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The Efficiency of *In vitro* Ovine Embryo Production Using an Undefined or a Defined Maturation Medium is Determined by the Source of the Oocyte

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Contents

In vitro oocyte maturation can be influenced by oocyte source and maturation media composition. The aim of the present study was to compare the efficiency of a defined *in vitro* maturation medium (TCM199 supplemented with cysteamine and epidermal growth factor (EGF); Cys + EGF) with an undefined medium (TCM199 supplemented with follicle-stimulating hormone and follicular fluid; FSH + FF) for *in vitro* production (IVP) of ovine embryos, using oocytes obtained by laparoscopic ovum pick-up from FSH-stimulated [n = 11; 158 cumulus-oocyte complexes (COCs)] and non-stimulated (n = 16; 120 COCs) live ewes, as well as abattoir-derived oocytes (170 COCs). The produced blastocysts were vitrified and some of them were transferred to synchronized recipients. The best and the worst final yields of embryo IVP observed in this study were obtained using oocytes from FSH-stimulated ewes matured in FSH + FF (41.3%; 33/80) and in Cys + EGF (19.2%; 15/78) medium, respectively ($p < 0.01$). No significant differences between both media were attained in the blastocyst development rate or in the final yield of embryo IVP using oocytes from non-stimulated ewes or abattoir-derived oocytes. The overall *in vivo* survival rate of the transferred vitrified blastocysts was 13.1% (8/61), without significant differences between oocyte sources or maturation media. In conclusion, under the experimental conditions of the present study, TCM199 supplemented with cysteamine and EGF is a convenient defined maturation medium for IVP of embryos from oocytes of live non-stimulated ewes or from oocytes of abattoir-derived ovaries. However, the best final yield of embryo IVP observed in this study was attained when oocytes came from FSH-stimulated donors and TCM199 was supplemented with FSH and follicular fluid.

Introduction

Based on the pioneer studies (Moor and Trounson 1977; Staigmiller and Moor 1984), procedures have been developed for ovine *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). However, in the ovine species, as in most mammalian species examined, oocytes matured *in vitro* are compromised in their developmental capacity compared with oocytes matured *in vivo* (Sutton et al. 2003). While the causes of these differences are not fully elucidated, it is known that the heterogeneous origin of the used oocytes (namely, different stages of the oestrous cycle and stage of folliculogenesis) makes oocyte quality variable (Lonergan and Fair 2008).

Oocyte developmental competence (or oocyte quality) may be defined as its ability to mature, be fertilized and give rise to normal and fertile offspring after normal gestation (Mermillod et al. 2008). The ability of oocytes to mature includes the successful completion of two cellular steps: nuclear maturation, i.e. reaching metaphase II stage, and cytoplasmic maturation, i.e. the molecular and structural changes that allow the oocyte to support fertilization and embryonic development (Bevers et al. 1997). *In vivo* acquisition of oocyte developmental competence occurs continuously throughout folliculogenesis and the influences of the follicular size and atresia on oogenesis have been reviewed (Mermillod et al. 1999). However, in addition to the heterogeneous nature of oocytes used for *in vitro* production (IVP), *in vitro* oocyte maturation can be influenced by culture media composition and the culture conditions used for IVM (Cognié et al. 2004). It was demonstrated that gonadotrophins, even without steroid supplementation, induces nuclear maturation of sheep oocytes and that the addition of oestradiol 17-β (E2) to medium containing follicle-stimulating hormone (FSH) and luteinising hormone (LH) increases blastocyst rates (Moor and Trounson 1977). As such, the most commonly used system for the maturation of oocytes outside the follicle (from now on, the standard maturation medium) is TCM199 medium supplemented with FSH, LH, oestradiol (E2) and 10% foetal calf serum (FCS).

On the other hand, pioneer experimental results demonstrated that follicular fluid (FF) is a useful supplement in IVM medium as an adjunct to, rather than replacement for, the standard serum and hormone supplements (Sun et al. 1994). It is known that FF contains high amounts of steroid hormones (Ainsworth et al. 1980), while concentrations of gonadotropins are similar to those found in plasma (Fortune and Hansel 1985). It also contains a range of proteins, growth factors, energy substrates and unknown compounds (Sutton et al. 2003). Unfortunately, FF and FCS are undefined components whose batch-to-batch consistency is low, thus contributing to the lack of reproducibility often observed in IVP laboratories. In this way, FF obtained from non-atretic sheep follicles supported oocyte developmental competence better than FF from atretic follicles when FF was used as substitute for serum in IVM media, although the size of the follicle from which the FF is sourced had little influence on embryo development (Cognié et al. 1995).

With the dual purpose of finding efficient defined media and elucidating the role of different factors in IVM, few authors made comparisons between some defined and undefined maturation media. Hence, epidermal growth factor (EGF), naturally present in FCS

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as well as in FF, has been shown to have a positive effect during IVM in bovine (Lonergan et al. 1996). In the same way, it was shown that EGF, FSH and estradiol play a key role in the nuclear and cytoplasmic maturation of oocytes, although the cleavage and blastocyst development rates were lower in defined media than when using FF and FSH. These authors hypothesized that while ovine FSH and EGF would increase the final embryo production by acting on nuclear maturation, FF would act by stimulating cytoplasmic maturation, through the presence of other regulatory molecules (Guler et al. 2000). One of them may be the novel peptide (Mr 26.6 kDa) isolated from buffalo ovarian FF, which is present in all categories of follicles. When the basic IVM medium is supplemented with this peptide, there is a dose-dependent increment in the cumulus expansion as well as *in vitro* maturation rates of ovine oocytes (Gupta et al. 2005).

Another experimental approach to improve the IVP results is the addition of glutathione synthesis promoters to IVM medium (recently reviewed by Deleuze and Goudet 2010). Glutathione is the major non-protein sulphydryl compound in mammalian cells and is known to play an important role in protecting cells from oxidative damage. In this way, it has been demonstrated that cysteamine (Cys) enhances glutathione synthesis, improving the efficiency of IVP when present during *in vitro* maturation of abattoir-derived ovine oocytes in the standard medium (De Matos et al. 2002). Moreover, the capacity for embryonic development and foetal development of IVP blastocysts proceeding from abattoir-derived sheep oocytes matured in the standard medium supplemented with cysteamine has been proved by transfer of both fresh and vitrified embryos (Dattena et al. 2007). In a recent report (Shabankareh and Zandi 2010), it has also been shown the beneficial effect of cysteamine, in addition to EGF and insulin-like growth factor I (IGF-I), on the blastocyst rates using abattoir-derived sheep oocytes matured in a defined medium, consisting essentially in the standard maturation medium in which the FCS was replaced by polyvinyl alcohol, compared to the same medium without Cys. Satisfactory IVP results were reported using a more simple defined medium consisting of TCM199, EGF and cysteamine for the maturation of abattoir-derived goat (Cognié et al. 2002) or sheep (Cognié et al. 2004) oocytes, as well as oocytes from FSH-stimulated live goats (Locatelli et al. 2008). Nevertheless, we have not found any reports about the use of this glutathione precursor in defined media for in vitro maturation of oocytes from live sheep.

Most studies carried out to identify the hormonal and cellular factors affecting *in vitro* maturation and subsequent embryonic development of sheep oocytes have been performed using abattoir ovaries. The aim of the present study was to compare the efficiency of a defined IVM medium (TCM199 + Cys + EGF) with an undefined medium (TCM199 + FSH + FF) for *in vitro* production of ovine embryos, using laparoscopic ovum pick-up (LOPU)-derived oocytes from FSH-stimulated, non-stimulated donor ewes and abattoir-derived oocytes.

Materials and Methods

Unless otherwise specified, all materials were obtained from Sigma-Aldrich (Madrid, Spain). All experimental procedures were performed in accordance with the current European Directive 86/609/EEC (DOCE number 358). All FSH used in the present study was highly purified porcine FSH, supplied by the Laboratory of Endocrinology, Faculty of Veterinary Medicine, University of Liege, Belgium. Quantities are expressed in mg of the NIH-FSH-P1 standard (1 mg NIH-FSH-P1 = 0.038 mg NIADDK-oFSH-17).

Experimental design

Cumulus-oocyte complexes (COCs) were obtained in two trials, carried out in the breeding season, using 27 ewes. Within each trial, ewes were randomly allocated into two groups: FSH-stimulated (LOPU-FSH group; n = 6 and n = 5 in trials 1 and 2, respectively) and non-stimulated (LOPU-C group; n = 8 in each trial). Within each trial, donor ewes were subjected to two LOPU sessions 1 week apart, as described thereafter. Coinciding with one LOPU session, abattoir-derived COCs were obtained from ovaries collected in a local slaughterhouse and processed for embryo IVP.

The COCs from the three sources studied (LOPU-FSH; LOPU-C; abattoir) were obtained and processed on the same days and with identical laboratory conditions. On each session, the recovered COCs from each source were pooled and randomly distributed between the two *in vitro* maturation media compared. Developmental competence of *in vitro* matured oocytes was assessed in terms of cleavage 48 h after IVF and blastocyst rates at days 7 and 8 post-fertilization. Embryos reaching the stage of blastocyst at these days and showing good morphological quality were vitrified. To assess that the morphological quality of the produced blastocysts matched to their survival after cryopreservation in all groups, a random sample of each group was warmed and transferred to synchronized recipients.

Recovery of oocytes

Ovaries were collected from slaughtered adult ewes at a local abattoir, washed and transported to the laboratory in two litres of physiological saline supplemented with one vial of IVENSALPEN-E1 (IVEN laboratories, Madrid, Spain), in an isothermal container at 32–35°C. Each vial contained 1 g streptomycin sulphate, 750 000 IU penicillin-G procaine (equivalent to 693.8 mg) and 300 000 IU penicillin-G sodium salt (equivalent to 179.6 mg). Follicle aspiration was performed between 2 and 4 h after slaughter, as described in the next paragraph.

Live donors were healthy, multiparous and dry Rasa Aragonesa ewes permanently housed in a pen and fed a maintenance diet. Fourteen days before LOPU, Fluorogestone Acetate (FGA) sponges (Chronogest® 40 mg; Intervet, Salamanca, Spain) were inserted in all donors and were replaced for new ones at the time of the first LOPU session. Sponges were withdrawn at the end

of the second session. FSH-stimulated donors additionally received 32 mg of pFSH (equivalent to 1.22 mg NIADDK-oFSH-17) in five im injections of 8, 8, 6, 6 and 4 mg at 60, 48, 32, 24 and 12 h before LOPU, respectively, as well as an injection of 100 μg of Cloprostenol, (Estrumate®; Schering-Plough Animal Health), coinciding with the first FSH dose.

Oocytes from live donors were obtained by LOPU as described previously (Alberio et al. 2002). In short, LOPU was carried out under general anaesthesia, donors being fasted for the previous 24 h. Three incisions were made in the ventral abdominal wall, approximately 10 cm cranial to the udder, to introduce an endoscope, a catheter to pass the pick-up needle and non-traumatic grasping forceps to fix the ovary. A video-camera was connected to the endoscope to visualize the ovary and to perform pick-up. After the puncture, ovaries were washed with heparinized saline (25 IU/ml) at 38°C.

Cumulus-oocyte complexes were aspirated from follicles of at least 2 mm in diameter with a 23G needle connected to an aspiration pump (VMAR 5100, Cook) adjusted to −25 mmHg. Cumulus-oocyte complexes from aspirated follicles of all sizes were collected altogether in a 15- ml Falcon® tube (Becton Dickinson, Madrid, Spain) with 1 ml of TCM199, supplemented with 40 μg/ml gentamicin sulphate, 10 mM HEPES and 10 IU/ml heparin to prevent clotting. During aspiration, the storage tube was maintained at 32°C. When aspiration was carried out in live donors, the total number of aspirated follicles per donor was recorded, as well as the number of those greater or equal to 4 mm in diameter. One collection tube was used for each donor.

In vitro maturation

Recovered COCs were washed four-times with TCM199 + 10 mM HEPES + 40 μg/ml gentamicin sulphate and morphologically scored in five categories (I to V) as previously described in full detail by Stangl et al. (1999). Cumulus-oocyte complexes from categories I, II and III were isolated and placed into maturation medium (30–40 COCs/500 μl) in four-well plates (Nunclon® Fisher Bioblock, Madrid, Spain). Two maturation media were compared: a defined medium consisting of TCM199 + 100 μM cysteamine + 10 ng/ml EGF + 40 μg/ml gentamicin sulphate (Cys + EGF medium) and a non-defined medium consisting of TCM199 + 132 μg/ml pFSH (equivalent to 5 μg/ml NIADDK-oFSH-17) + 10% (v/v) Follicular Fluid (FSH + FF medium) + 40 μg/ml gentamicin sulphate. Follicular fluid was prepared as described previously (Guler et al. 2000), although it was not charcoal-treated. Briefly, medium-sized follicles (4–5 mm) were aspirated from abattoir adult sheep ovaries collected during the breeding season and transported at 4°C to the laboratory. After collection and pooling, 100 IU/ml heparin was added to avoid coagulation. Follicular fluid was centrifuged at 10000 × ***g*** for 3 min at 4°C and stored at −20°C until use. Plates were incubated at 38.5°C in 5% CO_2 in humidified air for 24 h.

In vitro fertilization

Mature oocytes were denuded by gentle pipetting with a small-bore pipette, washed three times and further morphologically evaluated (Stangl et al. 1999). Only oocytes graded I to III were used for IVF. These oocytes were placed into equilibrated fertilization medium composed of synthetic oviductal fluid (SOF) + 20% (v/v) of heat-inactivated oestrous sheep serum as capacitating agent. The SOF used in the present study contained both essential and non-essential amino acids, but no glucose, as previously described in detail (Takahashi and First 1992). Oestrous sheep serum was selected for best sheep embryo IVP performance among 10 individual samples from ewes showing standing oestrus after FGA synchronization for 14 days in the breeding season. Ten to 15 oocytes were placed into 50 μl droplets of IVF medium on polystyrene petri dishes under washed mineral oil. Oil was washed with SOF at a 5 : 3 (v/v) ratio.

A pool of fresh semen from three Rasa aragonesa tested rams was used in all experiments. The same rams were used in both trials. Capacitation was carried out by a slight modification of the method described previously (Ptak et al. 1999). Briefly, semen was kept at room temperature in the dark for 2 h after collection. Afterwards, semen was diluted 1 : 10 (v/v) in SOF + 0.3% BSA + 40 μg/ml gentamicin sulphate and then centrifuged at 200 × ***g*** for 5 min. Two aliquots were taken for counting and progressive individual subjective motility evaluation, respectively. As semen showed a high quality, selection of spermatozoa was not performed. The pellet was diluted with fertilization medium to a concentration approximately 100×10^6 total sperm/ml. One microlitre of this suspension was introduced into 49 μl drops containing a maximum of 15 oocytes, under mineral oil, in four-well dishes (Nunclon®, Fisher Bioblock, Madrid, Spain). Incubation was carried out for 20–24 h at 38.5°C in 5% CO_2 humidified air.

In vitro culture

Presumptive zygotes were washed by gently pipetting and cultured in equilibrated SOF + 0.3% BSA + 40 μg/ml gentamicin sulphate under washed mineral oil (30–40 zygotes/50 μl droplets) in four-well plates at 38.5°C in a 5% CO_2/5% O_2/90% N_2 humidified atmosphere for 7 days. At days 2 and 5 from IVF, half of the media (25 μl) was replaced with 25 μl of SOF supplemented with either, 20% or 10% (v/v) FCS, respectively, to obtain a FCS concentration of approximately 10% (v/v) from day 2 onwards. Cleavage and blastocyst rates were assessed at 48 h, 7 and 8 days after IVF, respectively.

Embryo vitrification

Embryos were cryopreserved when they reached the blastocyst stage (days 7 or 8). Embryos proceeding from the same oocyte source and maturation medium group were vitrified in 0.25 ml straws (IMV; HUMECO, Huesca, Spain) in groups of two blastocysts per straw,

following the procedures described previously (Guignot et al. 2009). Briefly, embryos were exposed at room temperature to the following glycerol (GLY) and ethylene glycol (EG) solutions in PBS + 20% FCS: 10% GLY for 5 min, 10% GLY and 20% EG for 5 min, and finally 25% GLY, 25% EG and 0.4 M sucrose for 30 s. Embryos were placed in the centre of the straw, separated by two air bubbles from two surrounding segments of PBS + 20% FCS containing 0.8 M galactose. After sealing, straws were immediately plunged into liquid nitrogen. For warming, straws were held 5 s in air followed by 15 s in a 22°C water bath. The straws contents were poured on a polystyrene petri dish and gently stirred. Afterwards, embryos were aspirated with a Tomcat catheter attached to a 1 ml syringe to be transferred.

Embryo transfer

Recipient ewes were synchronized by 40 mg FGA sponges (Chronogest® 40 mg; Intervet, Salamanca, Spain) for 14 days and 400 IU eCG (Foligón®; Intervet, Salamanca, Spain) at sponge withdrawal. Eight days after withdrawal, laparoscopic ovulation rate assessment and embryo transfer were carried out. Two blastocysts were placed into the uterine horn ipsilateral to an ovary showing a morphologically healthy *corpus luteum* using a Tomcat catheter. The number of lambs born was recorded.

Statistical analyses

Data were analysed by ANOVA for categorical variables using the CATMOD procedure of SAS (SAS Institute Inc., 2004). For each variable, the following factors were included in the model: trial (two levels), session within trial (two levels), oocyte source (three levels: FSH-stimulated ewes, non-stimulated ewes and abattoir ovaries), maturation medium (two levels: defined: TCM199 + Cys + EGF and undefined: TCM199 + FSH + FF), and their interaction. As no significant differences were observed between trials or sessions in the studied variables (i.e. lower than 18%), these factors were excluded from the final model, which was analysed as a 3×2 factorial design. Rates were compared between groups by pre-planned contrasts. The mean numbers of follicles per ewe and session were compared by the Mann–Whitney *U* test, using the NPAR1WAY procedure of SAS.

Results

The number of aspirated follicles per ewe and session were 26.9 ± 1.8 and 12.5 ± 0.7 (means ± SEM) in FSH-stimulated and non-stimulated donors, respectively ($p < 0.001$). The rate of aspirated follicles greater or equal to 4 mm in diameter relative to the total number of aspirated follicles was higher in FSH-stimulated than in non-stimulated ewes (77.5% vs 59.4%; $p < 0.0001$). Recovery rate of oocytes (all grades) was 1.2 times higher in non-stimulated than in FSH-stimulated donors (29.9 vs 36.1%; $p < 0.05$). The rate of oocytes selected for IVF relative to the total number of oocytes recovered was similar in both groups (89.3 vs 83.3%, respectively; NS; data not shown).

Results of IVP of embryos are shown in Table 1. In abattoir-derived oocytes, no significant differences between IVM media were found in cleavage rate, blastocyst development rates at days 7 and 8, or *in vitro* final yield. In abattoir group, a few blastocysts were produced from day 7 to day 8 in both media (+5.8% in Cys + EGF and +3.7% in FSH + FF), although significance was not achieved.

When comparing *in vivo*-derived oocytes, the cleavage rate of oocytes derived from FSH-treated donors (LOPU-FSH) was higher when *in vitro* maturation was carried out in the undefined medium (FSH + FF) than in the defined medium (Cys + EGF) (+14.8%; $p < 0.05$). Likewise, the blastocyst development rates at days 7 and 8 were significantly higher in FSH + FF than in Cys + EGF medium (both, +23.6%; $p < 0.05$). Lastly, the *in vitro* final yield was more than double in FF + FSH medium (41.3 vs 19.2%; $p < 0.01$). Within the LOPU-FSH group, the blastocyst development rates obtained in each IVM media were the same at days 7 and 8, as no new blastocysts were observed from day 7 to day 8 (Table 1).

As in LOPU-FSH group, the cleavage rate of control ewe-derived oocytes (LOPU-C group) was higher when *in vitro* maturation was carried out in FSH + FF than in Cys + EGF medium (+17.1%; $p < 0.05$). However, no significant differences between both IVM media were achieved in the blastocyst development rate or in the final yield of IVP in this group. Embryo development to blastocyst was slower in oocytes matured in Cys + EGF compared to FSH + FF medium, as 18.2% new blastocysts were observed from day 7 to day 8 in Cys + EGF group, while no new blastocysts were observed from days 7–8 in FSH + FF group.

Table 1. Embryo *in vitro* production results in relation to oocyte source and maturation medium

	Cleavage rate (%)		Day 7 blastocyst rate (%)		Day 8 blastocyst rate (%)		*In vitro* final yield (%)	
Maturation medium	Cys + EGF	FSH + FF	Cys + EGF	FSH + FF	Cys + EGF	FSH + FF	Cys + EGF	FSH + FF
Oocyte source								
LOPU-FSH	57.7^{c} 45/78	72.5^{d} 58/80	33.3^{c} 15/45	56.9^{ad} 33/58	33.3^{c} 15/45	56.9^{ad} 33/58	19.2^{a} 15/78	41.3^{bc} 33/80
LOPU-C	54.1^{c} 33/61	71.2^{d} 42/59	39.4^{c} 13/33	42.9^{ab} 18/42	57.6^{d} 19/33	42.9^{ab} 18/42	31.1^{a} 19/61	30.5^{cd} 18/59
Abattoir	74.3^{d} 52/70	80.0^{d} 80/100	34.6^{c} 18/52	30.0^{b} 24/80	40.4^{cd} 21/52	33.7^{b} 27/80	30.0^{a} 21/70	27.0^{d} 27/100

Cleavage rate, number of cleaved ova/oocytes used for *in vitro* fertilization (IVF); Blastocyst development rate, number of blastocysts/cleaved ova; *In vitro* final yield, number of blastocysts at day 8/oocytes used for IVF; Cys, cysteamine; EGF, epidermal growth factor; FSH, follicle-stimulating hormone; FF, follicular fluid; LOPU, laparoscopic ovum pick-up.
Significance between media or between oocyte sources within each variable: a,b: $p < 0.01$; c,d: $p < 0.05$.

Table 2. *In vivo* survival rate of vitrified blastocysts produced *in vitro* using oocytes of three sources, matured in two different media

	Lambs born/transferred %	
Maturation medium	Cys + EGF	FSH + FF
Oocyte source		
LOPU-FSH	16.7 (1/6)	8.3 (1/12)
LOPU-C	25.0 (2/8)	14.3 (2/14)
Abattoir	9.1 (1/11)	10.0 (1/10)

Cys, cysteamine; EGF, epidermal growth factor; FSH, follicle-stimulating hormone; FF, follicular fluid; LOPU, laparoscopic ovum pick-up.

When comparing the sources of oocytes, cleavage rate was significantly higher in oocytes derived from abattoir ovaries than in those derived from either FSH-treated (+16.6%; $p < 0.05$) or control donors (+20.2%; $p < 0.05$) when IVM was performed in Cys + EGF medium. However, when oocytes were matured in FSH + FF medium, these differences were not significant (+7.5 and +8.8%, respectively; both, NS).

When FSH + FF medium was used, blastocyst development rates at days 7 and 8 were higher in LOPU-FSH than in abattoir group (+26.9 and +23.2%, for days 7 and 8, respectively; both, $p < 0.01$). When maturation was carried out in Cys + EGF medium, the blastocyst development rate at day 7 in LOPU-FSH group was similar to that of LOPU-C group, whereas at day 8 it was lower in LOPU-FSH than in LOPU-C group (−24.3%; $p < 0.05$).

The final yield of embryo IVP (based on total blastocysts observed at day 8) was significantly higher in oocytes from FSH-treated donors than in abattoir-derived oocytes (+14.3%; $p < 0.05$) when FSH + FF was used as the IVM medium. On the contrary, when using Cys + EGF medium, it was lower in FSH-treated donors than in abattoir-derived oocytes, although not statistically different (−10.8%; NS).

Survival rate of the vitrified and transferred blastocysts is shown in Table 2. The percentage of lambs born ranged from 8.3 to 25.0%, without significant differences between either maturation media or oocyte sources. The overall survival rate was 13.1% (8/61).

Discussion

Most reports concerning *in vitro* production of embryos from *in vivo*-derived oocytes have been carried out using undefined maturation media, containing different combinations of sera and gonadotropins (Czlonkowska et al. 1991; Huneau et al. 1994; Baldasarre et al. 1996; Berlinguer et al. 2004; Cox and Alfaro 2007; Morton et al. 2008). To our knowledge, this is the first report in which IVP results of a defined (Cys + EGF) and an undefined (FSH + FF) maturation medium have been simultaneously compared using oocytes from three sources: FSH-stimulated live ewes, non-stimulated live ewes and abattoir-derived sheep ovaries.

The maturation media compositions used in the present study are based on previous research developed with abattoir-derived sheep oocytes. The undefined medium is based on results, demonstrating that FF enhances the maturation and fertilizability of sheep follicular oocytes *in vitro* (Sun et al. 1994), and on additional results demonstrating the beneficial effects of FF and FSH on embryo developmental ability in sheep (Cognié 1999). As far as we know, the defined medium used in this study has never been tested in oocytes aspirated from live ewes, although satisfactory *in vitro* production results have been described in abattoir-derived goat and sheep oocytes (Cognié et al. 2002, 2004), as well as in oocytes aspirated from FSH-stimulated goats (Locatelli et al. 2008), confirming the efficiency of EGF (Guler et al. 2000) and cysteamine (De Matos et al. 1999, 2002; Cognié et al. 2004; Shabankareh and Zandi 2010) as additives during *in vitro* maturation in the ovine species.

In the present work, when using abattoir-derived COCs, cleavage and blastocyst development rates were similar for both maturation media. This result is similar to that reported in a previous study comparing maturation media in abattoir-derived caprine oocytes (Cognié et al. 2002). The cleavage rate reported here when using FF and FSH in maturation medium (80%) is also in accordance with that previously reported in abattoir-derived sheep oocytes (Guler et al. 2000).

In contrast to that observed in abattoir-derived oocytes, *in vivo*-derived oocytes resulted in higher cleavage rates when matured in FSH + FF than in Cys + EGF medium. However, the effect of maturation medium on the final embryo yield depended on the treatment of donors. Thus, in oocytes from non-stimulated ewes, the final embryo yield was only slightly higher, although not significantly, in defined (31.1%) than in undefined medium (30.5%), and both yields were somewhat higher than those reported by others (Berlinguer et al. 2004) using the standard maturation medium (20.4%). Nevertheless, in oocytes recovered from FSH-stimulated donors, final embryo IVP yield was significantly higher in our undefined (FSH + FF) than in the defined (Cys + EGF) medium. This result is in agreement with a recently published work comparing these maturation media for IVM of oocytes from FSH-stimulated goats (Locatelli et al. 2008). The fact that medium supplemented with FSH + FF performed better than defined medium when using oocytes from FSH-stimulated ewes demonstrates that these oocytes have maturation requirements different from oocytes coming from non-stimulated ovaries. As opposed to the rest of groups, where no new blastocysts were observed from day 7 to day 8 post-IVF, in the groups of abattoir-derived oocytes (irrespective of the maturation medium), or of oocytes from non-stimulated donors matured in medium containing cysteamine and EGF, a part of embryos reached the blastocyst stage from day 7 to day 8. This fact reveals a delayed development in these groups when compared to the remainder ones. As embryos reaching the blastocyst stage earlier are considered to have higher potential viability, our results would suggest a better adequacy of FSH + FF medium than Cys + EGF medium for the maturation of oocytes obtained from live non-stimulated donors. It would also suggest a better developmental competence of oocytes obtained from FSH-stimulated donors, independently of the maturation medium.

Although the modest number of transferred embryos did not allow us to assess potential differences in survival rates between oocyte sources or maturation media, *in vivo* survival served as an objective assessment of the quality of those blastocysts produced in the present study that showed a good morphological quality. In fact, approximately 13% of the produced blastocysts were able to resist cryopreservation and gave viable offspring; this *in vivo* survival rate is comparable to previously reported results using abattoir-derived sheep oocytes (21.7%; Dattena et al. 2000) or oocytes from FSH-stimulated ewes (23.8%; Ptak et al. 1999). The different survival rates observed between laboratories could be because of the different vitrification protocols used. As lambs born were obtained in every combination of oocyte source and maturation media, there is no evidence of a complete inadequacy of any medium for the maturation of the oocytes from a particular source.

While the different performance of the maturation media tested in oocytes from different sources cannot be definitively explained by the present experimental results, the different follicular populations observed in the ovaries of FSH-treated and untreated donors may be involved. In fact, although all oocytes obtained from donors within each treatment group were manipulated together and were randomly assigned to maturation media groups, the percentage of larger aspirated follicles (>4 mm) of the total aspirated follicles was 18.1% higher in FSH-stimulated than in non-stimulated donor ewes. Many studies on the effect of follicle size and quality on the oocyte developmental competence have been reported (Moor and Trounson 1977), and it is known that competence is acquired throughout folliculogenesis (Mermillod et al. 1999). Pioneer experiments also demonstrated that *in vitro* maturation and blastocyst development rates are higher in FSH-stimulated goat oocytes from larger follicles (≥5 mm) than in those from small ones (2–5 mm) (Crozet et al. 1995). Similarly, in bovine, follicular size has been related to oocyte quality and its developmental competence (Lonergan et al. 1994). Recently, it has been reported that embryo IVP yield in sheep is higher when abattoir-derived oocytes come from ovaries having a higher number of follicles (≥8) than from ovaries with <8 follicles (Mossa et al. 2008).

The best result reported here (*in vitro* final yield: 41.3%) was obtained using oocytes from FSH-stimulated ewes matured in the undefined medium (FSH + FF). It should be noted that this medium had not been previously used for IVM of oocytes from FSH-stimulated ewes. Unfortunately, follicular fluid is subjected to the unavoidable batch-to-batch variability observed in undefined additives. Knowing the substance(s) responsible of the satisfactory blastocyst yield observed in the present work will help towards the formulation of a defined medium that would be more appropriate for in vitro maturation of oocytes from FSH-stimulated ewes. Follicular fluid is thought to contain factors, apart from EGF and FSH, which improve the cytoplasmic maturation of the oocyte. Thus, there were observed higher cleavage and blastocyst development rates using maturation medium TCM199 supplemented with FSH + FF than when using the same medium supplemented with FSH and/or EGF, while rates of nuclear maturation were similar in both media (Guler et al. 2000).

Under our experimental conditions, while the defined maturation medium used (Cys + EGF) is suitable for IVM of oocytes from non-stimulated ewes as well as from abattoir-derived oocytes, results of the present study show that this medium is not appropriate for the maturation of oocytes from FSH-stimulated ewes. In fact, the worst blastocyst development rate (33.3%) and the worst *in vitro* final yield (19.2%) observed in the present study were found in the group of oocytes from FSH-stimulated ewes matured in Cys + EGF medium. The addition of cysteamine (Ptak et al. 1999) or EGF (Grazul-Biska et al. 2003) to the standard maturation medium was reported to increase the efficiency of *in vitro* production of embryos using oocytes from FSH-stimulated ewes, obtaining *in vitro* final yields of 21.0 and 31.5%, respectively. These results are similar to ours, taking into account the high variability usually observed between laboratories of embryo IVP. Moreover, it should be noted that the above-mentioned results were obtained using only one component (EGF or cysteamine) added to the standard maturation medium (also containing FSH, LH, E2 and FCS), while in the present study both components were added to a defined medium lacking hormones and serum.

In conclusion, under our experimental conditions, results show that the defined and the undefined in vitro maturation medium tested in this work perform equally well for in vitro embryo production using oocytes from live non-stimulated ewes or from abattoir-derived ovaries. However, undefined medium performs better than defined medium when using oocytes from FSH-stimulated ewes. Further explanation of these findings would require evaluation of the putative effects of *in vivo* FSH treatment before LOPU on the quality of the recovered oocytes, as well as further research towards knowing which component used to supplement the IVM medium is responsible for the observed differences.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

All authors partly contributed to the research work presented in this article and to the preparation of the final version of the manuscript.

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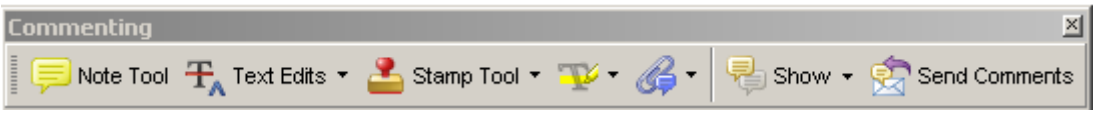

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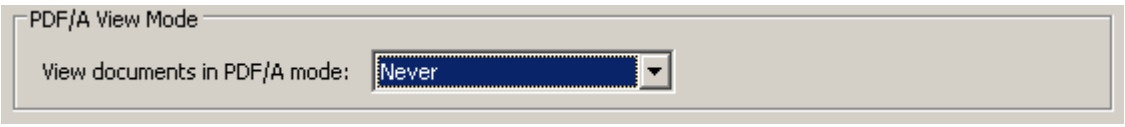

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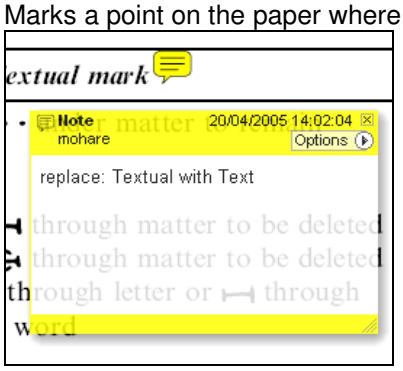

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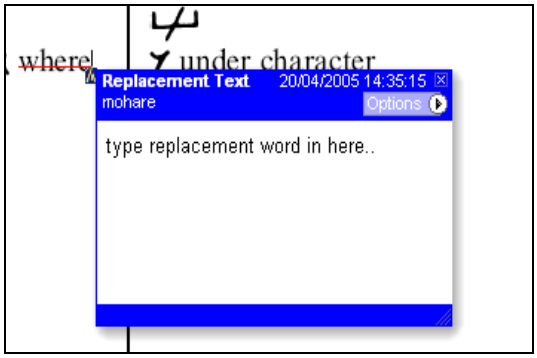

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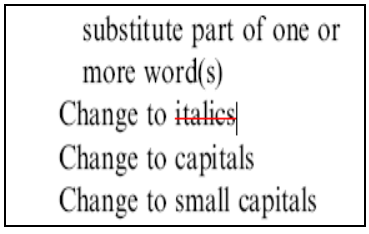

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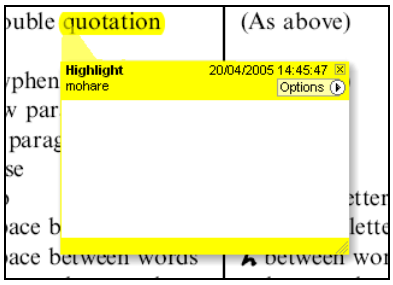

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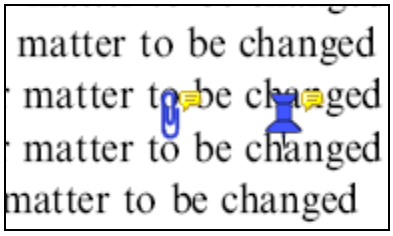

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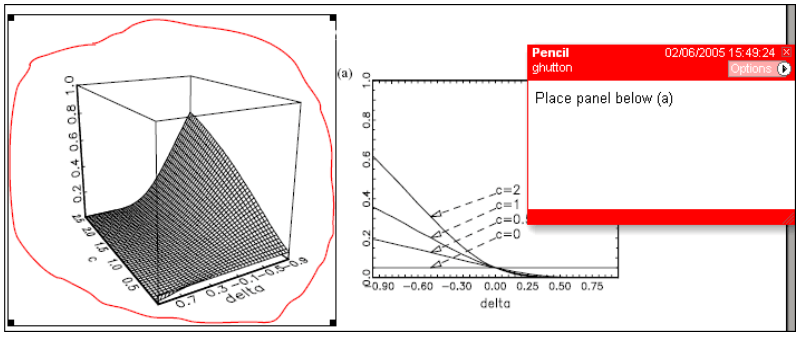

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