shot group (Bragança *et al.* 2018). The best protocol achieved in this experiment is shown in Fig. 3*a*.

There is considerable evidence that P4 has an important role in oocyte maturation, although its direct effect on oocyte quality has not yet been accurately determined (Lonergan 2011). When intravaginal P4 devices were inserted during FSH stimulation, sheep embryo yield was improved in both MOET (Cuadro *et al.* 2018) and IVEP (Menchaca *et al.* 2018) systems. Previously, it was demonstrated in sheep that the presence of a corpus luteum had a role in both MOET (improving the number of transferable embryos from 4.1 to 7.4) and IVEP (increasing rates of IVF from 45% to 73%, blastocyst formation from 19% to 36% and hatching after vitrification from 25% to 80%; Gonzalez-Bulnes *et al.* 2005). When providing exogenous devices, it is important to consider that because of variations in the chemical structure between P4 and its analogues, different actions at the cellular level may occur (Lieberman and Curtis 2017). Thus, we verified whether insertion of a P4-releasing device during antral follicular growth would have a similar role on sheep COC gene expression to that of a medroxyprogesterone acetate (MAP)-releasing device (Bragança *et al.* 2021). In that study, we assessed the abundance of mRNA encoding for proteins expressed in the oocyte (*MATER*, *ZAR1*, *BMP15* and *GDF9*), CCs (reelin (*RELN*), LDL receptor–related protein 8 (*LRP8*), FSH receptor (*FSHr*), LH receptor (*LHR*), *ERα*, and steroidogenic acute regulatory protein (*STAR*)) or both (*BAX* and *BCL2*). Gene expression was affected in both the P4- and MAP-treated groups compared with the non-progestogen-treated control (only corpus luteum (CL)): the expression of genes related to steroidogenic pathway receptors (*FSHR*, *LHR*, *ERα*) and markers of oocyte quality (*BCL2*, *ZAR1* and *GDF9*) was upregulated in the P4-treated compared with control group, whereas expression of the *FSHR*, *LHR* and *RELN* genes was upregulated in the P4-treated compared with MAP-treated group. We concluded that exogenous P4 seems to positively affect oocyte competence, probably due to its chemical structure (identical to endogenous P4) and the higher greater serum P4 concentrations in ewes (Bragança *et al.* 2021). The best protocol achieved in this experiment, which is the one we currently use for ovarian stimulation, is shown in Fig. 3*b*.

Oocyte collection by LOPU

For an experienced team/group, oocyte collection by LOPU takes nearly 20 min per donor. As part of the LOPU procedure, female donors should be deprived of food and water for 24 h before laparoscopy. During the procedure, the animals are administered a general anaesthetic and are restrained in an inverted position on a cradle or laparoscopy table at a 45° angle. Lidocaine is injected locally around three puncture sites (Souza-Fabjan *et al.* 2014*a*). The LOPU technique involves the use of

laparoscopy equipment with a 5-mm diameter endoscope, two trocar sets (endoscope and forceps), one thinner trocar set for the aspiration pipette, atraumatic grasping forceps, a fibre optic cable and a light source (Baldassarre 2012; Menchaca *et al.* 2016). Each step of the LOPU procedure has been reviewed elsewhere (Souza-Fabjan *et al.* 2014*b*). A schematic representation of the LOPU system is shown in Fig. 2*b*.

To maintain the integrity of COCs and optimise their recovery rate, the materials used and the aspiration conditions during LOPU are of critical importance. Overall, needle diameter can vary between 16G and 22G, and the pressure of the connected vacuum pump is adjusted from 25 to 70 mmHg for both goats and sheep. Under these conditions, oocyte recovery rates range from 40% to 90%, with the number of structures retrieved frequently reaching around 8–10 oocytes per ewe (Baldassarre 2012; Bragança *et al.* 2018) and 10–15 per doe (Baldassarre 2012; Sanchez *et al.* 2014). In small ruminants, there is a positive correlation between COCs derived from medium-sized follicles (diameter 3–5 mm) and better IVM, cleavage and blastocyst rates, making these follicles the preferential size for aspiration (Lahoz *et al.* 2013).

The time between each LOPU session in successive treatments in the same animal varies between 4 days (Gibbons *et al.* 2007) and 21 days (Sanchez *et al.* 2014). We have assessed the ovarian response of goats undergoing eight repeated hormone stimulation treatments and LOPU within 2- or 3-week intervals. The number of COCs recovered, cleavage and blastocyst rates and embryo quality (blastomere count) (Sanchez *et al.* 2014). It is important to note that repeated LOPU did not trigger painful discomfort to the ewes and no complications were encountered, such as adhesions and fibrosis of the ovaries (Teixeira *et al.* 2011), confirming that LOPU is a minimally invasive procedure. Indeed, repeated LOPU did not affect the fertility of donor ewes, even when repeated up to 20 times (Stangl *et al.* 1999).

Oocyte selection and IVM

The first step of successful IVEP is the collection of high-quality oocytes (i.e. those with high developmental competence). Overall, there are five levels of oocyte competence, which are related to an oocyte's ability to: (1) resume meiosis; (2) cleave after being fertilised; (3) develop to the blastocyst stage; (4) establish a pregnancy; (5) deliver a healthy progeny in the expected time (Sirard *et al.* 2006). This competence is acquired during folliculogenesis and is characterised by the ability of the oocyte to undergo chromosomal segregation (nuclear maturation), reorganisation of organelles, storage of mRNAs, proteins and transcription factors that act in the general process of maturation, fertilisation and early embryogenesis (cytoplasmic maturation). The mRNA content of mammalian oocytes is affected by several factors, such as animal nutrition (Pisani *et al.* 2008), follicle diameter (Caixeta *et al.* 2009), IVM culture media (Salhab *et al.* 2011), *in vivo* and *in vitro* conditions (Wells and Patrizio 2008) and apoptosis (Li *et al.* 2009). For example, inadequate oocyte IVM was the main reason for the occurrence of polyspermy when developmental competence was compared between MOET and IVEP-derived oocytes (Maalouf *et al.* 2009).

It is widely recognised that the relatively lesser efficiency of IVM is largely due to spontaneous oocyte meiotic resumption after physical removal of COCs from the antral follicle and subsequent culture (for a review, see Gilchrist and Thompson 2007). After such procedure, intra-oocyte cAMP concentrations decrease and meiotic resumption starts spontaneously due to the depletion of inhibitory factors from the follicle. This often leads to asynchrony between cytoplasmic and nuclear maturation, reducing oocyte developmental competence (Zhu *et al.* 2018). Therefore, attempts to maintain relatively greater cAMP concentrations before IVM have been reported to enhance oocyte competence. This increase in oocyte cAMP concentrations may be achieved by different approaches, including invasive adenylate cyclase (AC), stimulators of AC activity, phosphodiesterase inhibitors and cAMP analogues. The use of cAMP modulators in a pre-IVM system and their effects on completion of meiosis and cytoplasmic reorganisation were recently reviewed (Ramos Leal *et al.* 2018). Nevertheless, compared with bovines, there are few reports of biphasic IVM in small ruminants and these involve the use of forskolin and 3-isobutyl-1-methylxanthine (IBMX) in peripubertal ewes (Rose *et al.* 2013) and C-type natriuretic peptide in adult (Zhang *et al.* 2015) or prepubertal (Soto-Heras *et al.* 2019*b*) goats. When roscovitine was tested, although it was efficient in reversibly inhibiting the meiosis of adult sheep oocytes, it did not affect embryo development or quality (Crocomo *et al.* 2016*a*).

Oocyte grade

A grade classification system, with oocytes graded from 1 to 3 (Souza-Fabjan *et al.* 2016) or from 1 to 4 (Almeida *et al.* 2011) based on the number of CC layers and ooplasm homogeneity, is currently used by many laboratories because it is the only 100% non-invasive way to select homogeneous COCs after collection (Fig. 2*c*). However, this strategy has been shown to be a weak predictor of oocyte competence (Almeida *et al.* 2011), resulting in considerable variability in IVEP outcomes. Brilliant cresyl blue (BCB) staining could be a complement to simple assessment of COC morphology because it indirectly estimates oocyte growth, selecting oocytes with a larger diameter in a heterogeneous pool. The mechanism of BCB staining is related to glucose-6-phosphate dehydrogenase (G6PD) activity, which is increased in growing oocytes and decreased when oocytes have finished growing. BCB staining divides the COC population into two groups, namely BCB positive (BCB+; fully grown oocyte) and BCB negative (BCB–; growing oocyte; Paramio and Izquierdo 2016), with BCB+ COCs being more competent than BCB– COCs. In sheep, the developmental competence of BCB+ COCs to the embryo stage was higher, with more active mitochondria and higher MPF activity, ATP content, two pronuclear (PN) zygote formation (monospermic penetration) and blastocyst cell number (Catalá *et al.* 2011, 2012). BCB has been used extensively in small ruminants to select a more homogeneous oocyte pool for IVEP (Catalá *et al.* 2011, 2012) or for gene expression analyses (Bragança *et al.* 2018, 2021).

When BCB is not used, usually only Grade 1 and 2 oocytes are used; oocytes that are partially denuded at the time of collection (DOC; Grade 3) are considered not suitable and thus routinely discarded. We assessed the possibility of using DOCs, collected

either by LOPU or after culling, for the production of additional embryos from genetically valuable females (Souza-Fabjan *et al.* 2016). Interestingly, coculturing DOCs with COCs in equal proportions during maturation had a beneficial effect on the developmental competence of DOCs and did not affect COCs (Souza *et al.* 2013; Souza-Fabjan *et al.* 2016). This strategy enables the greater use of genetic material from females of high economic value and could certainly be a benefit to the industry.

Culture media for oocyte IVM

The basic maturation medium used in most laboratories is tissue culture medium (TCM)-199 supplemented with different types of serum, either fetal calf serum (FCS), sheep or goat serum, follicular fluid or bovine serum albumin (BSA; Tables 1, 2). The IVM medium is often supplemented with hormones such as FSH, LH and/or 17β-oestradiol (Paramio and Izquierdo 2014; Zhu *et al.* 2018). However, although serum can have beneficial effects during oocyte IVM (Shabankareh and Zandi 2010), it is well known that it makes the interpretation of results more difficult, hampers reproducibility and is associated with sanitary risk (Souza-Fabjan *et al.* 2016). For these reasons, there has been a trend to use more defined maturation media.

To make IVM simpler and repeatable, we have proposed a simplified maturation medium that contains only TCM-199, epidermal growth factor (EGF) and cysteamine (Rodríguez-Dorta *et al.* 2007). It is of note that reliable results in cleavage and embryo development rate were obtained for abattoir-derived COCs over many years in goats when using this simplified maturation medium (Rodríguez-Dorta *et al.* 2007; Souza *et al.* 2013), with no differences observed compared with COCs matured in media containing either FCS or BSA (Souza-Fabjan *et al.* 2014*a*). However, LOPU-derived COCs may have different requirements during IVM and/or IVF (Souza-Fabjan *et al.* 2014*a*), indicating that more complex media would be needed to achieve high IVEP rates (Souza-Fabjan *et al.* 2013). Interestingly, these two sources of oocytes (i.e. abattoir and LOPU) may also affect IVM kinetics: abattoir-derived COCs were found to mature significantly faster than LOPU-derived COCs after 18 and 22 h in simplified medium (Souza-Fabjan *et al.* 2014*a*). Currently, we generally use the simplified maturation medium for abattoir-derived COCs and undefined medium with 10% FCS for LOPU-derived COCs (Souza-Fabjan *et al.* 2019).

Additives to enhance IVM

In oocytes from prepubertal females, the harmful effect of the ROS can be reduced by the addition of antioxidants to the IVM medium. The high levels of ROS in these oocytes are due to a reduced ability to synthesise reduced glutathione (GSH; Jiao *et al.* 2013). Thus, lower GSH concentrations have been found in oocytes from juvenile goats during IVM compared with oocytes ovulated from adults (5.6 vs 23.7 pmol per oocyte). The use of cysteamine in the IVM medium for oocytes from prepubertal goats has improved intracytoplasmic GSH concentrations, the formation of the male PN in zygotes and blastocyst development (Rodríguez-Gonzalez *et al.* 2003). Cysteamine has become the conventional antioxidant in the IVM medium for small ruminants. Another antioxidant, melatonin, has been tested with positive results in the IVEP of sheep (Tian *et al.* 2017) and goats (Saeedabadi *et al.* 2018). In prepubertal goat oocytes, the addition of 10^{-7} M melatonin significantly increased blastocyst yield and quality compared with the control group (28% vs 12% blastocysts; Soto-Heras *et al.* 2018). Moreover, melatonin not only reduces intracytoplasmic ROS levels, but also reduces mitochondrial activity and ATP content in prepubertal goat oocytes (Soto-Heras *et al.* 2019*a*).

Natural antioxidants have also been tested to improve oocyte competence during IVM. The addition of 1 μM resveratrol (a compound found in grapes) to the IVM medium of prepubertal goat oocytes improved blastocyst yield up to 20% compared with 7% in the control group (Piras *et al.* 2019). The addition of another natural antioxidant crocetin, an active constituent of saffron, to IVM medium reduced intracytoplasmic ROS levels in prepubertal goat oocytes but did not improve blastocyst production (Menéndez-Blanco *et al.* 2020).

Based on the results of these experiments with antioxidants, it can be concluded that they are useful for improving oocyte competence at specific and defined concentrations. High concentrations of antioxidants or a mixture of them in IVM media may be harmful for the fragile COCs. When using COCs with low developmental competence, such as those from prepubertal females, the IVM medium should be supplemented with more compounds than used in IVM medium for more robust and competent COCs. These supplements include hormones, growth factors and antioxidants, all added to the medium at appropriate concentrations (Table S1).

Other substances have been tested as supplements of IVM media. Supplementation of IVM media with insulin–transferrin–selenium and ascorbic acid did not improve the blastocyst rates for BCB– COCs lamb oocytes (Catalá *et al.* 2013). Curiously, supplementation of IVM medium with 200 μM mL^{-1} α-linoleic acid, which is normally found in high concentrations in the follicular fluid, decreased both nuclear maturation and CC expansion in prepubertal sheep oocytes (Ghaffarilaleh *et al.* 2014) and blastocyst yield in adult ovine oocytes (Amini *et al.* 2016). Conversely, sericin had a significant effect on nuclear and cytoplasmic maturation and enhanced the development of ovine embryos (Aghaz *et al.* 2015). Finally, a low concentration of cerium dioxide nanoparticles enhanced IVEP outcomes for prepubertal sheep oocytes (Ariu *et al.* 2017).

Regarding IVM conditions, the literature does not vary substantially. We usually perform IVM by incubating groups of COCs (40–60) in 4-well plates with 500 μL medium, under 5% CO_2 in air at 38.8°C with maximum humidity for 22–24 h (Souza-Fabjan *et al.* 2019).

In vitro fertilisation

During fertilisation, spermatozoa must traverse the CC surrounding the oocyte, bind to a mature oocyte, penetrate the zona pellucida (ZP) and fuse with the oolemma. Subsequently, the oocyte activates, allowing the spermatozoa process within oocyte cytoplasm, culminating in PN formation (Swain and Pool 2008). To reproduce this *in vitro*, most IVEP laboratories separate these processes into three parts: (1) sperm selection;

(2) sperm capacitation; and (3) coculture of spermatozoa and an oocyte.

Sperm selection, capacitation and media

In both goats and sheep, spermatozoa are usually selected using a Percoll gradient (45%/90%) in the case of frozen–thawed spermatozoa (Garcia-Garcia *et al.* 2007; Wan *et al.* 2009; Heidari *et al.* 2013; Wang *et al.* 2013; Souza-Fabjan *et al.* 2016) or the swim-up method in the case of fresh semen (De *et al.* 2011; Shabankareh and Akhondi 2012; Wani *et al.* 2012; Shirazi and Motaghi 2013; Hammami *et al.* 2014). However, comparing both techniques, Rho *et al.* (2001) demonstrated that the total number of goat spermatozoa recovered using a Percoll gradient is approximately fourfold greater than that recovered by the swim-up technique, and with no difference in the progression of the acrosome reaction after capacitation. Presumptive zygotes were examined for PN formation 6, 12, 18 and 24 h post insemination. At 12 h, the rate of PN formation was greater in Percoll-selected spermatozoa than in spermatozoa selected using the swim-up technique (Rho *et al.* 2001), suggesting that the former is a superior technique for the separation of spermatozoa. However, it is important to highlight that Rho *et al.* (2001) used frozen–thawed goat semen. Therefore, additional studies with fresh semen are needed to confirm these findings.

Sperm capacitation occurs *in vivo* following removal of the decapacitating factors (basically proteins and other substances that cover the sperm membrane) present in the seminal plasma. During this process, the main changes are biochemical and consist of cholesterol removal, which increases the fluidity of the sperm membrane (Langlais *et al.* 1988), entry of Ca^{2+} into the cell (Singh *et al.* 1978), an increase in intracellular cAMP concentrations (White and Aitken 1989) and changes in enzyme activities (e.g. protein kinase C involved in the signal transduction mechanism that triggers the acrosome reaction; Florman and First 1988). These biochemical changes promote a transient change in the pattern of sperm motility, called hyperactivation (Yanagimachi 1994). To complete the process, hyperactivated spermatozoa bind via plasma membrane receptors to specific proteins in the oocyte ZP area to induce an acrosome reaction (Florman and First 1988). In the case of small ruminants, to induce these modifications *in vitro*, several agents are used, such as oestrus sheep serum (ESS) or oestrus goat serum, heparin, ionomycin and PHE (penicillamine, hypotaurine and epinephrine), before incubation of spermatozoa for 15–60 min and/or during the coculture of spermatozoa with oocytes (for a review, see Paramio and Izquierdo 2014). We routinely use 10% ESS with heparin (5 μg mL^{-1}) during goat IVF. The combination of these agents has a beneficial effect on the blastocyst production rate (with (54%) vs without (42%) heparin; $P < 0.05$), but has no effect on cleavage (Souza *et al.* 2013).

For IVF success, it is essential that the medium used is able to provide the secondary oocyte and spermatozoa with ideal conditions for penetration to occur as quickly as possible (Gordon 1994). Currently, most ovine IVEP laboratories use synthetic oviductal fluid (SOF) for IVF (Leoni *et al.* 2007; Bai *et al.* 2008; Wang *et al.* 2013), whereas the Tyrode's albumin–lactate–pyruvate (TALP) is widely used for IVF in goats (Kątska-Książkiewicz *et al.* 2007; Hammami *et al.* 2013). However, our results demonstrate the possibility of also using SOF in goats and achieving high cleavage (72–88%) and blastocyst (27–51%) rates (Souza *et al.* 2013; Souza-Fabjan *et al.* 2016).

Sperm concentration and coincubation with COC

To maximise the fertilising capacity of the semen sample while minimising polyspermy, tests with different sperm concentrations should be performed to determine the ideal concentration for each animal. Currently, the sperm concentration used varies between 0.5 and 4.0×10^6 spermatozoa mL^{-1} (Table 3), calculated based on motility and the live sperm population obtained after selection, but high concentrations have also been reported, such as 10×10^6 spermatozoa mL^{-1} (Menchaca *et al.* 2018) or even 80×10^6 spermatozoa mL^{-1} (Khatun *et al.* 2011). In a recent study in goats, we did not observe any effect of different sperm concentrations (1, 2 or 4×10^6 cells mL^{-1}) on the penetration rate (67%, 69% and 74% respectively); however, using 4×10^6 cells mL^{-1} significantly decreased rate of monospermy rate (>20% of difference), with no effect on IVF efficiency (G. M. Bragança, A. S. Alcantara-Neto, R. I. T. P. Batista, F. Z. Brandão, V. J. F. Freitas, P. Mermillod, J. M. G. Souza-Fabjan, unpubl. data).

The IVF step usually takes between 16 and 24 h in different laboratories (Table 3). However, Beilby *et al.* (2011) demonstrated that 5 h was long enough to reach a 63% cleavage rate and a 57% rate of blastocyst formation (as a percentage of oocytes cleaved). In addition, Beilby *et al.* (2011) confirmed that the current sexing procedures using flow cytometry do not compromise embryo development *in vitro* or the expression of genes associated with epigenetic changes (e.g. DNA methyltransferase 3a (*DNMT3*), suppressor of variegation 3–9 homologous 1 (*SUV39H1*)), cell stress (e.g. heat shock protein 70 (*HSP70*)), oxidative stress (e.g. *G6PD*) and cell metabolism (e.g. solute carrier family 2 member 3 (*SLC2A3*). Nevertheless, IVEP rates in bovines are often lower after IVF with sexed spermatozoa (Morton *et al.* 2007; Palma *et al.* 2008).

Polyspermy (i.e. penetration of the oocyte cytoplasm by more than one spermatozoon) is a pathological condition in placental mammals that usually results in early embryo death. In general, polyspermy is related to lower-quality oocytes, and this is the main reason why it occurs more frequently in prepubertal goats. Polyspermy is common in IVF due to the low competence of IVM oocytes to ensure monospermic fertilisation and flaws in the process of mimicking the oviductal environment. Research has shown that the exposure of pig oocytes to oviduct fluid (OF) before (Coy *et al.* 2008) or during (Batista *et al.* 2016) IVF reduces the incidence of polyspermy. In this sense, we have assessed the role of OF at three sperm concentrations (1, 2 and 4×10^6 cells mL^{-1}). The medium consisted of 10% ESS (control) with the different concentrations of spermatozoa (CTRL1, CTRL2 and CTRL4 respectively) or the same medium plus 10% OF (OF1, OF2 and OF4 respectively). When comparing OF vs CTRL at the same sperm concentration, OF had no effect on the penetration and monospermy rates, or on cleavage and blastocyst rates. However, when data were plotted regardless of sperm concentration, OF increased the production of monospermic zygotes and tended to enhance IVF efficiency in

Table 3. Recent results of parameters related to fertilisation, polyspermy and pregnancy of oocytes recovered from adult and prepubertal goats and sheep

Note, the cleavage rate represents the number of cleaved oocytes relative to the number of oocytes undergoing maturation. Only studies reporting either polyspermy or pregnancy rates are included in the table. COCs, cumulus–oocyte complexes for IVF; DOP, oocytes denuded on purpose before IVF; LOPU, laparoscopic ovum pick-up; OVX, ovariectomy; Prep, prepubertal; [Sperm], sperm concentration

	Oocyte origin and type	IVF (h)	[Sperm] ($\times 10^6$ mL^{-1})	No. oocytes	[Sperm] (mL^{-1} per oocyte)	IVF medium (μL) per oocyte	Cleavage rate (%)	% Blastocysts		Polyspermy rate (%)	Pregnancy rate (%)	References
								From cleaved oocytes	From COCs			
Goat												
Adult	Abattoir											
	COC	18	2	25	8×10^3	4	–	–	–	–	20–60	Ferreira-Silva *et al.* (2017)
		22	2	45–50	$2–2.2 \times 10^4$	10–11	66–77	60–70	43–47	28–37	–	Souza-Fabjan *et al.* (2014*a*)
		27	80	5	8×10^5	10	–	–	–	16–22	–	Khatun *et al.* (2011)
	DOP	5	4	20	2×10^4	5	75–80	9–30	7–24	30–47	–	Han *et al.* (2008)
		18	1	20–30	$1.7–2.5 \times 10^4$	17–25	63–83	28–90	16–66	–	14–92	Rodríguez-Dorta *et al.* (2007), Wang *et al.* (2017)
Prep	Abattoir											
	COC	12–28	0.5	14–16	3.5×10^3	6–7	–	–	–	0.5–2	–	Palomo *et al.* (2010)
			1	14–16	7×10^3	6–7	–	–	–	1–2	–	
			2	14–16	1.4×10^4	6–7	–	–	–	2–12	–	
			4	14–16	2.8×10^4	6–7	–	–	–	4–15	–	
		20	4	25–30	$1.3–1.6 \times 10^4$	3–4	51–88	15–46	9–36	16–39	–	Soto-Heras *et al.* (2018, 2019*a*)
Sheep												
Adult	LOPU											
	DOP	22	1	5	1×10^4	10	83	–	–	–	41	Fang *et al.* (2016)
		20–24	1	40–50	$2–2.5 \times 10^4$	10–12	84–88	26–38	22–33	–	31–50	Lahoz *et al.* (2013)
			100	15	6.6×10^3	3	54–73	33–58	19–43	–	16–25	Cocero *et al.* (2011)
	Abattoir											
	COC	24	0.5	30–40	$6.3–8.3 \times 10^3$	12–16	88	50	44	3–6	–	Bai *et al.* (2008)
		12	1	10–20	$5–10 \times 10^3$	5–10	41–72	21–40	9–29	11–33	–	Davachi *et al.* (2014)
		20	1	15	3.3×10^3	3	–	–	–	–	53	Wei *et al.* (2016*b*)
		22	1	25–30	$3.3–4 \times 10^3$	3–4	–	–	–	–	30–43	dos Santos-Neto *et al.* (2017)
		16–18	2	40–50	$2–2.5 \times 10^4$	10–12	10–90	5–59	1–45	4–17	–	Moawad *et al.* (2012, 2013)
		24	2–4	40–50	$2–5 \times 10^4$	10–12	23–68	15–46	4–31	4–16	–	Maalouf *et al.* (2009)
	DOP	20	1	10–15	$3.3–5 \times 10^4$	3–5	79–84	37–52	31–43	–	83–85	Mara *et al.* (2014)
		24	2–4	40–50	$2–5 \times 10^4$	10–12	46–73	19–38	12–28	8–21	–	Maalouf *et al.* (2009)
		20–24	100	15	6.6×10^3	3	74–80	34–40	27–30	–	9	Cocero *et al.* (2011)
Prep	LOPU											
	COC	24	2	30	3.3×10^4	16	39–75	–	–	–	30–39	Gou *et al.* (2009)
	DOP	24	1	5	1×10^4	10	59	–	–	–	24	Fang *et al.* (2016)
	Abattoir											
	COC	20	1	15	3.3×10^3	3	58–79	7–26	4–21	5–6	–	Catalá *et al.* (2011)
	OVX											
	COC	24	0.5	30–40	$6.3–8.3 \times 10^3$	12–16	80–84	25–34	21–27	4–26	–	Bai *et al.* (2008)

terms of the production of normally fertilised zygotes. It is of note that analysis of OF protein composition revealed the presence of the main proteins involved in modulating fertilisation, namely oviduct-specific glycoprotein 1 (OVGP1), annexin A1 (ANXA1), HSP70, myosin-9 (MYH9) and heat shock protein-A8 (HSPA8) (G. M. Bragança, A. S. Alcantara-Neto, R. I. T. P. Batista, F. Z. Brandão, V. J. F. Freitas, P. Mermillod, J. M. G. Souza-Fabjan, unpubl. data).

Other factors during IVF that affect IVEP output

An important parameter that can compromise IVEP is the presence or absence of CCs during IVF. Souza *et al.* (2013) and dos Santos-Neto *et al.* (2020) demonstrated a beneficial effect of CCs during caprine and ovine IVF respectively. In sheep, the presence of CCs during IVF resulted in a greater cleavage rate (85% vs 75%) and enhanced development (36% vs 16%) compared with oocytes denuded before IVF. Similar improvements have been reported in goats, with a higher blastocyst yield (48% vs 37%) when CCs were maintained during IVF, regardless of whether they were in intimate contact with the oocyte or had only been added to the well (Souza *et al.* 2013). These data suggest that in addition to its role as an additional barrier in controlling polyspermy, the presence of CCs may contribute to beneficial factors for the interaction between spermatozoa and oocytes or minimise the negative effects of high oxygen tension during IVF. Corroborating this hypothesis, Leoni *et al.* (2007) demonstrated that a low (5%) O_2 atmosphere during IVF positively affected the production of high-quality sheep blastocysts when compared with 20% O_2. The IVF conditions we routinely use have been described in detail elsewhere (Souza-Fabjan *et al.* 2019).

***In vitro* development**

In the final step of IVEP, which takes 6–8 days after IVF in ruminants, the zygotes undergo significant transformations that enable them to implant themselves in the uterine cavity. Morphologically, these transformations involve cleavage, compaction and embryo cavitation, whereas biochemically the transformations are associated with changes in protein synthesis, energy demand and amino acid absorption. To drive these transformations, *in vitro* culture environments need to reproduce the environment found in the oviduct, including osmolality, ion composition, temperature, pH, CO_2, oxygen, carbohydrates, amino acids, lipids, fatty acids, proteins, growth factors and cytokines. Currently, 50–60% of presumed zygotes do not reach the blastocyst stage due, in part, to a failure to adequately mimic oviduct conditions. In ruminants, the block in embryo development occurs at the 8- to 16-cell stage, which corresponds to the period of the maternal–zygotic transition (Gordon 1975; Fehilly *et al.* 1984).

Embryo culture media

In the late 1980s, Gandolfi and Moor (1987) showed that the coculture of presumed zygotes with oviductal epithelial cells (OEC) drives the development of sheep embryos beyond the 8- to 16-cell stage in a 20% O_2 atmosphere. Later studies tested several cell types and proved beneficial effects during IVD (Rho *et al.* 2001; Kątska-Książkiewicz *et al.* 2007), with interspecific effects (Izquierdo *et al.* 1999). The most commonly used somatic cells were OEC, granulosa cells and Vero cells, among others (Gordon 1994). The benefit of adding somatic cells to the culture is the production of growth factors (EGF, tumour growth factors α and β1) and the removal of inhibitory components from the culture medium, such as free radicals, cell metabolites and ammonia, among others (Thompson 1996, 2000). However, the concentrations of cell-secreted compounds differ depending on the physiological status of the cell, meaning that the results are not always predictable. Moreover, it is not always possible to identify the compounds, which prevents determination of exact embryo requirements (Gordon 1994). For this reason, in the 1990s, the coculture system was replaced by other systems. SOF (Tervit *et al.* 1972) is based on sheep OF and is currently the primary medium used for IVD, often supplemented with serum for small ruminants. However, as noted above with regard to IVM, the composition of the serum is undefined and variable. In addition, serum significantly reduces the morphofunctional, cellular and molecular competence of embryos, which reflects their ability to establish pregnancy (Young *et al.* 1998).

The presence of serum in the embryo culture medium can also induce the accumulation of cytoplasmic lipid droplets, making the embryos darker and less tolerant to cryopreservation (Barrera *et al.* 2018; Davachi *et al.* 2018). Rodríguez-Dorta *et al.* (2007) compared two undefined IVD systems, namely control (SOF with 10% fetal calf serum (FCS) in a 5% O_2 atmosphere) and a coculture of a goat OEC (GOEC) monolayer in SOF with 3 mg mL^{-1} BSA in a 20% O_2 atmosphere, and observed a greater blastocyst rate in the control system (28% vs 20%). However, when embryo quality was challenged by vitrification followed by transfer, Rodríguez-Dorta *et al.* (2007) observed greater rates of pregnancies and offspring born for GOEC embryos (56% and 33% respectively) than SOF embryos (14% and 9% respectively). These results indicate that GOEC embryos acquired a better cryotolerance than those developed in SOF under 5% O_2. Despite collateral effects of serum, most laboratories still work with an undefined system for small ruminant IVD (Tables 1, 2). An alternative to replacing serum has been supplementation with BSA, a system known as 'semi-defined'. Albumin appears to play an important role in the nutrition of the developing embryo, especially after compaction. When SOF supplementation with BSA was compared with supplementation with 5% FCS, a similar blastocyst rate was reported in both systems (22% vs 24% respectively); however, the hatching capacity was greater for blastocysts in the FCS-supplemented system (44% vs 87%; Garcia-Garcia *et al.* 2007).

The evidence suggests that the exposure of zygotes to serum during IVD may induce the large offspring syndrome (LOS). *In vitro* culture of sheep zygotes derived from maturation and fertilisation *in vivo* revealed that the manifestation of excessive fetal size depended on the serum source and the presence or absence of granulosa cells (Sinclair *et al.* 1997, 1998). LOS is characterised by the manifestation of phenotypes such as macrosomia (increased body size), macroglossia (enlarged tongue), omphalocoele (umbilical hernia), abnormal organ growth and abnormal placental development (Young *et al.* 1998). Evidence for serum as the source of the LOS-inducing factor(s) came from the transfer of embryos grown *in vitro* to

the blastocyst stage in defined and serum-free media. When SOF supplemented with BSA and amino acids was used, the mean birth weight and incidence of abnormalities were similar to those of controls derived from embryos *in vivo* (Thompson *et al.* 1995; Sinclair *et al.* 1997). Molecular studies suggest that epigenetic changes are responsible for the manifestation of LOS. Young *et al.* (2001) reported that ovine fetuses exposed to the *in vitro* environment show a reduction in methylation and expression of the insulin-like growth factor 2 receptor (*IGF2R*) imprinting gene.

Unlike IVD static models, embryo development during the preimplantation period occurs in a dynamic fluid, which changes as the embryo moves towards the uterus. These changes coincide with changes in the nutritional demands of the embryo. For example, amino acid requirements change as the embryo develops from the cleavage to the blastocyst stage, as demonstrated in sheep (Gardner *et al.* 1994). In the precompaction stages, when the embryo resides in the oviduct, the internal fluid is characterised by relatively high concentrations of pyruvate and lactate and a low concentration of glucose. Conversely, uterine fluid is characterised by relatively low levels of pyruvate and lactate and a higher concentration of glucose (Gardner *et al.* 1996; Leese *et al.* 2001). Therefore, by the time the embryo is in the reproductive tract, there is a decreasing gradient of pyruvate and lactate from the oviduct to the uterus, whereas the reverse is true for glucose. These changes in carbohydrate levels reflect the shift from a carboxylic acid-based metabolism to a glucose-based metabolism in the embryo. Thus, sequential media have been formulated to reflect carbohydrate levels, which change throughout the reproductive tract (Lane *et al.* 2003). In small ruminants, several studies have shown that this strategy can support embryo development up to the blastocyst stage in both goats (Koeman *et al.* 2003) and sheep (Garcia-Garcia *et al.* 2007), although it could compromise hatching rates (Garcia-Garcia *et al.* 2007).

Other embryo culture conditions

The O_2 concentration in mammal oviducts is around 2–8% (Mastroianni and Jones 1965; Yedwab *et al.* 1976; Fischer and Bavister 1993). This relatively low concentration compared with the atmospheric concentration (20%) can result in minimal ROS levels and thus protect gametes and embryos against stress (Catt and Henman 2000). Therefore, to prevent the deleterious effects associated with oxidative stress, when working in a 20% O_2 atmosphere many authors report supplementing the IVD media with antioxidants such as cysteamine (De *et al.* 2011), α-tocopherol (Natarajan *et al.* 2010*a*) or L-ascorbic acid (Natarajan *et al.* 2010*b*). However, supplementation of IVD media with exogenous antioxidants under 5% O_2 tension has no effect on blastocysts (Amiridis and Cseh 2012). Currently, most laboratories are performing embryo culture using semi-defined media with little or no serum and low O_2 tension (unlike for IVM and IVF), without cell coculture. However, research using defined media and replacing the protein source has remained a target of interest since the early 1990s. The IVD conditions we routinely use are described in detail elsewhere (Souza-Fabjan *et al.* 2019).

Cryopreservation of IVP embryos

Mammalian embryos can be cryopreserved by either conventional slow freezing (SF) or vitrification (VIT). SF is characterised by a progressive lowering of temperature and the use of low concentrations of cryoprotectants, which are toxic. VIT is a fast technique that uses high concentrations of cryoprotectants (therefore high toxicity) before placing embryos in liquid nitrogen. The first kid born after transfer of an embryo cryopreserved using SF was reported by Bilton and Moore (1976), whereas the first kid born from a vitrified embryo was reported 14 years later (Yuswiati and Holtz 1990). VIT has become a useful method for embryo cryopreservation because it is faster, simpler, does not require expensive equipment and is more effective than SF (El-Gayar and Holtz 2001).

VIT has also been tested for goat IVEP. Ferreira-Silva *et al.* (2017) reported similar pregnancy (60% and 50%) and kidding (40% and 35%) rates for SF and VIT with dimethylsulfoxide, whereas VIT with dimethylformamide showed poorer results (pregnancy, 20%; kidding, 12.5%). However, Yacoub *et al.* (2010) reported significantly better kidding rates after the transfer of VIT rather than SF blastocysts, although the superiority of VIT did not apply to caprine morulas and hatched blastocysts. In sheep, Bettencourt *et al.* (2009) compared SF and VIT for the preservation of Portuguese merino sheep embryos and observed no difference in embryo survival and pregnancy rates. Recently, dos Santos-Neto *et al.* (2017) reported greater pregnancy rates for MOET- than IVEP-derived embryos in sheep (69% vs 30%) and improved birth rates after VIT with Cryotop compared with SF. The same authors reported that, with the Cryotop method, a pregnancy rate of >50% was achieved in ewes receiving IVEP embryos (Menchaca *et al.* 2016).

Different embryo stages can exhibit different degrees of cryotolerance. For example, in the goat, the survival of morulas is lower after both SF (Li *et al.* 1990) and VIT (Yacoub *et al.* 2010; Gibbons *et al.* 2011) compared with that of blastocysts. Yacoub *et al.* (2010) did not find any difference in kidding rates between not hatched and hatched blastocysts after either VIT or SF. In sheep, the survival of morulas and 16- to 32-cell embryos was significantly lower than those of more advanced embryos in IVP-sheep embryo subjected to VIT (Li *et al.* 2020). Therefore, expanded blastocysts are the most suitable stage for embryo VIT (Li *et al.* 2020). In general, it is well accepted that as the embryo develops, its viability after freezing increases.

Studying different parameters affecting embryo cryopreservation, Morató *et al.* (2011) tested the effects of oocyte donor age (adult vs prepubertal goat) and blastocyst stage (non-expanded, expanded, hatching and hatched) on the ability of the blastocyst to survive the VIT and warming procedures. Total blastocyst survival was not affected by age (41% in adults vs 52% in prepubertal goats). However, expanded and hatched blastocysts showed the greatest *in vitro* embryo survival rates from adult goat COCs, whereas hatching blastocysts were the most cryotolerant from prepubertal goat COCs (Morató *et al.* 2011). In sheep, Li *et al.* (2020) recently tested the antifreeze protein (AFP) from *Anatolica polita* as cryoprotectant in SF and VIT protocols and observed better cryopreservation survival in high-speed embryos (i.e. embryos reaching the blastocyst stage

6–6.5 days after IVF) than slow-speed embryos (reaching the blastocyst stage 7–8 days after IVF). Li *et al.* (2020) concluded that the AFP they used did not increase the freezing efficiency of the high-speed embryos, but the addition of 10 μg mL^{-1} AFP increased the hatching rate of slow-speed embryos.

Future developments in small ruminant IVEP

Regardless of the IVEP system used, surely the only ideal environment for embryo preimplantation development is in the oviduct and uterus (Rizos *et al.* 2002). The development of techniques that combine the advantages of *in vivo* and *in vitro* systems, such as the intrafollicular transfer of immature oocytes, has been proposed to increase embryo quality in bovine species (Kassens *et al.* 2015); however, to date, there are no reports of such techniques in small ruminants. Curiously, we first demonstrated that the caprine uterine horn may support the nuclear maturation of bovine oocytes (*ex situ* maturation) in addition to reducing the amount of lipids in the cytoplasm compared with IVM oocytes (Fonseca *et al.* 2019*b*). This technique still needs to be improved and used in a greater number of animals, but hypothetically it may represent an important alternative for enhancing oocyte quality before using the oocytes in IVF or subjecting them to cryopreservation.

IVEP media consisting of completely defined or semi-defined reagents have been developed for small ruminants. However, as evidenced in this review, improvements are still needed to increase the maturation and blastocyst formation rates. Follicular fluid proteomics could be an important approach to achieving these objectives. Follicular fluid makes up the microenvironment in which the oocyte develops (Sirard *et al.* 2003), and can provide insights into the processes occurring during follicle development because of its intimate proximity with maturing oocytes. The effects of follicular fluid on the IVM, IVF and IVD have been studied in domestic animals, especially cattle (Lopes *et al.* 2019). In goats, a study was conducted to characterise the major proteome of ovarian follicular fluid, with the authors reporting that protein concentrations were similar in fluid from small, medium and large follicles (Paula Junior *et al.* 2018). However, the zinc-α-2-glycoprotein-like, complement factor B and complement C3 concentrations were greater in the fluid from large than medium follicles (Paula Junior *et al.* 2018). In sheep follicular fluid, Wu *et al.* (2018) observed 243 differentially expressed proteins between lambs and ewes, including 155 that were downregulated and 88 that were upregulated. The abundance of many of the proteins known to affect follicle development was lower in lambs (e.g. ADAM metallopeptidase with thrombospondin type 1 motif 9 (ADAMTS 9), CD14 molecule (CD14), catenin beta 1 (CTNNB1), follistatin (FST), glutamate-cysteine ligase catalytic subunit (GCLC), heparan sulphate proteoglycan 2 (HSPG2), insulin-like growth factor-binding protein 2 (IGFBP2), insulin-like growth factor-binding protein 6 (IGFBP6), inhibin subunit beta A (INHBA), prolactin (PRL), pappalysin 1 (PAPPA), periostin (POSTN), peroxiredoxin 1 (PRDX1), serpin family A member 1 (SERPINA1), superoxide dismutase 3 (SOD3), stanniocalcin 1 (STC1) and vascular endothelial growth factor C (VEGFC)). The proteomics studies led to two main conclusions: (1) the molecules described may be potential biomarkers of oocyte competence; and (2) the requirements for oocyte IVM could be used in the future as a guide for inexpensive and more appropriate formulation of oocyte culture conditions.

Conclusions

The need to accelerate the breeding of and genetic improvements in small ruminants worldwide, as well as the possibility of using these animals for biomedical research, highlights the role of IVEP. Although the logistics for COC recovery by LOPU are more complex and expensive in small ruminants than in bovines, the overall IVEP outcomes in small ruminants are similar to those reported in bovines. In this context, there is no technical reason for such a low number of IVP embryos worldwide, but this low number is probably related to demand. Surely it is important to increase the LOPU recovery rate of COCs with high developmental competence to achieve a positive benefit : cost ratio. Ideally, efforts to use more defined media should be increased. Despite the recent progress in small ruminant IVEP in recent years, if only the blastocyst rate is considered, then the overall efficiency in recent studies is remarkably similar to that reported by research groups two to three decades ago. However, it should be noted that the quality of the embryos is increasing, resulting in IVP embryos with greater cryotolerance, which is extremely important for the industry.

Conflicts of interest

The authors declare no conflicts of interest.

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