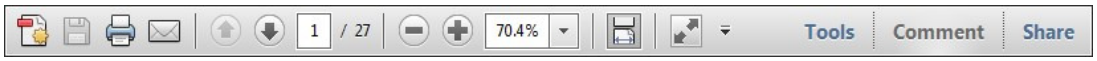
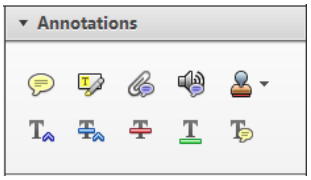
Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:




This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

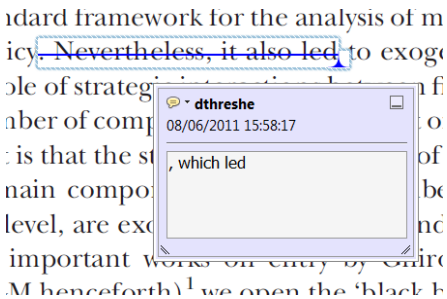



1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

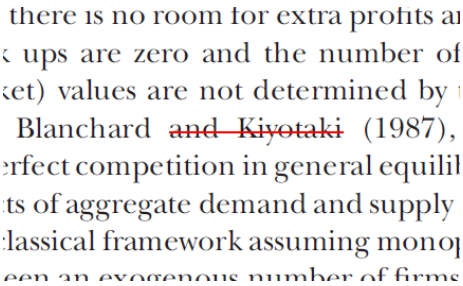



2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

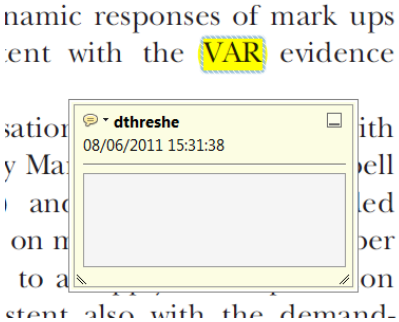


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

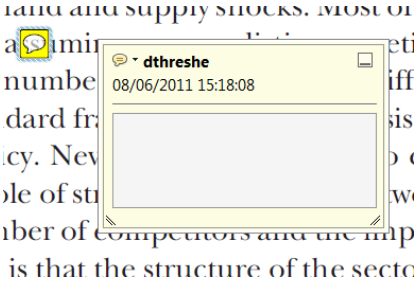



4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

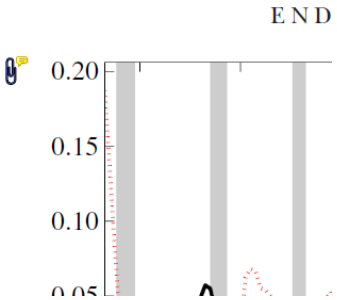


5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

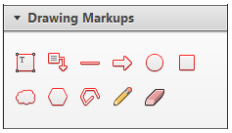
How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



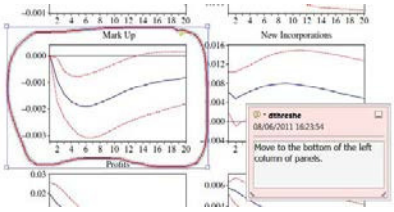

6. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.




How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



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# Current Status of *In Vitro* Embryo Production in Sheep and Goats

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**Contents**

Sheep and goat production is an important economic activity in Spain with an increasing interest in milk production. Multiovulation and Embryo Transfer (MOET) and *In vitro* Embryo Production (IVEP) are assisted reproductive technologies aimed at increasing the genetic diffusion of females. *In vitro* embryo production is a multi-step methodology comprising the following procedures: (i) *In vitro* Maturation (IVM) of oocytes recovered directly from the follicles, (ii) *In vitro* Fertilization (IVF) or co-incubation of capacitated spermatozoa with *in vitro* matured oocytes and (iii) *In vitro* culture (IVC) of zygotes up to the blastocyst stage. *In vitro* embryo production from oocytes recovered from prepubertal females is called JIVET (Juvenile *in vitro* Embryo Transfer) and allows shortened generation intervals and increased genetic gain. Embryo production together with embryo cryoconservation would allow large-scale embryo marketing, a pathogen-free genetic movement and easier and cheaper germplasm commercial transactions. Commercial Embryo activity in small ruminants is low compared to cows in the European Union (data from the European Embryo Transfer Association) and in the world (data from the International Embryo Transfer Association). There is less IVEP ~~Research~~ in small ruminants compared to other livestock species. The aim of this review was to provide an overview of the current status of IVEP of small ruminant with an emphasis on (i) description of the main methodologies currently used for IVM, IVF and IVC of embryos (ii) comparing procedures and outputs from JIVET and IVEP of adult females and (iii) the future research perspectives of this technology.

## Introduction

Multiovulation and Embryo Transfer (MOET) and *In vitro* Embryo Production (IVEP) are assisted reproductive technologies aimed at increasing the genetic diffusion of females. Both, *in vivo* derived (IVD) embryos and *in vitro* produced embryos together with cryoconservation techniques will be essential to increase sheep and goat productivity (Paramio 2010). The most critical factor affecting the whole efficiency of MOET is the large variation in embryo response to superovulation treatments, the early regression of *corpora lutea* and the traumatic surgical procedure of embryo recovery. However, in IVEP procedure superovulation is not needed because oocytes are recovered directly from the follicle in hormonally or not stimulated females. Furthermore, IVEP allows the production of progeny from non-fertile females, prepubertal, pregnant, lactating even dead or slaughtered females. Embryo production from oocytes of prepubertal females is a technology called Juvenile *in vitro* Embryo Transfer (JIVET) and it permits shortening generation interval and increases genetic gain. Thus, 3-month-old females in a JIVET scheme reduces this interval to 7 months and using oocytes obtained from 3- to 4-week-old females to 6 months with an increases in the rate of genetic gain of approximately 5% (reviewed by Morton 2008). *In vitro* embryo production is a multi-step methodology comprising the following procedures: (i) *In vitro* Maturation (IVM) of oocytes recovered directly from the follicles, (ii) *In vitro* Fertilization (IVF) or coincubation of capacitated spermatozoa with *in vitro* matured oocytes and (iii) *In vitro* culture (IVC) of zygotes up to the blastocyst stage. At this stage, the blastocyst could be directly transferred to a recipient female or cryopreserved for future use. According to the European Embryo Transfer Association (AETE) in cattle, *in vitro* produced embryos increase at a higher rate than *in vivo* derived (IVD) embryos. In 2012 in Europe, the total number of transferable IVD embryos was 105 212 with a mean of embryos recovered per cow of 5.8. Total IVEP was 9500, of which 8200 were produced by oocytes recovered by Ovum Pick Up (OPU) from live cattle and 1300 embryos produced from oocytes of ovaries obtained from slaughterhouses. The mean embryo production was 1.5 embryos per OPU session (AETE Newsletter 40, December 2013). In goats and sheep, the numbers of transferable embryos were 406 and 265, respectively, France being the country with the highest number recorded. Data from the International Embryo Transfer Society (IETS) confirmed this embryo activity trend throughout the world. The low commercial embryo activity of small ruminants observed in Europe is also reflected at IETS data. According to IETS, only twelve countries reported embryo transfer in sheep with Australia leading the way strongly with over 1000 flushes and over 7000 embryos collected. South Africa, Mexico and Argentina all reported over 100 flushes each. Globally, 13633 IVD embryos were collected and 12458 transferred. Only six countries reported embryo transfer in goats, with 1805 IVD embryos collected and 1013 embryos transferred (IETS Newsletter, December 2013).

There is less research on assisted reproductive technologies in small ruminants compared to other livestock species such as cattle and pigs. However, in the last few years, there has been significant research in small ruminant embryo studies due to the rising importance of these animals in economically fast developing countries such as China and India and also because of the growing interest in small ruminants, mainly goats, as animals to express recombinant proteins in milk (Paramio and Izquierdo 2014). *In vitro*



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embryo production also is a key area of research for studies on somatic cell nuclear transfer (SCNT) in order to create cloned animals. Using ovaries recovered from slaughterhouses as a source of oocytes, IVEP allows the production of a high and cheap number of embryos essential for studies such as sexing, stem cells, cloning, and in general studies needing a high number of embryos.

Goat and sheep production in Spain is economically and socially important. Spanish Agriculture Ministry data (MAGRAMA, 2013) show 16.6 million sheep and 2.8 million goats with an increasing interest in milk production. The economic values of this products are 537 and 75 USD millions for meat and 569 and 315 for milk in sheep and goats respectively. According to FAO (Faostat, 2012), the number of sheep and goats in the world was ~~1.169~~, and 996 million respectively. The number of sheep in Africa, America, Asia, Europe and Oceania was 322, 87, 526, 129 and 106 million, respectively, and goats, 334, 35, 595, 17 and 4 million respectively. The economic value in the world for milk of sheep and goats was 5.6 and 6.4 billons USD and for meat was 37 and 25 billons. In these species, Artificial Insemination is the most used reproductive technology. To our knowledge, no data on embryo activity has been recorded. However, we have several scientific groups researching MOET and IVEP in small ruminants in different regions of Spain. In our laboratory at the University Autonoma of Barcelona, we have been using goat oocytes from 1-month old and lamb oocytes of 3-month-old females to produce embryos after IVF and ICSI (Intracytoplasmic Sperm Injection). Embryo production together with embryo cryopreservation would allow large-scale embryo marketing, a pathogen-free genetic movement and easier and cheaper germplasm commercial transactions. These technologies are important tools for the dissemination of valuable or endangered breeds.

Despite research efforts over the last 30 years, results on *in vitro* embryo production are still unpredictable and variable which is an important limitation to its commercial application. Better knowledge of gamete and embryo physiology is needed to use IVEP on a large scale commercial level.

The aim of this review was to provide an overview of the current status of IVEP of small ruminants with an emphasis on (i) description of the main methodologies currently used for IVM- IVF and IVC of embryos, (ii) comparing procedures and outputs from JIVET and IVEP of adult females and (iii) the future research perspectives of this technology.

## *In Vitro* Embryo Production Procedures

### *In vitro* maturation

*In vitro* maturation (IVM) is the most critical part of the whole process of *in vitro* embryo production. For successful IVM, oocytes must undergo synchronically nuclear and cytoplasmic maturation. Due to the heterogeneous nature of immature oocytes (oocytes from a wide range of follicle stages) used for IVEP, their response to the same IVM condition is different and unpredictable (reviewed by Cognie et al. 2004; Tibary et al. 2005). Thus, the percentage of oocytes that resume nuclear maturation and reach the metaphase II stage can be more than 80% both in sheep (Guler et al. 2000; Bai et al. 2008; Shirazi et al. 2010; Shabankareh et al. 2011) and goat (Izquierdo et al. 2002; Rodriguez-Gonzalez et al. 2003a,b; Urdaneta et al. 2003; Souza-Fabjan et al. 2014a). Conventionally, oocytes of sheep and goat are *in vitro* matured in groups (25–50 oocytes with a ratio of 1 oocyte/2–5 μl medium) and incubated at 38–39°C in humidified atmosphere of 5% $CO_2$ in air for 24–27 h.

The culture systems used for *in vitro* embryo production (IVEP) can be classified according to their formulation in (i) undefined media, where serum and/or a somatic cells co-culture is used; (ii) semi-defined, without somatic cells and serum which is replaced albumin; (iii) defined, where albumin is replaced by macromolecules, such as polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP) (Farin et al. 2001; Vanroose et al. 2001).

Several IVM media have been proposed for small ruminant oocytes (MEM, Waymouth, Ham-F10, etc.). However, the most widely used is the TCM199 medium, bicarbonate buffered and containing minerals, carbon and energy sources (glucose, glutamine) as well as vitamins and amino acids (reviewed by Mermillod et al. 2006), supplemented with L-glutamine, pyruvate, hormones (FSH, LH, 17β-$E_2$,) plus complex fluids such as heat-treated serum and follicular fluid recovered from healthy non-atretic follicles (reviewed by Cognie et al. 2003; Tibary et al. 2005; Paramio 2010). Nevertheless, there are some research teams using SOF medium instead of TCM1999 in sheep (Shabankareh et al. 2012) and goat (Ongeri et al. 2001; Bormann et al. 2003; Herrick et al. 2004) oocytes.

The atmospheric conditions of the IVM are air with 5% $CO_2$ in air. It means 3–4 times more $O_2$ than in the oviduct (Mastroianni and Jone 1965) resulting in increased reactive oxygen species (ROS) production (Luvoni et al. 1996; Agarwal et al. 2006) in the *in vitro* culture harmful for oocytes. Compound thiols such as cysteamine, 2-mercaptoethanol, cysteine, and cystin and glutathione (GSH) added to IVM media protect the oocytes from ROS (de Matos et al. 2002). These thiols also increase Intracytoplasmic GSH concentration which has a positive effect on fertilization and male pronucleus formation in oocytes from adult (de Matos et al. 2002) and prepubertal (Rodriguez-Gonzalez et al. 2003a,b; Bai et al. 2008) females. Addition of 100 μM cysteamine to the IVM medium improves blastocyst yield in goats (Rodriguez-Gonzalez et al. 2003a,b; De et al. 2011). Moreover, Wani et al. (2012) adding 200 μM cysteamine enhances *in vitro* embryo development rates in sheep. On the other hand, lamb oocytes matured in presence of 100 μM 2-mercaptoethanol and 600 μM cysteine produce embryos that reach the blastocyst stage earlier (Bai et al. 2008). Vitamins are one of the components of many defined media. Vitamins have been shown to increase glucose metabolism and to act as an antioxidant for developing embryos (Gardner et al. 1994). The effects of vitamins have been tested on oocyte

maturation and embryo culture and have indicated some benefits for oocyte maturation and embryonic development in goat (Bormann et al. 2003) and sheep (Shabankareh et al. 2012). The minimal essential medium (MEM) contain six water-soluble vitamins (thiamine hydrochloride, riboflavin, pyridoxal hydrochloride, folic acid, d-calcium pantothenate and nicotinamide) and two quasi-vitamins (myoinositol and choline chloride). Also, TCM-199 contains the same vitamins that MEM. Using semi-defined media, the addition of vitamins improves embryo development. Thus, the addition of MEM vitamins to a SOF maturation medium plus BSA increases the percentage of blastocysts in goat (Bormann et al. 2003) and sheep (Shabankareh et al. 2012).

Gonadotrophin hormones and 17β-estradiol are generally used in *in vitro* maturation protocols to improve nuclear and cytoplasmic oocyte maturation as well as expansion of the surrounding cumulus cells in ovine (Guler et al. 2000) and caprine (Pawshe et al. 1996) oocytes. The concentration of FSH, LH and 17β-estradiol vary among laboratories. Gonadotrophin concentration commonly added to IVM medium are 10 μg/ml LH, 10 μg/ml FSH and 1 μg/ml 17β-$E_2$ (goat: Cognie et al. 2004; Hammami et al. 2013; sheep: Leoni et al. 2007; Wang et al. 2013) and 5 μg/ml FSH, 5 μg/ml LH and 1 μg/ml 17β – estradiol (sheep: Loi et al. 2008; Catalá et al. 2012; goat: Kharche et al. 2009). In prepubertal goats, supplementation with high concentrations of 17β-estradiol (10 and 100 μg/ml) was found to be inhibitory on the meiotic progression up to metaphase II compared with 1 μg/ml (Lv et al. 2010). Human chorionic gonadotrophin (hCG) and equine chorionic gonadotrophin (eCG) also are added to IVM, in goats (reviewed by Rahman et al. 2011) and sheep (Shabankareh et al. 2012).

Conventionally, IVM media are supplemented with Serum and Follicular Fluid (FF), both of them with unknown composition. Their actions are not fully understood, but it is believed that they provide proteins and/or some growth factors that contribute to the success of *in vitro* maturation and subsequent development. Despite the undefined and variable nature of serum composition, this supplementation is widely generalized in a conventional concentration of 10–20% v/v. The 4 sera mainly used are (i) Foetal Calf Serum (FCS) (goat: Cognie et al. 2004; Zhang et al. 2013; sheep: Garcia-Garcia et al. 2007; Wani et al. 2012), (ii) Steer Serum (SS) (goat: Urdaneta et al. 2003; Jimenez-Macedo et al. 2006; Romaguera et al. 2010), (iii) Ovariectomized goat serum (OGS) (goat: Malakar et al. 2011; sheep: Shabankareh et al. 2011) and (iv) Oestrous Sheep Serum (OSS) (goat: Tajik and Esfandabadi 2003; sheep: Shabankareh et al. 2011).

Follicular fluid is also used as a supplement in the IVM media in goat and sheep (Cognie et al. 2004). The FF constitutes the microenvironment of the oocyte during follicular maturation and contains molecules involved in nuclear and cytoplasmic maturation, ovulation and fertilization (Yoshida et al. 1992). The supplementation of IVM media with FF from non-atretic healthy or gonadotrophin stimulated large follicles (>4 mm) resulted in beneficial effects in both sheep and goat oocytes (reviewed by Tibary et al. 2005). However, Shabankareh et al. (2011) have compared the use of IVM media supplemented with human menopausal serum (HMS), OGS, OSS, bovine follicular fluid (BFF) and ovine follicular fluid (OFF) on *in vitro* maturation of sheep oocytes and have observed that HMS, OSS and OGS in the presence of FCS and hormones increased cleavage and blastocyst rates (90 and 18%, 87 and 43%, 86 and 38% respectively) compared with media containing FCS only or OFF and BFF (56 and 18.4%, 53 and 14%, 54 and 15% respectively).

Must be highlighted that both oestrous serum and follicular fluid need to be tested before use because both compounds present high chemical variations between samples. These variations are one of the causes contributing to the lack reproducibility of results often observed in IVEP laboratories.

Growth factors also have been added to IVM to improve embryo results. Epidermal growth factor (EGF) is the most of the used. Thus, EGF is routinely added to IVM medium plus serum in goat (Zhang et al. 2013) and sheep (Shabankareh et al. 2012; Wani et al. 2012). EGF is added to IVM without serum supplementation in goat (Bormann et al. 2003; Souza et al. 2013; Souza-Fabjan et al. 2014a) and sheep (de Matos et al. 2002). A used IVM defined medium of TCM199, EGF and cysteamine is used with good results in goat (Cognie et al. 2004; Locatelli et al. 2008; Souza et al. 2013; Souza-Fabjan et al. 2014b) and sheep (Cocero et al. 2011). Insulin-like growth factor-I (IGF-I) has also been used to stimulate oocyte maturation and promoting blastocyst development in sheep (Guler et al. 2000) and goat (Magalhães-Padilha et al. 2012). In sheep, Shabankareh and Zandi (2010) have studied the addition of EGF plus IGF-I plus cysteamine in a defined-IVM, semi-defined-IVM, and undefined-IVM medium on the cleavage and embryo rates concluding that these supplements added to undefined-IVM produced a higher percentage of morula and blastocysts than in a defined and semi-defined media. Also, the positive influence of growth hormone (GH) on oocyte maturation has been reported. In ovine (Shirazi et al. 2010), addition of 300 ng/ml GH in serum-containing maturation medium during IVM has significantly increased embryo development in terms of cleavage, blastocyst and hatching rates compared to the control. In contrast, GH supplementation in serum-free oocyte maturation medium had no effect on subsequent cleavage, blastocyst and hatching rate.

Finally, some laboratories have studied other molecules to try to find a defined medium for IVM. Thus, Herrick et al. (2004) have obtained good results in embryonic development using for IVM goat oocytes hyaluronate and citrate in SOF medium (22% blastocysts) instead of SOF supplemented with BSA (19% blastocysts) and TCM199 with 10% OGS (5% blastocysts).

In conclusion, the aim of the research about IVM media was to find a defined and standardized media for small ruminant oocytes. However, the high heterogeneity of oocytes recovered from Ovum Pick Up (OPU) in live females or recovered from ovaries of

slaughterhouse, made difficult to achieve a standardized culture medium. Thus, different laboratories use different protocols according to their specific oocyte quality. In general, oocytes from adult females, recovered of healthy and non-atretic follicles bigger than 3 mm diameter, are able to be matured in a semi o defined IVM such as TCM199 plus EGF plus cysteamine. Thus, Souza-Fabjan et al. (2014a) testing three different IVMs showed no differences in blastocyst production between (i) defined medium (TCM199 plus cysteamine plus EGF), (ii) TCM199 with FCS and (iii) complex medium TCM199 with serum, hormones, EGF, Insulin Like Growth Factor, vitamins, thiol compounds and ITS with 46% and 45% and 45% of blastocysts respectively. Nevertheless, these authors use 10% Oestrus Sheep Serum (OSS) in the fertilization medium and 10% FCS for embryo culture medium.

However, laboratories using prepubertal females with oocytes recovered from small follicles or oocytes from ageing discarded females, they need a more complex and undefined IVM such as TCM 199 with hormones, antioxidants and serum. Oocyte quality is the key factor to produce blastocysts; however, the quality of these blastocysts is affected mainly by the embryo culture conditions (Rizos et al. 2002).

### *In vitro* fertilization

*In vitro* fertilization (IVF) is a complex procedure whose success requires appropriate oocyte maturation, sperm selection, sperm capacitation and IVF media.

Thus, before fertilization, male ejaculates need to be prepared to inseminate the oocytes. The first step is to select the most motile and viable spermatozoa from the whole fresh ejaculate or the frozen-thawed sperm.

The ejaculate comprises of a mixture of seminal plasma, mature and immature spermatozoa, non-reproductive cells, various microorganisms and non-specific debris. The most common method used for separating the sample into motile and non-motile fractions from fresh ejaculate is the swim-up technique (sheep: Shahkhareh and Akhondi 2012; Wani et al. 2012; Shirazi and Motaghi 2013; goat: Jimenez-Macedo et al. 2007; Arteaquita et al. 2007a,b; Romaguera et al. 2010; De et al. 2014; Pradeep et al. 2011; Hammami et al. 2014). Thus, an aliquot of sperm suspension (50–100 µl) is layered under 2 ml of capacitation medium in several tubes at 38.5°C and after 0.5–1 h the top medium (0.4–1 ml) is collected from each tube. The pooled medium containing highly motile spermatozoa is centrifugated (200 × g) for 10 min. For frozen-thawed semen, conventionally, motile spermatozoa are obtained by centrifugation on a discontinuous Percoll gradient (sheep: Li et al. 2006; Garcia-Garcia et al. 2007; Wan et al. 2009; Heidari et al. 2013; Wang et al. 2013; goat: Bormann et al. 2003; Herrick et al. 2006; Souza et al. 2013). Percoll consists of colloidal silica particles of 15–30 nm diameter (23% w/w in water) which have been coated with polyvinylpyrrolidone (PVP). Normally two densities (45% and 90%) of Percoll solutions are used. The gradient is formed by pipetting 1.5 ml of 90% Percoll solution into a 15 ml conical tube and then overlaying it with 1.5 ml of 45% Percoll solution. Semen is placed onto the top of the 45% layer and then centrifuged at 600–1000× g at room temperature for 10–15 min through the gradient. After removal of supernatant, the resulting pellet is washed twice by centrifugation and resuspended in 0.5–2 ml of medium.

Rho et al. (2001) have compared selection of frozen-thawed buck spermatozoa by swim-up, glass-wool filtration and Percoll density gradient centrifugation and the percentages of both cleavage and blastocyst formation in the Percoll density-gradient group (62% and 18% respectively) were significantly higher ($p < 0.05$) than in the swim-up (50% and 11% respectively) and glass-wool filtration (44% and 8% respectively) groups.

Once the most viable and motile spermatozoa are selected, sperm capacitation is carried out *in vitro*. Several capacitating agents have been used to capacitate spermatozoa and to yield good fertilization and cleavage rates, such as (i) Oestrous sheep serum (OSS) (ovine: Berlinguer 2012; Catalá et al. 2011, 2012; Forcada et al. 2013; Lahoz 2013; Shirazi and Motaghi 2013; Wang et al. 2013), (ii) oestrous goat serum (OGS) (goat: Koeman et al. 2003; Katska-Ksiazkiewicz et al. 2007; Kharche et al. 2009), (iii) heparin (goat: Izquierdo et al. 1999; Hammami et al. 2014; ovine: Wani et al. 2012; Heidari et al. 2013; Sreenivas et al. 2013), (iv) heparin and ionomycin (Wang et al. 2002; Urdaneta et al. 2004), (v) heparin and PHE (penicillamine, hypotaurin and epinephrine) (ovine: Shabankareh and Akhondi 2012), (vi) heparin and serum (ovine: Cox and Alfaro 2007; goat: Bormann et al. 2003; Cox and Alfaro 2007) and (vii) heparin, PHE and serum (ovine: Wan et al. 2009; goat: Katska-Ksiazkiewicz et al. 2007). These capacitating agents can be used previously to the IVF, incubating the spermatozoa with them 15 min to 1 h, or to be presents in the fertilization medium.

The use of oestrous serum instead of BSA in the IVF medium significantly increases the cleavage rate of sheep (78% and 0% respectively; Li et al. 2006) and goat (35% and 12% respectively; Kharche et al. 2009) oocytes. Moreover, the addition of heparin in the IVF medium supplemented with 5–10% OSS does not seem to enhance the cleavage rate by improving the efficiency of ram sperm capacitation (Li et al. 2006).

Regarding the fertilization media used for IVF in small ruminants, the Synthetic Oviductal Fluid (SOF) medium is the most used in ovine (Leoni et al. 2007; Bai et al. 2008; Wan et al. 2009; Catalá et al. 2012; Wang et al. 2013), whereas the Tyrode's Albumin Lactate Pyruvate (TALP) medium is widely used as IVF medium in goat (Katska-Ksiazkiewicz et al. 2007; Hammami et al. 2013).

For IVF, groups of 15–30 oocytes are transferred to drops of 50–100 µl of IVF medium where are inseminated. The final sperm concentration used in the IVF drop is $1 \times 10^6$ spermatozoa/ml but it can vary from $0.5 \times 10^6$ cells/ml (Bai et al. 2008) to $12–15 \times 10^6$ cells/ml (Herrick et al. 2004, 2006) depending on the male and IVF system used. Sperm and oocytes are coincubated for 16–24 h at 38–39°C. Concerning the atmosphere used during IVF, most laboratories use a humidified atmosphere of 5% $CO_2$ in air (reviewed by Cognie et al. 2003). However, Leoni et al. (2007)

suggest that low oxygen atmosphere during the IVF period improves the number of high quality sheep blastocysts as evaluated through kinetic development to blastocyst stage and cryotolerance to vitrification procedures. Semen can be sex-sorted before IVF. Beilby et al. (2011) did not find differences between X- or Y-chromosome enriched ram sperm and non-sorted ram sperm.

### *In vitro* embryo culture

The last step of *in vitro* embryo production is the culture of the presumptive zygotes to reach the stage of blastocysts at 6–8 days after *in vitro* fertilization in ruminant (Gardner et al. 1994). The post-fertilization culture is the period having the greatest impact on the blastocyst quality (Rizos et al. 2002). Regardless of the culture media conditions, the percentage of blastocysts produced is highly variable between laboratories and experiments. The success of IVC depends on the various factors such as osmolarity and ionic composition, temperature, pH and $CO_2$, oxygen, carbohydrates, amino acids, lipids and fatty acids, proteins, growth factors and cytokines. Any deviation from the appropriate environment could lead to embryonic arrest at any stage of development.

The composition of the mammalian embryo environment *in vivo* changes from the early to late cleavage stages. Embryonic genomic activation, which occurs at the 8- to 16-cell stage in ruminants, coincides with an increase in metabolic activity (Rieger et al. 1992), oxygen consumption and uptake of carbohydrates (Thompson et al. 1996) to the blastocyst stage. Thus, early embryos cultured *in vitro* fail to develop past the 8- to 16-cell stages in traditional culture media. This block occurred around time of activation of the embryonic genome. Serum and cells have been added to the culture to avoid this block. Thus, since Gandolfi and Moor (1987) showed that co-culture with epithelial oviduct cells could support sheep embryos past the 8- to 16-cell stage, a large number of teams have used this undefined system, especially in laboratories with incubator atmosphere of 20% of $O_2$. Co-culture cells allow reduce $O_2$ tension to less toxic levels. Moreover, somatic cells used in co-culture may produce unknown embryo growth promoting factors or delete embryo toxic factor from culture medium (reviewed by Bavister 1995). Although a wide variety of different cell types have been used, the most widely employed are the oviductal epithelium cells (Ko et al. 2001; Katska-Ksiazkiewicz et al. 2007). In our laboratory, with prepubertal goat oocytes, we compared embryo development in 4 co-culture systems, oviductal epithelial cells (OEC) and cumulus cells (CC), both caprine and bovine, and the best results in blastocyst yield was obtained with OEC co-culture regardless of the species from which the cells are taken (Izquierdo et al. 1999).

Co-culture with somatic cells presents several disadvantages such as contamination risks, dubious quality control and long preparation time. Moreover, the results are not predictable because of the unknown physiological status of the cells. To avoid the inconveniences of co-culture in atmospheres 20% $O_2$ a strategy for decreasing oxidative stress and oxidative damage is to add antioxidants to the media. Thus, supplementation of IVC medium with 200 μM cysteamine (De et al. 2011) has improved the blastocyst yield (33% and 49%, without and with cysteamine respectively) in goats. A smaller concentration (100 μM cysteamine) has not been sufficient to alleviate the oxidative stress (Urdaneta et al. 2003; De et al. 2011). In sheep, α-tocopherol (Natarajan et al. 2010a) or L-ascorbic acid (Natarajan et al. 2010b) added to SOF medium has enhanced embryo development at 20% $O_2$ (18% and 8% blastocysts, with and without α-tocopherol respectively; and 14% and 9% blastocysts, with and without L-ascorbic acid respectively). However, at 5% oxygen concentrations, exogenous antioxidants do not influence bovine blastocyst formation rate or quality (reviewed by Amiridis and Cseh 2012).

Several studies have carried out to test biologically derived components, such as serum and albumin, added to the IVC medium in order to avoid co culture cells media. However, as mentioned previously, the composition of serum is also undefined and variable and it has been shown that exposure to serum can greatly alter embryo morphology, developmental rates, newborn weight and gestation length (Thompson et al. 1995). The developmental abnormalities associated with IVC, collectively termed 'large offspring syndrome' (LOS), include a range of features and phenotypes, for example, increased early embryo losses, large foetuses, disproportionate foetal organ growth and abnormalities of placental development. Thus, LOS and several other developmental abnormalities have been reported also in lambs produced *in vitro* (reviewed by Tibary et al. 2005). Rooke et al. (2007) concluded that the presence of serum during the first 2 days of IVC resulted in increased weights of gravid uterus, placenta, foetus, foetal heart and liver of lambs. However, even when serum was absent throughout IVC, there was still an infrequent incidence of foetal weights greater than control foetus. Thus in sheep, pre-compaction development was more sensitive to environmental stress which leads to LOS. The cell cycles during this sensitive window in the sheep cover the period from which the embryo is dependent on maternal RNA and proteins through to the onset of major genome activation. In goats, we have not found studies about LOS after IVEP. But, Wilmut et al. (2002) concluded that in cloning goats, placenta-abnormalities, LOS, respiratory or cardiovascular dysfunction, organ dysplasia, high perinatal mortality or abnormal postnatal development have not been observed as it was observed in calves and sheep.

Different culture media have been successfully used for small ruminant embryo development such as TCM 199 (Wani et al. 2012), B2 (Katska-Ksiazkiewicz et al. 2007) and 'Sydney IVF Blastocyst' medium (Beilby et al. 2011). However, the most widely used medium is the synthetic oviduct fluid (SOF). Tervit et al. (1972, 1974) were the first to report successful culture of ruminant zygotes to the blastocyst stage *in vitro* using SOF medium, which was based on the composition of ovine oviduct fluid. Subsequently, changes to the original composition have been made. Some

laboratories routinely supplement SOF medium with 5–10% FCS (goat: Hammami et al. 2013; sheep: Catalá et al. 2012) but others only add BSA (sheep: Leoni et al. 2007; Shabankareh and Akhondi 2012; Wang et al. 2013).

With sheep and goat oocytes recovered by LOPU, Cox and Alfaro (2007) have obtained a high blastocyst rate (50% and 61.5%, goat and sheep respectively) culturing the embryos in SOF medium plus BSA 5 days and then 2 days more in TCM199 plus BSA. Recently, it has published some works testing the effect of some molecules until now no proved in culture media of embryos of small ruminants. Ghrelin is a widespread hormone that several studies have linked with reproductive physiology (Garcia et al. 2007; Tena-Sempere 2008). In sheep (Wang et al. 2013), the blastocyst rate, total cell number of blastocysts and the expression levels of the GLUT1 and IFNT genes were increased when 50 ng/ml ghrelin was added during IVC to the SOF medium. On the other hand, activin is an important member of the transforming growth factor β (TGFβ) superfamily. Expression of protein and mRNA for activin-A and activin receptors has been localized in both oocyte and granulosa cells of follicles at various developmental stages of ovine (Thomas et al. 2003); and caprine (Silva et al. 2004). Supplementation of SOF medium with 10 ng/ml activin-A has enhanced embryo development of prepubertal goat oocytes (Hammami et al. 2014).

In goat, Rodríguez-Dorta et al. (2007) have compared the effect of two culture conditions, SOF in a humidified atmosphere of 5% $O_2$, 5% $CO_2$ and 90% $N_2$ vs goat OEC (GOEC) co-culture in a 5% $CO_2$ in air, on the pregnancy rate, embryo survival rate and offspring development after direct transfer of vitrified/thawed caprine IVP embryos. The percentage of blastocyst in SOF (28%) was higher than in GOEC (20%). However, the percentage of pregnancy and kids born were significantly higher in embryos from GOEC (56% and 33% respectively) than SOF (14% and 9% respectively). These results indicate that embryos cultured with GOEC acquired a better quality in terms of cryotolerance and embryo survival rate than embryos developed in SOF in anaerobiosis conditions.

The progress in understanding of the requirements of the developing embryo resulted in the development of sequential media where components change according to the needs of the embryo (reviewed by Thompson 2000). These sequential media would mimic the change in environment experienced by the developing embryo *in vivo*, enabling the biochemical and morphological changes of embryos. Thus, physiological sequential media are formulated to reflect the carbohydrate levels of the reproductive tract and reduce cellular stress on the embryo (Lane et al. 2003). In goat, the sequential G1.2/G2.2 media supplemented with BSA have been shown to support embryo development until blastocyst stage (Ongeri et al. 2001; Bormann et al. 2003; Koeman et al. 2003). In sheep, Garcia-Garcia et al. (2007) have tested sequential G1.3/G2.3 media supplemented with BSA compared to SOF plus 5% FCS and they have obtained a similar blastocyst rate (21.5% and 24.1% respectively) in both IVC systems. However, blastocysts developed in G1.3/G2.3 serum-free were of lower quality in terms of hatching (44.3% and 86.6% respectively).

Finally, embryos are routinely cultured in groups. In fact, most researchers report higher blastocyst rates and improvement embryo quality following group, compared to single culture, because oocytes and embryos stimulate reciprocally during their *in vitro* development (Gardner et al. 1994). From a practical point of view, an individual oocyte culture system would be very useful when working with endangered species or other animals of high genetic value, and/or when LOPU-sessions, or other retrieval methods yield only one or a few cumulus oocyte complexes. Methods of culturing embryos individually, or in groups, but in an individually identifiable manner using microfluidics (Krisher and Wheeler 2010), wells-in-wells (Vajta et al. 2008) or on adhesive substances (Matoba et al. 2010) are being studied. More recently, innovative ways of bar-coding individual embryos have even been reported (Novo et al. 2013).

In conclusion, embryo culture media are divers and also according with the experimental conditions of the laboratory. In goat and sheep routinely presumptive zygotes are cultured for 8 days after insemination in an atmosphere of 5% $O_2$ using SOF medium plus serum. Addition of serum increases blastocyst yield because its effect in the mitosis activation but also serum have a significant effect on the incidence of chromosomal abnormalities in the resulting blastocysts (Lonergan et al. 2004). In our laboratory assessing chromosomal anomalies in blastocysts obtained of oocytes from prepubertal and adult females an cultured in SOF plus 10% FCS we observed that 90% of blastocyst showed mixoploidy with an average of 23.68% of abnormal blastomere per embryo and without differences between prepubertal and adult blastocysts (Romaguera et al. 2011). A chemical defined serum-free media is also an important aim to reach in IVEP of small ruminants.

## *In Vitro* Embryo Production in Juvenile and Adult Goat and Sheep

The incorporation of Juvenile *in vitro* Embryo Technology (JIVET) into breeding programmes is advantageous because it can reduce the generation interval and increase the rate of genetic gain. Another advantage of oocyte collection from prepubertal ovaries is the early availability of large numbers of oocytes per female (Koeman et al. 2003). Thus, this technology would allow a fast multiplication of unique and expensive animals such as endangered species (Tervit 1996). Nevertheless, the greatest limitation of JIVET is that the production of embryos and their development to term after transferring to recipient females is lower in comparison to their adult counterparts. This fact has been shown in cattle 1.2 vs 2.2 blastocysts per animal in calf and cow (Palma 1993), in sheep 20% vs 40% blastocysts in lamb and ewe (Ledda et al. 1997), in pig 21% vs 34% blastocysts in prepubertal and adult females (Marchal et al. 2001) and in goats stimulated hormonally 24% vs 34% blastocysts in prepubertal and adult females (Leoni et al. 2009). This low embryo development of oocytes obtained from prepubertal

females might be caused because these ovaries present a low number of follicles larger than 3 mm. In goats, we have observed 1.1 follicles larger than 3 mm per ovary in prepubertal females (Martino et al. 1994). In adult goats, Crozet et al. (1995) obtained a percentage of blastocysts of 6%, 12%, 26% and 41% of oocytes recovered from small (2–3 mm), medium (3.1–5 mm), large (5 mm) follicles and ovulated oocytes, respectively. The positive and direct relationship between follicle size, oocyte diameter and embryo development is well-known (Gandolfi et al. 2005). Studies in our laboratory have shown ultrastructural and functional deficiencies in prepubertal goat oocytes such as altered distribution of cortical granules (Velilla et al. 2004) and mitochondria (Velilla et al. 2006), disorganization of microtubules and microfilaments (Velilla et al. 2005) and alteration in total RNA content, p34 (cdc2) and cyclin B1 expression and maturation promoting factor (MPF) activity (Anguita et al. 2007a,b, 2008). However, when selecting prepubertal oocytes from follicles larger than 3 mm, the percentage of blastocysts obtained after IVF was similar to those obtained from adult females by LOPU (Laparoscopy Ovum Pick Up) (18 vs 20% respectively) (Romaguera et al. 2011). Ovaries from prepubertal animals have a high percentage of antral follicles with a diameter smaller than 3 mm (Martino et al. 1994), making it difficult to release oocytes by follicular aspiration or select oocytes by their follicular diameter as is conventionally done in adult ovaries. Prepubertal female oocytes are routinely obtained by slicing the ovary surface recovering a pool of oocytes with a heterogeneous degree of growth and atresia and coming from unknown follicles. In this case, oocytes are selected by diameter and the morphological appearance of cumulus cells and oocyte cytoplasm. Thus, Anguita et al. (2008) classifying prepubertal goat oocytes diameters in 4 categories: <110 μm; 110–125 μm; 125–135 μm and larger than 135 μm observed a blastocyst development of 0%, 0%, 7% and 10%, respectively, after IVF. Using Intracytoplasmic Sperm Injection (ICSI), Jimenez-Macedo et al. (2006) found significant differences between oocytes 125–135 μm diameter and larger than 135 μm in cleavage (67% and 75% respectively) but blastocyst yield was not different (16% and 11% respectively). Assessment of oocyte diameter is a time-consuming procedure which affects IVEP output because the long exposure of oocytes to light and harmful atmosphere. In order to select the larger and more competent oocytes avoiding this inconvenience we have tested staining oocytes with Brilliant Cresyl Blue (BCB) to select full-growth oocytes. This stain is based on the ability of the BCB dye to be reduced by glucose-6-phosphate dehydrogenase (G6PDH) activity; thus, oocytes that have reached their growth phase and have low G6PDH activity cannot reduce BCB to a colourless compound exhibiting a blue-coloured cytoplasm (BCB+) and the growing oocytes with high G6PDH activity are able to reduce the blue compound, which results in a colourless oocyte cytoplasm (BCB−). The absence of enzymatic activity of G6PD can be an indirect measure of fully-grown oocytes that have finished their intraovarian growth phase. Rodriguez-Gonzalez et al. (2002) using BCB showed that the mean diameter of BCB+ oocytes (136.6 ± 6.3 μm) was larger than BCB− oocytes (125.5 ± 10.2 μm) in prepubertal goats. In prepubertal sheep oocytes, BCB+ oocytes were also larger (123.66 ± 2.72 μm) than BCB- (106.82 ± 2.82 μm) with a blastocyst development after IVF of (21% and 8% respectively) (Catalá et al. 2011). However, these significant differences in blastocyst yield were not observed after BCB selected oocytes fertilized by ICSI (14% and 11% for BCB+ and BCB- oocytes) (Catalá et al. 2012). Intracytoplasmic Sperm Injection (ICSI) has allowed an increase in the percentage of small oocytes, both goat and sheep, developing to blastocyst stage. A possible explanation of the better embryo development of BCB− and small oocytes after ICSI procedure could be the accurate selection of metaphase II oocytes before sperm injection and the lack of polyspermic zygote formation.

Our results of JIVET using oocytes from 30- to 45-day-old goats and 90- to 120-day-old lamb show yields of 18% and 21% of blastocysts/total oocytes respectively. In a recent review by Souza-Fabjan et al. (2014b) summarized a production of blastocysts in goats from 7% to 55% and in sheep from 10 to 42% with oocytes from adult females. The high variability of *in vitro* blastocyst production observed between studies and research groups is due mainly to the heterogeneous and unknown quality of the oocytes used in these experiments but also because of the undefined culture media of IVM-IVF and IVC procedures.

Oocyte quality (also called oocyte competence) is the ability of the oocyte to resume meiosis, be correctly fertilized, develop to a viable embryo and produce normal offspring after normal gestation.

Adult females oocytes are recovered from LOPU or recovered from ovaries at slaughterhouses. In ovaries from adult sheep and goat oocytes are retrieved by aspiration of healthy, non-atretic and larger than 3 mm follicles. Even so, immature oocytes are still heterogeneous in atresia and quality. Conventionally, these oocytes are selected by visual assessment of morphological appearance. Katska-Ksiazkiewicz et al. (2007) tested the BCB stain to select goat oocytes for blastocyst production concluding that the percentage of blastocysts was significantly higher in BCB+ (13%) and control (19%) than BCB- oocytes (0%). In ovine oocytes, Wang et al. (2012) observed significantly higher percentages of blastocyst (34%) of BCB+ oocytes than BCB- (6%) and Mohammadi-Sangcheshmeh et al. (2012) described 34%, 4% and 20% of blastocyst rate for BCB+, BCB- and control oocytes respectively.

In conclusion, staining oocytes with BCB allows selection of a pool of larger and more homogeneous oocytes. However, the total number of blastocysts produced per ovary is significantly smaller despite the higher percentage of blastocysts produced from BCB+ oocytes because of the low number of BCB+ stained oocytes compared to the number of conventionally morphologically selected oocytes.

Variability in IVEP output has also been observed according to season. In our laboratory with prepubertal goat oocytes and fresh semen capacitated with heparin we have observed a different blastocyst production in summer, autumn, winter and spring with 13%, 2%,

17% and 25% respectively (Catala et al. 2013). In adult females, Souza-Fabjan et al. (2014a) also found higher blastocyst production of goat oocytes recovered at the slaughterhouse during the anoestrus season. In contrast, Mara et al. (2013) in sheep testing during 3 years the output of IVEP programme concluding that the percentage of blastocysts was higher during the breeding season, but there were no differences in pregnancy and lambing rates among blastocysts produced throughout the year.

In conclusion, *in vitro* blastocyst production in goat and sheep is highly variable with many factors affecting these results. Assessment of the oocyte competence is one of the key points in IVEP programmes. Today an important research is being undertaken to establish markers of oocyte competence that can help to design better *in vitro* conditions for the physiological needs of the embryos.

## Future Research Perspectives

This manuscript shows the high variation among IVEP results. A better and deeper knowledge of preovulatory oocyte physiology and the follicular fluid composition at this stage will allow us to design culture media more suitable for *in vitro* embryo production. Several studies in humans, but also in livestock species, are carried out in order to find biomarkers of oocyte competence. A competent oocyte is not only capable of successful fertilization and transmission of the female genetic contribution but also has responsibility for supporting and controlling the first few mitotic divisions, until the activation of the embryonic genome. Sustaining early embryo development depends on stored mRNA transcripts and proteins accumulated by the oocyte during its maturation period prior to ovulation. Follicular environment has a great impact on the viability of the oocyte and also likely to affect the cumulus cells surrounding the oocytes. Both, cumulus cells and follicular fluid are attractive targets for the development of non-invasive biomarkers of oocyte competence without compromising oocyte viability for IVEP programmes. 'Omic' technologies are aimed to detection of mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) for biomarker discovery studies.

### Biomarkers in granulosa and cumulus cells

Transcriptomic profiling of follicular cells (granulosa and cumulus cells) may help identify biomarkers of oocyte and embryo competence. In a revision from Uyar et al. (2013) described the study made of the follicular cells from oocytes that resulted in a positive pregnancy and the follicular cells from oocytes resulting in embryos that represent developmental failure. Among the 115 differentially expressed genes, altered expression of 3-beta-hydroxysteroid dehydrogenase (*HSD3β1*), ferrodoxin 1 (*FDX1*), serine (or cysteine) proteinase inhibitor clade E member 2 (*SERPINE2*), cytochrome P450 aromatase (*CYP19A1*) and cell division cycle 42 (*CDC42*) were significantly associated with pregnancy outcome. Using the findings of this initial study, the investigators subsequently identified phosphoglycerate kinase 1 (*PGK1*), *RGS2*, regulator of G-protein signalling 3 (*RGS3*), *CDC42*, and UDP-glucose pyrophosphorylase 2 (*UGP2*), and pleckstrin homology-like domain, family A, member 1 (*PHLDA1*), as potential follicular markers associated with embryo quality resulting in a successful pregnancy.

### Metabolomic in follicular fluid

Follicular fluid contains metabolites important for oocyte growth and development and hence reflects embryo viability and oocyte quality. Nuclear magnetic resonance (NMR) spectroscopy is ideal for the analysis of biofluids, because it allows the simultaneous identification of all the low-molecular-weight metabolites in the intact FF sample with little sample preparation. Metabolite profiling approach is called metabolomics or metabonomics biomarkers. Metabolite analysis, specifically by NMR spectroscopy, has great potential for the possibility of identifying candidate biomarkers that may allow the assessment of oocyte competence (McRae et al. 2012). In human, analysis of the fatty acid composition revealed differences between follicular fluid from the cleaved and the non-cleaved oocyte sample groups. Of particular interest were the higher concentration of total saturated and the lower concentration of total polyunsaturated fatty acids in the non-cleaved sample group (O'Gorman et al. 2013). In our laboratory, analysing PUFA (Poly Unsaturated Fatty Acids) in follicular fluid of goats we found significant differences among PUFAs according to follicular size, female age an year seasons (not published data). Also in bovine, Orsi et al. (2005) analysing carbohydrate and amino acids profile in preovulatory follicular fluid (FF) concluded that *in vitro* maturation medium had higher pyruvate and lower lactate concentrations than preovulatory FF. In porcine, Bertoldo et al. (2013) using NMR spectroscopy that the concentrations of glucose, lactate, hypoxanthine and five amino acids were lower in large follicles compared with small ones, except for glucose.

### Proteonomic in follicular fluid

Follicular fluid (FF) includes various biologically active proteins which can affect follicular growth and maturation. Certain proteins could reflect the physiological and pathological status of follicles. The composition of FF is related to the metabolism of follicular cells and the permeability of the follicular wall; hence, FF proteome could reflect the physiological and pathological status of follicles during development. Therefore, the number of proteins transported into the FF increases as follicle develops. In humans, Severino et al. (2013) using iTRAQ (for relative and absolute quantitation), a non-gel based multiplexed protein quantitation technique that provides relative and absolute measurements of in theory all peptides from different samples/treatments, concluded that quantification of 89 proteins, 30 of which were differentially expressed in follicular fluid with successful compared to unsuccessful IVF outcome. The targeted study, based on multiplexed antibody protein arrays, allowed the simultaneous quantification

of 27 low abundance proteins, including growth factors, chemokines and cytokines endowed with pro- and anti-inflammatory activity. In porcine Ducolomb et al. (2013) observed that protein fraction composed of immunoglobulin fragments, cytokeratin, transferrin and plasminogen precursor were specific indicators of oocytes with increased *in vitro* maturation and *in vitro* fertilization.

In conclusion, 'omic' techniques will allow a better knowledge of oocyte competence and environmental follicular conditions to improve *in vitro* maturation of oocytes. Studies on oocyte oviduct interactions will provide information to design culture media to avoid polyspermic and abnormal fertilized zygotes.

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## Conflict of Interest

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

## Author contributions

Both authors have done an equal contribution in this manuscript.

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