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In vitro embryo production from prepubertal goat oocytes in different culture media

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À ma mère,
À mon frère
À mes soeurs

Abstract

The prepubertal goat ovary shows a large number of small oocytes with a compromised competence to develop up to blastocyst stage following IVM, IVF and IVC. Culture conditions used to support *in vitro* embryo production appears to be an important factor to suit the requirements of these oocytes and to increase the blastocyst yield. In the present thesis work, we have carried out two studies to evaluate the effects of addition of different supplements to *in vitro* maturation and culture media on embryo development and the quality of resultant blastocyst in prepubertal goat oocytes.

In oocytes from prepubertal females, different types and concentrations of substances including gonadotrophic hormones, growth factors, protein sources and antioxidants, such as insulin-transferrin-selenium (ITS) and L-ascorbic acid (AA), have been shown to improve oocyte cytoplasmic maturation and embryo development. The aim of the first study was to assess the effects of adding low level of hormones and/or ITS plus AA to IVM medium through the developmental competence and embryo quality of small oocytes from prepubertal goats. Specifically, we tested four maturation media: conventional IVM medium (CM), CM+ITS+AA and low level of hormones (named as Growth Medium; GM), CM with low level of hormones (modified CM; mCM) and CM+ITS+AA and normal level of hormones (modified GM; mGM). Cumulus-Oocyte Complexes (COCs) were classified into two categories according to oocyte diameter: small (<125 μm) and large (≥125 μm) oocytes. Large oocytes were matured in CM and small oocytes were matured in different combination of CM, GM, mCM or mGM. After IVM, oocytes were fertilized and after 24h presumptive zygotes were cultured for 8 days. Results of this study showed that using different combinations of CM, GM, mCM, and mGM for IVM of prepubertal goat small oocytes did not have a beneficial effect on the percentage of blastocysts. The high blastocyst rate was only related to oocyte diameter. However, the culture of small oocytes in GM improved the quality of blastocysts and enhanced their survival post-warming compared to CM (53.3% vs 30.8%, respectively; $P<0.05$).

The second study examined the effect of activin-A during IVM and embryo culture on the blastocyst rate of prepubertal goat oocytes. Activin is a member of the transforming growth factor β superfamily that plays a functional role in the process of cellular proliferation and differentiation. In experiment 1, with a view to search for optimal

concentration of recombinant human activin-A during IVM, three concentrations (0, 10 and 100 ng/mL) were tested on oocytes meiotic maturation and subsequent *in vitro* embryo development. The experiment 2 was performed to evaluate the effect of adding 10 ng/mL activin-A at the IVM and/or IVC media on embryo development and blastocyst quality. Results of this study demonstrated that the addition of activin-A to IVM yielded similar percentages of maturation and blastocyst. Moreover, the addition of 10 ng/mL of activin-A throughout the whole embryo culture significantly increased rates of development to the blastocyst stage as compared with the control group (19.5 ± 2.21% vs 13.1 ± 2.37%, respectively; $P<0.05$). Blastocyst quality was not improved in the presence of activin-A neither during IVM evaluated after Hoechst 33342 staining, nor during IVM and/ or IVC evaluated by differential staining.

In conclusion, IVM of prepubertal goat small oocytes in GM would be useful to improve the quality of blastocysts produced *in vitro* and the presence of activin-A during IVC enhances embryo development of prepubertal goat oocytes.

Resumen

Los ovarios de cabras prepúberes proporcionan una población de ovocitos de pequeño diámetro con una menor competencia para el desarrollo hasta blastocisto después de la maduración, fecundación y posterior cultivo *in vitro* (MIV, FIV y CIV). Las condiciones del cultivo *in vitro* aportan los requerimientos necesarios a los ovocitos e influyen sobre su posterior desarrollo embrionario. En esta tesis hemos llevado a cabo dos estudios para evaluar los efectos de la adición de diferentes suplementos a los medios de MIV y CIV sobre el desarrollo embrionario y la calidad de los blastocistos obtenidos a partir de ovocitos de cabras prepúberes.

En ovocitos de hembras prepúberes, la adición de sustancias, incluyendo las hormonas gonadotrópicas, factores de crecimiento, fuentes de proteínas y antioxidantes, como son el complejo insulina-transferrina-selenio (ITS) y el L-ácido ascórbico (AA), han demostrado producir una mejora en la maduración citoplasmática de los ovocitos y su subsiguiente desarrollo embrionario *in vitro*. El objetivo del primer estudio fue evaluar los efectos de la adición de AA, ITS y un bajo nivel hormonal en el medio de MIV sobre el desarrollo embrionario y la calidad de los blastocistos obtenidos. En concreto, hemos utilizado 4 medios maduración: Medio Tradicional (MT), Medio de Crecimiento (MC): MT + ITS + AA + bajo nivel hormonal, Medio Tradicional modificado (MTm): MT + bajo nivel hormonal y Medio de Crecimiento modificado (MCm): MT + ITS + AA + nivel hormonal normal. Los complejos–ovocito-cúmulus (COCs) se clasificaron en dos grupos según el diámetro ovocitario: pequeños (<125 μm) y grandes (≥125 μm). Los ovocitos ≥125 μm fueron madurados en MT mientras que los ovocitos <125 μm fueron madurados en combinaciones de MT, MC, MTm y MCm. Después de la MIV, los ovocitos fueron fecundados *in vitro* y posteriormente los embriones fueron CIV durante 8 días. En este estudio se observó que el desarrollo embrionario de los ovocitos <125 μm no mejoró en las diferentes combinaciones de medios de MIV estudiadas (MT, MC, MTm y MCm). El mayor desarrollo hasta blastocisto se obtuvo en el grupo de embriones procedentes de ovocitos ≥125 μm. Sin embargo, cuando los ovocitos de menor diámetro se maduraron en MC, se observó una mejoría significativa en la calidad de los blastocistos obtenidos, optimizando la supervivencia de los embriones tras la descongelación comparado con el MT (53.3% vs 30.8%, respectivamente; $P<0.05$).

En el segundo estudio se analizó el efecto de la suplementación de los medios de MIV y CIV con activina-A sobre el desarrollo embrionario. La activina-A es un miembro de la superfamilia del factor de crecimiento transformante β (TGF-β) y cumple un papel importante en la proliferación y diferenciación celular. Con el fin de determinar el efecto de la activina-A sobre los ovocitos de cabras prepúberes, se realizaron 2 experimentos, analizando la maduración nuclear de los ovocitos, su posterior desarrollo embrionario y calidad de los blastocistos obtenidos. En el primer experimento se estudió la suplementación del medio de MIV con varias concentraciones de activina-A (0, 10 y 100 ng/mL) y en el segundo experimento se evaluó la adición de 10 ng/mL de activina-A a los medios de MIV y CIV. Los resultados de este estudio demostraron que la suplementación del medio de MIV con diferentes concentraciones de activina-A proporciona similares porcentajes de maduración nuclear y de blastocistos comparado con la MIV sin activina-A. Además, la adición de 10 ng/mL de activina-A durante el CIV aumentó significativamente las tasas de desarrollo hasta blastocisto en comparación con el grupo control (19.5 ± 2.21% vs 13.1 ± 2.37%, respectivamente; $P<0.05$). Sin embargo, no se observó una mejora en la calidad de los blastocistos cuando se añadió activina-A en el medio de MIV (evaluada por la tinción de Hoechst 33342) y/o en el de CIV (evaluado por la tinción diferencial).

En conclusión, la maduración *in vitro* de los ovocitos de cabras prepúberes de diámetro <125 μm en medio de crecimiento sería una alternativa útil para mejorar la calidad de los blastocistos producidos *in vitro,* mientras que la presencia de activina-A durante el cultivo *in vitro* incrementa el porcentaje de embriones desarrollados hasta el estadío de blastocisto.

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List of abbreviations

µg	Microgram
µm	Micrometer
AA	Ascorbic Acid
AI	Artificial Insemination
ARTs	Assisted Reproductive Technologies
AI-TI	Anaphase I- Telophase I
BCB	Brilliant Cresyl Blue
CM	Conventional Medium
COCs	Cumulus–Oocyte Complexes
DNA	Desoxyribonucleic Acid
E_2-17β	Estradiol-17β
EGF	Epidermal Growth Factor
ET	Embryo Transfer
FCS	Fetal Calf Serum
FF	Folicular Fluid
FSH	Follicle Stimulating Hormone
GDF9	Growth-Differentiation Factor 9
GH	Growth Hormone
GM	Growth Medium
GnRH	Gonadotrophin-Releasing Hormone
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
IGF-I	Insulin-Like Growth Factor-I
ITS	Insulin-Transferrin-Selenium
IU	International Unit
IVC	*In Vitro* Culture
IVEP	*In Vitro* Embryo Production
IVF	*In Vitro* Fertilization
IVM	*In Vitro* Maturation
IVP	*In Vitro* Production

JIVET	Juvenile *In Vitro* Embryo Technology
LH	Luteinizing Hormone
LOPU	Laparoscopic Ovum Pick-Up
MI	Metaphase I
MII	Metaphase II
mL	Milliliter
mm	Millimeter
mM	Millimolar
MOET	Multiple Ovulation and Embryo Transfer
MPF	Maturation Promoting Factor
ng	Nanogram
OPU	Ovum Pick-Up
PN	Pronuclei
PUFAs	Polyunsaturated Fatty Acids
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SR	Serum Replacer
TE	Trophectoderm
TGFβ	Transforming Growth Factor β
ZP	Zona Pellucida

INTRODUCTION

Introduction

In recent years, demand for goat products has increased in both developing and developed countries; the population of goats in the world has increased by nearly 100% over the past four decades. In 2012 there were more than 921 million goats around the world: Asia had the greatest number, followed by Africa, South America, Europe, North America and Oceania (http://www.fao.org/). Goats have always had a major role to play in developing countries due to their adaptability under harsh and marginal rural conditions, predominantly in the tropical and subtropical zones of the world.

A growing interest in assisted reproductive technologies (ARTs) has occurred. ARTs are being used for enhancement of reproductive performance and genetic improvement in sheep and goats. In addition, they can have substantial contribution in preservation of endangered species or breeds (Cognié *et al.*, 2004). While their applications are widespread in cattle, in small ruminants it is almost restricted to artificial insemination. Nonetheless, during the last 30 years, considerable progress has been made in sheep and goat embryo technologies.

Furthermore, *in vitro* embryo production (IVEP) in small ruminants provides an excellent source of low cost embryos for basic research on developmental biology and physiology and for commercial application of the emerging biotechnologies such as nuclear transfer, transgenesis, embryo sexing and stem cells. Thus, goats offer a good model for the development of these technologies (reviewed by Cognié *et al*., 2003).

In the laboratory, embryos can be routinely produced and developed up to the blastocyst stage using three subsequent techniques: *in vitro* maturation (IVM) of oocytes recovered from ovaries, followed by sperm capacitation and *in vitro* fertilization (IVF) of matured oocytes and then *in vitro* culture (IVC) of the fertilized oocytes up to the blastocyst stage that can be transferred to recipient females or cryopreserved for future use. However, *in vitro* embryo production still is not very efficient due to several limiting factors affecting the outcome of each step of the process.

The use of prepubertal females as oocyte donors for *in vitro* embryo production (juvenile *in vitro* embryo technology or JIVET) offers considerable potential for accelerating genetic gain in domestic livestock through reduced generation interval as compared to classical approach (Armstrong *et al*., 1997). However, reduced

developmental competence in oocytes of juvenile animals as compared to their adult counterpart when matured *in vitro*, was addressed in numerous studies on farm animals including bovine (Presicce *et al*., 1997), ovine (Ledda *et al*., 1997), caprine (Leoni *et al*., 2009) and porcine (Marchal *et al*., 2001).

Evidence suggests that the low number of embryos obtained from prepubertal females is mainly due to the low competence of these oocytes, and this competence is defined by Sirard *et al*., (2006) as the ability to: 1) resume meiosis; 2) cleave following fertilization; 3) develop to the blastocyst stage; 4) induce pregnancy; and 5) bring offspring to term in good health, and it is acquired progressively during oocyte growth (Sorensen and Wassarman, 1976; Eppig, 1996).

Embryo development is influenced by events occurring during oocyte maturation. For successful IVM, oocytes must undergo nuclear and cytoplasmic maturation. Therefore the efficiency of embryo development from these oocytes is mainly attributed to limitations induced by two factors: failure to select the most competent oocytes for *in vitro* production and an inadequate understanding of oocyte maturation and culture conditions to provide an optimized environment, necessary to support the physiological changes associated with oocytes maturation and required for attaining developmental competence (Rizos *et al*., 2002a; Rizos *et al*., 2002b; Morton *et al*., 2008).

To overcome the reduced maturational competence of oocytes from juvenile females, several factors and strategies have been shown to affect the oocyte competence such as: selection follicles and oocytes diameters (Anguita *et al*., 2007; Romaguera *et al*., 2010a), donor's age (Ledda *et al*., 1997; Baldassarre and Karatzas, 2004), hormonal stimulation (Koeman *et al*., 2003), *in vitro* maturation protocol (Wu *et al*., 2006) and supplementation to oocyte maturation media (Mertens *et al*., 2005; Romaguera *et al*., 2010b; Córdova *et al*., 2011).

Numerous research groups have reported a positive relationship between follicle size and the ability of its oocyte to develop to blastocyst stage in cows (Lonergan *et al*., 1994), ewes (Cognié *et al*., 1998), sows (Motlik *et al*., 1984), buffalos (Raghu *et al*., 2002a) and goats (Martino *et al*., 1994; Crozet *et al*., 1995). The developmental competence of an oocyte is obtained progressively as follicular diameter increases to maximum size, suggesting that the follicle size and oocyte diameter are closely related, and as both increase oocyte competence is gradually acquired within the ovary.

According to oocyte diameter, previous studies in our laboratory concluded that a higher blastocyst rate is obtained in oocytes larger than 135 µm (12.5%) compared to oocytes of 125 to 135 µm diameter (1.95%) after IVF (Anguita *et al*., 2007). However, when ICSI was used to fertilize oocytes, Jiménez-Macedo *et al*., (2006) did not find differences between these two oocytes categories.

A range of oocyte maturation media and additives have been tried to accomplish with the *in vitro* maturation of oocytes, and their developmental competence. Reports in the literature have described the effect of maturing oocytes *in vitro* in culture medium supplemented with different types and concentrations of substances including gonadotrophic hormones, antioxidants, growth factors and protein sources. The addition of gonadotropins (FSH and LH) and estradiol-17β (E_2-17β), to the maturation medium significantly has improved maturation rates (nuclear, cytoplasmic maturation and cumulus expansion) in mammalian oocytes (Sanbuissho and Threlfall, 1990; Mattioli *et al*., 1991; Singh *et al*., 1993; Guler *et al*., 2000; Abdoon *et al*., 2001; Roberts *et al*., 2005; Alvarez *et al*., 2009), has promoted the ability of the oocytes to form a male pronucleus after *in vitro* fertilization (Thibault *et al*., 1975; Funahashi and Day, 1993) and their developmental competence up to blastocyst stage (Men *et al*., 2002). The concentration of gonadotropins used during the process of IVM of oocytes has been showed to determine the results of oocyte maturation and developmental competence. In bovine, Liu *et al*., (2011) reported that extremely high concentrations of gonadotropins have detrimental effects on oocyte nuclear maturation and embryo development and increase apoptosis in cumulus cells. In porcine, Bing *et al*., (2001) have reported that using a low concentration of FSH (1 µg/mL) is not sufficient to induce full nuclear maturation of oocytes, compared with 10 µg/mL FSH. On the other hand, some studies have shown that lower concentrations of hormones are enough for IVM in mice (Peluso, 1988; Byskov *et al*., 1997). Also, in prepubertal gilts Wu *et al*., (2006) have observed a significant increase in blastocyst yield derived from oocytes obtained from small follicles by reducing 250-fold the concentration of the hormones in a protocol of IVM medium (2-step culture system). Results of those studies suggest the importance of judicious use of gonadotropins to enhance *in vitro* oocyte maturation and embryonic development, depending on the species and the donor age.

During *in vitro* culture, cells are exposed to higher concentrations of oxygen than those that occur *in vivo* and this causes the constant production of free radicals. High levels of free radicals cause damage to cell components by lipid peroxidation, protein modification, and DNA damage resulting in impaired cell function and subsequently can affect oocyte maturation and further embryonic development (Cetica *et al*., 2001). Thus, to optimize embryo production, oocytes need to be protected against oxidative stress during *in vitro* culture. Several antioxidants have been added to maturation media (Ali *et al*., 2003). Insulin-transferrin-selenium (ITS) combination is routinely used in culture systems to promote the development of oocytes because of its protecting effects from oxidative damage by reducing free radical production and inhibiting lipid peroxidation, and the promoting effect of the uptake of glucose and amino acids (Tatemoto *et al.,* 2004; Ebert *et al*., 2006). Studies performed in several species during *in vitro* maturation, showed that ITS addition during IVM, improved cytoplasmic maturation and decreased polyspermic fertilization rate in porcine oocytes (Jeong *et al*., 2008). Similarly, in bovine, supplementation with ITS (Jaakma *et al*., 1997; Bowles and Lishman, 1998) showed a higher blastocyst rate. In mice, an increase on blastocyst rate (Zhang and Armstrong, 1990) as well as their quality (Jeong *et al*., 2008) was observed when IVM medium was supplemented with ITS. Moreover, the ascorbic acid is an anti-oxidant that has been shown to support cytoplasmic maturation of porcine oocytes relating to developmental competence after fertilization by alleviating oxidative stress (Tatemoto *et al*., 2001). In addition, the presence of ascorbic acid in the culture medium has been shown to significantly reduce the level of apoptosis in mouse oocyte-associated granulosa cells although no improvement of oocyte developmental competence was detected (Eppig *et al*., 2000). Ascorbic acid addition plus the ITS complex to the culture medium also enhances the blastocyst production of gilts (Wu *et al*., 2006) and bovine oocytes (Córdova *et al*., 2010).

A second approach to improve the developmental competence of oocytes after their recovery from the follicle has been the addition of growth promoting substances to *in vitro* maturation and culture media, such as epidermal growth factor (EGF), growth hormone (GH), inhibin, activin and follistatin. Those growth factors are generally added to the basal medium to increase cell proliferation and to stimulate specific cell functions (Van der Valk *et al*., 2010) despite modest improvements in development have been achieved in this way. Activin-A is an important member of the transforming growth

factor β (TGFβ) superfamily. In the ovaries of vertebrates, activin-A is expressed predominantly in the granulosa cell layer of follicles, suggesting important roles in processes such as folliculogenesis, steroid hormone production, and oocyte maturation as paracrine or autocrine factors (Peng and Mukai, 2000). Studies *in vitro* showed that exposure of oocytes during maturation and/or culture to different concentrations of exogenous activin-A, stimulates the resumption of meiosis of oocytes in several species (mice (Itoh *et al*., 1990); cattle (Stock *et al*., 1997; Park *et al*., 2010; Trigal *et al*., 2011); rhesus monkey (Alak *et al*., 1996) and human (Alak *et al*., 1998)). Moreover, a positive effect of activin-A on embryo development were shown when the protein was added at the earliest stages of bovine (Yoshioka and Kamomae, 1996, Yoshioka *et al*., 1998a, Lee *et al*., 2009) embryo culture.

The basal components of the TGFβ superfamily signalling pathways are the type II and type I transmembrane serine and threonine kinase receptors and the cytoplasmic Smad proteins (Heldin *et al*., 1997). Activins signal through type I and type II receptor proteins, both of which are serine/threonine kinases. Subsequently, signals such as Smad proteins are phosphorylated (Peng and Mukai, 2000). Activin receptors are represented by two isoforms: activin receptors type IA (ActR-IA), IB (ActR-IB), IIA (ActR-IIA) and IIB (ActR-IIB). Both protein and mRNA for activin-A and activin receptors type I and II have been localized in both oocyte and granulosa cells of follicles at various developmental stages in a variety of species, including human (Eramaa *et al*., 1995; Mather *et al*., 1997; Sidis *et al*., 1998), rodent (Zhao *et al*., 2001), porcine (Van den Hurk and Van de Pavert, 2001), and bovine (Hulshof *et al*., 1997) follicles and caprine ovary and follicles (Silva *et al*., 2004; 2006). These facts suggest that activin-A may play a role in development of prepubertal goat oocytes, and investigation about the mechanism of action of this protein in oocytes from prepubertal goat during *in vitro* maturation and during embryo *in vitro* culture is required because, to date, no studies on the effects of activin-A on *in vitro* developmental potential of prepubertal goat oocytes have been reported.

In summary, juvenile donors will have an important place in livestock-improvement as well as in biotechnology programs. However, further investigations on molecular and cellular approaches will increase our understanding of cytoplasmic maturation in prepubertal oocytes allowing a more efficient *in vitro* production of embryos by IVM/IVF, ICSI and nuclear transfer, and the production of offspring from juveniles to

reduce the generation interval and increase the rate of genetic progress in breeding schemes.

All these points above mentioned need to be addressed in future studies to find the most efficient *in vitro* embryo production to achieve high rates of oocyte maturation and embryo development from prepubertal goats.

Continuing efforts of previous research performed in our laboratory with the aim to improve the *in vitro* embryo yield of prepubertal goat oocytes, the present thesis work is designed to evaluate the effects of addition of different supplements to *in vitro* maturation and culture media on embryo development.

LITERATURE REVIEW

Literature Review

Over recent years, considerable research into *in vitro* embryo production (IVEP) technology has been undertaken in an attempt to determine which conditions are needed during the three subsequent steps: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). These *in vitro* processes are intended to mimic the processes *in vivo*, for improving animal breeding programs and biomedical field. These steps will be discussed in more detail in subsequent sections of this thesis.

2.1. *In vitro* maturation (IVM)

Full maturation involves both nuclear and cytoplasmic events that confer on the oocyte the capacity for supporting normal fertilization and early embryonic development (Thibault and Gerard, 1973; Moor and Trounson, 1977). Mammalian oocytes arrested at the germinal vesicle (GV) stage acquire the capacity to resume meiosis during growth of the ovarian follicle from the primary to the preovulatory stage. As removed from antral follicles of females ovaries, cumulus–oocyte complexes (COCs) can be induced to undergo *in vitro* the sequence of events found during *in vivo* maturation. Matured oocytes reach metaphase II stage (nuclear maturation), accompanying by the molecular and structural changes that allow them to support normal fertilization and embryonic development (cytoplasmic maturation) (Bevers *et al*., 1997; Watson, 2006).

2.1.1. Nuclear maturation

Oocytes that are collected from growing follicles for IVEP are blocked at the prophase of the first meiotic division. As soon as they are removed from follicles and transferred to a culture medium, the oocytes can spontaneously resume the meiotic division (Edwards, 1965). The resumption of meiosis is characterized by dissolution of the nuclear membrane and a condensation of the chromatin, a nuclear stage referred as germinal vesicle breakdown (GVBD). Chromosomes are then arranged in metaphase I (MI) stage which is followed by anaphase I to telophase I (AI-TI) transition and oocytes progress to metaphase II (MII) where they remain until their fertilization. Nuclear maturation can be visualized by the extrusion of the first polar body. Studies *in vitro* (Samake *et al*., 2000; Bormann *et al*., 2003), showed that under specific conditions, pre-

selected oocytes can resume the nuclear maturation reaching 70–90% of the oocytes the MII stage. Prepubertal goat oocytes recovered from follicles larger than 3 mm (Martino *et al*., 1994), were able to reach the MII stage at a rate of 72 % after IVM.

2.1.2. Cytoplasmic maturation

Embryo development is strongly influenced by events occurring during oocyte maturation. IVM seems to be the limiting factor, because even after selection of a homogenous population of cumulus-oocyte complexes, only 35% will attain full cytoplasmic maturation and possess the competence to produce a viable, transferable blastocyst (Blondin and Sirard, 1995). Cytoplasmic maturation covers all morphologic and molecular events accompanying nuclear maturation after LH surge in preovulatory follicles and preparing oocyte cytoplasm to successful fertilization and embryo development. As reviewed by Mermillod *et al.,* (2006), cytoplasmic maturation includes well known morphological modifications, such as the migration of cortical granules in the cortical region of the ooplasm. These granules are stored during oocyte growth and release their enzymatic content in the perivitellin space after fertilization. Cytoplasmic maturation also includes the accumulation of mRNA, proteins, alterations to Golgi complexes, mitochondrial accumulation in the ooplasm, and cumulus expansion (Sirard, 2001). Cumulus expansion can importantly be used to microscopically assess the *in vitro* maturation rate of oocytes (Gupta *et al.,* 2005). This store of maternal RNA instill upon the oocyte a capacity to decondense and remodel the sperm head, to form the pronuclei (PN), zygote formation, early embryogenesis and normal fetal development (Mermillod *et al*., 1999; Watson, 2006).

2.1.3. Factors affecting oocyte quality

Good quality oocyte maturation is an essential prerequisite for embryo development. Naturally the oocyte quality is determined by the oocyte's ability to mature, be fertilized and give rise to normal offspring after gestation (Sirard *et al*., 2006). This capacity is acquired during folliculogenesis, as the oocyte grows and during the period of oocyte maturation (Krisher, 2004). Proper oocyte selection in the laboratory is crucial for successful embryo production. A study performed in bovine (Blondin and Sirard, 1995)

showed that the best indicators of an immature oocyte ability to undergo maturation and embryonic development were the presence of an intact complement of cumulus cell layers surrounding the oocyte and a homogenous appearing cytoplasm. Also, the follicular and oocyte sizes, as well as several factors, such as age of the donor animal, and the media used for maturing the oocytes are some factors that have been linked to the maturational competence of oocytes and have therefore been proposed as selection criteria for oocyte quality, as a key factor in determining the development of embryos to the blastocyst stage (Lonergan *et al*., 2003).

2.1.3.1. Effect of follicle and oocyte size

The size of the follicle seems to be an important factor in the selection of potential oocytes (reviewed by Sirard *et al*., 2006), involving RNA or protein stores as factors involved in oocyte competence. Increased developmental competence of oocytes has been associated with increased follicular diameter as reported in several studies in various species. In cattle, Lonergan *et al*., (1994) reported a higher proportion of blastocysts obtained from follicles > 6 mm compared to 2–6 mm follicles. Similarly, in calves, Kauffold *et al*., (2005) showed an increase in blastocyst production in oocytes coming from follicles with diameter > 8 mm than from follicles of < 8 mm. It has been suggested that the reason for the differences between the follicular diameters on oocyte quality is due to their content. The follicular fluid (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). Thereby, Ali *et al*., (2004) illustrated the effect of follicle diameter by the use of bovine follicular fluid obtained from large (> 8 mm) and small follicles (2–5 mm) as a supplement of *in vitro* maturation media of bovine oocytes. Results of this study showed that following fertilization and embryo culture, more oocytes reached the blastocyst stage when oocytes were cultured with FF from large follicles compared with FF derived from small follicles. Similarly, in buffalo (Raghu *et al*., 2002a), sheep (adult: Cognié *et al*., 1998; prepubertal: Ledda *et al*., 1999b), goats (adult: De Smedt *et al*., 1994; Crozet *et al*., 1995; prepubertal: Romaguera *et al*., 2010a) and pigs (Marchal *et al*., 2002) has been reported that the acquisition of meiotic competence as well as the ability to develop up to the blastocyst stage is acquired sequentially as the follicle

enlarges. In prepubertal goats, Romaguera *et al,*. (2010a) demonstrated that oocytes from follicles of ≥ 3 mm showed greater mean oocyte diameter, higher percentages of fragmented DNA cells, higher cleavage rates and greater developmental competence to the blastocyst stage than oocytes from follicles of < 3 mm.

The comparison of the oocyte diameter is often used as a marker for oocyte maturity or meiotic competence, able to attain their full developmental competence to blastocysts *in vitro*, as there is an intensive synthesis of RNA during this phase that causes an increase in size. According to Brevini *et al*., (2007), during the oocytes growth, messenger RNAs and proteins of maternal origin are accumulated into the oocyte throughout its growth in the ovary, upon fertilization, several mechanisms are activated that control the appropriate use of such material and prepare for the synthesis of new products supporting fertilization and initiating embryo development. The association between the oocyte diameter and its ability to resume and complete meiotic maturation *in vitro* has been described in several farm animals. In bovine, Otoi *et al*., (1997) classified oocytes in six categories according to oocyte diameter (< 110 µm, 110 to < 115 µm, 115 to < 120 µm, 120 to < 125 µm, 125 to < 130 µm and ≥ 130 µm), and concluded that bovine oocytes have acquired full meiotic competence at a diameter of 115 µm but not yet attained full developmental competence to blastocysts, and that oocytes have acquired full developmental competence at a diameter of 120 µm. In ovine, Shirazi and Sadeghi (2007) reported no significant differences in the percentage of oocytes that reached the MII stage (81, 82, and 84%) with diameters of < 110 µm, 110–150 µm, > 150 µm, respectively. In buffalo, the rate of *in vitro* blastocyst production was significantly higher in oocytes with diameters greater than 145 µm (Raghu *et al*., 2002a). In prepubertal goats, oocytes were classified by Anguita *et al*., (2007) and Jiménez-Macedo *et al*., (2006), into 4 categories of diameter: < 110 µm; 110 to 125 µm; 125 to 135 µm and > de 135 µm, results of those studies showed that oocytes smaller than 125 µm fertilized by IVF (Anguita *et al*., 2007) and ICSI (Jiménez-Macedo *et al*., 2006), were unable to develop up to blastocyst stage. Anguita *et al*., (2007) observed that the oocyte diameter was positively related to the percentage of oocytes at MII after IVM (0, 21, 58 and 78%, respectively) and the percentage of blastocysts obtained at 8 days postinsemination (0, 0, 1.95 and 12.5%, respectively). However, using ICSI to fertilize these oocytes categories, the percentage of ICSI derived blastocysts (blastocysts/injected oocytes) obtained from oocytes of 125–135 µm diameter had

similar blastocyst development to oocytes larger than 135 µm (15.9 and 11.1%, respectively) (Jiménez-Macedo *et al*., 2006). This difference between oocyte categories after IVF and ICSI protocols could be explained by the fact that oocytes selected to perform ICSI have completed their nuclear maturation. Also, the lack of polyspermic zygotes by the microinjection could be one other explanation.

2.1.3.2. Age of the donor

There is a general agreement upon the fact that the use of prepubertal females as oocytes donors plays an important role on *in vitro* embryo production programs, by the reduction of the generation interval and consequently increasing the intensity of breeding. However, oocytes derived from juvenile females show a reduced developmental competence as reported in numerous studies on farm species including bovine (Revel *et al*., 1995; Damiani *et al*., 1996; Khatir *et al*., 1998), ovine (Ledda *et al*., 1997; O'Brien *et al*., 1997b), and porcine (Marchal *et al*., 2002). In caprine, using *in vitro* produced zygotes from laparoscopic ovum pick-up (LOPU), both cleavage and blastocyst development rates of embryos from adult donors have been higher than those from prepubertal donors (90 and 16% vs. 82 and 6%, respectively) (Wang *et al*., 2002a).

Many factors may reduce the development competence of oocytes collected from prepubertal animals. In bovine, the lower developmental ability of oocytes from 3-month-old calves compared with that of cyclic cow oocytes may depend on some defective endocrine environment encountered *in vivo* before the onset of puberty (Revel *et al*., 1995). Gandolfi *et al*., (1998) observed differences in size between calf and cow oocytes (118 µm and 123 µm, respectively) and showed that the difference in the developmental competence may be induced because of difference in gene expression abundance between adult and the prepubertal oocytes, showing a reduced protein synthesis in oocytes and cumulus cells from calves. It has been also reported that in oocytes from prepubertal donors, structural changes are delayed and incomplete and may contribute to failures of appropriate zona pellucida (ZP) changes (reviewed by Slavik *et al*., 2005). In prepubertal goats, it has been revealed functional deficiencies in cytoplasmic maturation of oocytes, such as altered distribution of cortical granules (Velilla *et al*., 2004) and mitochondria (Velilla *et al*., 2006), disorganization of microtubule and microfilament (Velilla *et al*., 2005) and alteration in total RNA content,

p34 (cdc2) and cyclin B1 expression as well as maturation promoting factor (MPF) activity (Anguita *et al*., 2007; 2008). After *in vitro* fertilization, a low incidence of sperm head decondensation at fertilization (Martino *et al*., 1995; Mogas *et al*., 1997b), a high percentage of haploid embryos (Villamediana *et al*., 2001) and, consequently, a low rate of blastocyst production (Izquierdo *et al*., 2002) has been also observed.

However, other reports suggest that donor age may not be the only important criterion, since oocytes coming from follicles (> 3mm diameter) of 45 days old goats have the capacity to *in vitro* develop to blastocyst stage as well as oocytes derived from adult goats (Romaguera *et al*., 2011). Thus, the low embryo development of prepubertal females oocytes could be related to the small number of large follicules in their ovaries, as reported by Martino *et al*., (1994) since only 1.1% of follicles were larger than 3 mm.

Studies in ovine (Earl *et al*., 1994; O'Brien *et al*., 1997a; Ledda *et al*., 1999a) prove a better effectiveness of using this source of oocytes applying a previous stimulation of prepubertal females with exogeneous gonadotropins. A positive effect of gonadotropins on follicular and oocyte size could be the cause of the lack of differences in embryo development between prepubertal and adult oocytes, which allow the oocytes to develop up to blastocyst stage similarly to that obtained when oocytes from adult sheep. Besides, these oocytes showed higher diameter than oocytes from unstimulated lambs. Also in caprine, following ovarian stimulation and laparoscopic recovery, prepubertal and adult goat oocytes cultured in semi-defined media presented a similar developmental competence to blastocyst stage (Koeman *et al*., 2003).

For all above mentioned and in order to make use of these prepubertal oocytes more efficiently, it is important to develop culture systems that permit oocytes to acquire the competence for undergoing maturation, fertilization, and development up to blastocyst stage *in vitro* in a similar way than their adult counterparts and those coming from *in vivo*.

2.1.3.3. Media and supplements used for IVM

In vitro maturation is the most critical part of the whole process of *in vitro* embryo production. Due to the heterogeneous nature of immature oocytes (oocytes from a range of follicle stages) used for IVEP, the *in vitro* maturation of oocyte can be influenced by

culture media components and culture conditions used for IVM (Cognié *et al*., 2004). Several culture media have been proposed for IVM (MEM, Waymouth, Ham-F12, etc.). However, the most widely used seems to be 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, FSH, LH, estradiol-17β (E_2-17β), plus complex fluids (heat-treated serum, follicular fluid) (reviewed by Cognié *et al*., 2003; Tibary *et al*., 2005; Paramio, 2010). Nevertheless, there are some research teams using synthetic oviductal fluid (SOF) medium instead of TCM199 in sheep (Shabankareh and Akhondi, 2012) and goat (Ongeri *et al*., 2001; Bormann *et al*., 2003; Herrick *et al*., 2004) oocytes.

2.1.3.3.1. Hormones

During *in vivo* follicular development, the mammalian follicular oocyte is arrested at diplotene stage of meiosis I, and resumption from this arrest depends upon the preovulatory surge of gonadotropins (Eppig, 1996). Gonadotropin hormones and E2-17β are generally used in *in vitro* maturation protocols. It has been shown to improve nuclear and cytoplasmic oocyte maturation as well as expansion of the surrounding cumulus cells of several species including porcine (Mattioli *et al*., 1991; Singh *et al*., 1993; Schoevers *et al*., 2003; Alvarez *et al*., 2009), ovine (Guler *et al*., 2000), murine (Roberts *et al*., 2005), bovine (Sanbuissho and Threlfall, 1990), equine (Tremoleda *et al*., 2003), buffalo (Abdoon *et al*., 2001) and caprine (Pawshe *et al*., 1996). Moreover, gonadotropins improve the ability of mammalian oocytes to form the male pronucleus after IVF (Thibault *et al*., 1975; Funahashi and Day, 1993) and their developmental competence up to blastocyst stage (Men *et al*., 2002). In goats, blastocyst formation is also improved by media containing 1 IU/ml FSH reporting a rate of 19.4% to 22.6% compared to a 12% blastocyst formation rate in media without FSH (Wang *et al*., 2007). Other effects of the presence of gonadotropins during IVM have been observed on gene profile expression of granulosa cells from bovine (Endo *et al*., 2013) and maternal mRNA levels in oocytes from buffalo (Nath *et al*., 2013), that have been shown to be gonadotropin (FSH and LH) dependents.

The concentration on gonadotropins used during the process of IVM of oocytes has been showed to determine the results of oocyte maturation and developmental

competence. In bovine, Liu *et al,*. (2011) reported that extremely high concentrations of gonadotropins have detrimental effects on oocyte nuclear maturation and embryo development and increase apoptosis in cumulus cells. In porcine, the use of a low concentration of FSH (1 μg/mL) seems not be sufficient to induce full nuclear maturation of oocytes, compared with 10 μg/mL FSH (Bing *et al*., 2001). However, and in contrast, some studies have shown that lower concentrations of hormones are enough for IVM of mice oocytes (Peluso, 1988; Byskov *et al*., 1997). Also in gilt, Wu *et al*., (2006) have observed a significant increase in blastocyst yield derived from oocytes obtained from small follicles by reducing 250-fold the concentration of the hormones in the protocol of IVM (2-step culture system).

Estradiol-17β is also involved in *in vitro* oocyte maturation in numerous species, including cattle (Sirotkin, 1992), sheep (Guler *et al*., 2000) and goats (Pawshe *et al*., 1996). In bovine, the supplementing of IVM medium with 1 μg/mL E_2-17β enhanced oocyte maturational success in some cases (Sirotkin, 1992), but was deleterious in others (Beker-van Woudenberg *et al*., 2004). In prepubertal goats, supplementation with high concentrations of E_2-17β (10 and 100 μg/mL) during IVM was found to be inhibitory on the progression to metaphase II of oocytes collected from prepubertal goats as compared with 1 μg/mL (Lv *et al*., 2010).

Results of those studies suggest the importance of judicious use of gonadotropins to enhance *in vitro* oocyte maturation and embryonic development, depending on the dose, specie and the donor age. Gonadotropic concentration commonly added to oocyte maturation of prepubertal and adult goats IVM medium is 10 μg/mL LH, 10 μg/mL FSH and 1 μg/mL E_2-17β (Mogas *et al*., 1997a; Cognié *et al*., 2004).

2.1.3.3.2. Antioxidants

The manipulation of gametes and embryos in an *in vitro* environment when performing assisted reproductive techniques carries the risk of exposure of these cells to supraphysiological levels of reactive oxygen species (ROS) (Agarwal *et al.*, 2006), especially when the oxygen concentration employed in most IVM protocols is approximately 20%. When the physiological balance between ROS production and antioxidant defences is lost during exposure to stressful stimuli, oxidative stress

subsequently results in damage to nucleic acids, proteins and lipids despite that they are produced endogenously and derived from external sources (Liu *et al.*, 2003). Studies performed *in vitro* showed that oxidative stress during *in vitro* culture provokes alterations in bovine (Fatehi *et al*., 2005), murine (Choi *et al*., 2007), porcine (Tatemoto *et al*., 2000) and goat (Rodriguez-Gonzalez *et al.,* 2003) oocytes, that impair their maturation and developmental competence.

Within cells, one of the means to control excessive ROS formation is their degradation by antioxidant enzymes. For the past few years, the regulatory role of antioxidants, during oocyte maturation and embryonic development has been a subject of great research interest for their potential use to improve oocyte nuclear and cytoplasmic competence and quality of *in vitro* produced embryos. An antioxidant is any substance that, when present at low concentrations compared to that of an oxidizable substrate can significantly delay or prevent the oxidation process (Halliwell and Gutteridge, 1989). Several previous studies have reported that the presence of non-enzymatic antioxidants, such as β-mercaptoethanol (Choe *et al.,* 2010), vitamin A (Rajesh *et al.,* 2010), vitamin C (Tao *et al*., 2010), vitamin E (Tareq *et al.,* 2012), cysteine/cysteamine (Zhou *et al*., 2008), hypotaurine (Mizushima and Fukui, 2001), taurine (Manjunatha *et al*., 2009) or melatonin (Cebrian-Serrano *et al*., 2013), enhance embryo development ability or quality after IVC. For instance, improvements in oocyte maturation, embryo development and quality, and embryo survival ability have been observed when oocytes and embryos are treated with these antioxidants in pig (Kitagawa *et al*., 2004; Hossein *et al*., 2007), cattle (Olson and Seidel, 2000; Takahashi *et al*., 2002; Wongsrikeao *et al*., 2007; Hosseini *et al*., 2009; Rooke *et al*., 2012, Korhonen *et al*., 2012), mouse (Lane *et al*., 2002; Wang *et al*., 2002b) and sheep (Miclea *et al*., 2012). In addition, gene expression profile has been shown to be altered when porcine embryos are cultured with vitamin C (Huang *et al*., 2011). Likewise, the presence of flavonoids or melatonin in the culture medium has also reported to affect the gene expression profile in bovine (Lee *et al*., 2011) and mouse (Wang *et al.,* 2013a) embryos. Thiol compounds, such as cysteamine, 2-mercaptoethanol, cysteine, 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 (Bai *et al*., 2008; Rodriguez-Gonzalez *et al*., 2003) females. In prepubertal

goats, Rodriguez-Gonzalez *et al*., (2003) showed that the addition of 100 μM of cysteamine to the maturation medium improves embryo development. The relationship between glutathione and development competence has also been previously demonstrated in bovine and sheep oocytes (De Matos *et al*., 1995; 2002).

One of the most important antioxidant in extracellular fluids, ascorbic acid (vitamin C), plays a key role in many biological processes such as the protection of lipid structures against peroxidation and the biosynthesis of collagen and other components of the extracellular matrix (Buettner, 1993; Rose and Bode, 1993). The antioxidant properties of ascorbic acid are attributed to its capacity to significantly reduce the damage from ROS, forming ascorbate as a stable free radical (Buettner, 1993). Although it is reasonable to hypothesise that ascorbic acid mediates this ROS-decreasing through altering the expression of genes related with antioxidant response. Several works emphasize the antioxidant effect of ascorbic acid in different species. In this way, studies in porcine (Tatemoto *et al*., 2001) showed that oocytes treated with 250 μM ascorbic acid during IVM displayed enhanced abilities to undergo the male pronucleus formation and to develop to the blastocyst stage after *in vitro* fertilization by prevention of oxidative stress. Similarly, after parthenogenetic activation, ascorbic acid promoted the subsequent development of porcine cumulus-denuded oocytes (Tao *et al*., 2010), and prevented cumulus cell DNA fragmentation (Tao *et al*., 2004). Also in mice, ascorbic acid has been shown to exhibit anti-apoptotic effects, when addition of ascorbic acid (0.5 mM) into the culture medium with no serum supplementation significantly reduced the level of apoptosis in the oocyte-associated granulosa cells (Eppig *et al*., 2000).

2.1.3.3.3. Insulin-Transferrin-Selenium

Insulin is a polypeptide hormone that promotes the uptake of glucose and amino acids and may have mitogenic effects (Spicer and Echternkamp, 1995). Selenium (Se) is an essential trace element. The predominant biochemical action of Se in both humans and animals is to serve as an anti-oxidant, via the Se-dependent enzyme glutathione peroxidase, and thus protect cellular membranes and organelles from peroxidative damage (Moslemi and Tavanbakhsh, 2011). Iron-mediated toxicity has been attributed to iron (Fe), which reacts with oxygen to generate free radicals that damage

macromolecules and cause cell death (Chamnongpol *et al*., 2002). Transferrin binds iron, making the Fe ions less toxic, but still available to the cell. Thus, transferrin exercises its supportive effect on *in vitro* embryo production via its chelating effects (reviewed by Bowles and Lishman, 1998).

Studies performed *in vitro* reported that the combination of Insulin–transferrin–selenium (ITS) had a positive effect on nuclear and cytoplasmic maturation that make an improvement of the post-fertilization and embryonic development in pig oocytes (Hu *et al*., 2011). In buffalo, the yield of blastocysts was higher in IVM media containing ITS as compared to control group (Raghu *et al*., 2002b). In bovine, Córdova *et al*., (2010) showed that the presence of ITS and ascorbic acid during the first 12h of IVM rendered significantly higher blastocyst rates. Whereas in lamb oocytes the supplementation of maturation medium with ITS and ascorbic acid have shown no effects on the percentage of *in vitro* embryos produced (Catalá *et al*., 2013). Moreover, the combination of L-ascobic acid with insulin-transferrin-selenium (ITS) in a growth-supporting maturation medium of gilt oocytes (Wu *et al*., 2006) has showed to improve the competence of oocytes isolated from small follicles.

2.1.3.3.4. Activin-A

The transforming growth factor-beta (TGF-β) superfamily is a large group of peptide growth and differentiation factors that have important functions in many physiological processes, including reproduction (Bilezikjian *et al*., 2006). The activins and inhibins are members of the TGF-β superfamily and, along with follistatin, a high affinity binding protein of activin, form a group of interrelated factors originally isolated for their role in regulating the release of follicle-stimulating hormone (FSH) (Phillips, 2005). As described by Knight and Glister (2003) (Figure 1), all three proteins are synthesized and secreted by granulosa cells in an FSH-responsive manner. Local actions of activins include promotion of granulosa cell proliferation, FSH receptor expression, higher levels of P450 aromatase expression and estrogen production, and inhibition of LH-induced androgen production by theca cells. Thus, granulosa cells are likely to be the main source of paracrine factors, and are crucial for oocyte maturation. Follistatin is structurally unrelated to the activins and inhibins, but binds with high affinity to the β-subunits and neutralize the activity of inhibin (Phillips, 2005). The inhibin α-subunit

exerts negative feedback on FSH secretion, and inhibits FSH activity by suppressing its receptor expression in granulosa cells.

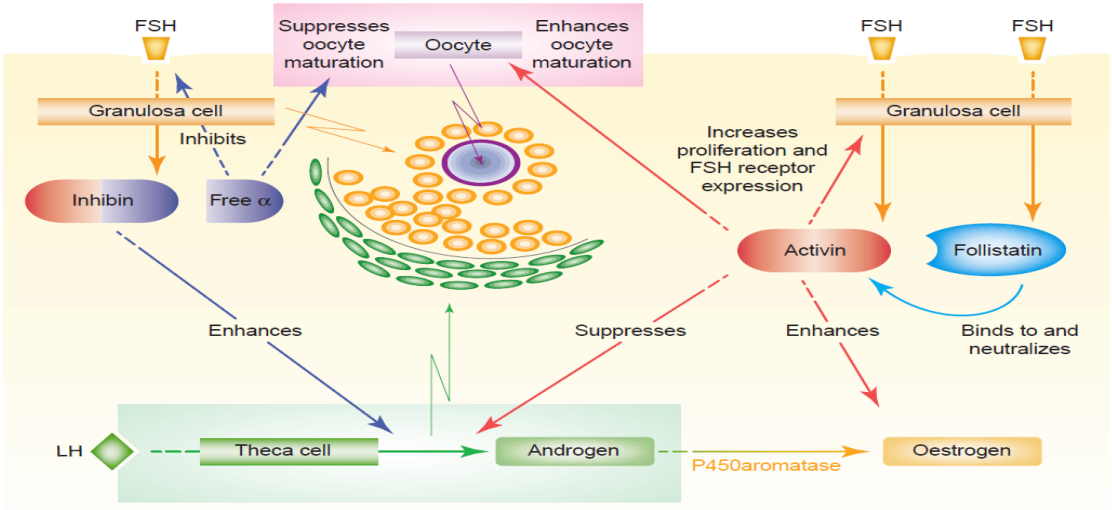

Figure 1: Schematic diagram summarizing potential intrafollicular roles of inhibins, activins and follistatin (Knight and Glister, 2003).

Various tissues express protein and mRNA for activin-A and activin receptors, among them the ovary is one of the representative tissues that expresses activin. Likewise, the 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 primates (Roberts *et al*., 1993; Sidis *et al*., 1998); swine (Van den Hurk and Van de Pavert, 2001); bovine (Gandolfi *et al*., 1995; Hulshof *et al*., 1997; Izadyar *et al*., 1998); ovine (Tisdall *et al*., 1994; Thomas *et al*., 2003); and caprine (Silva *et al*., 2004). The presence of activin receptors on oocytes and granulosa cells suggests that activin and its binding protein, follistatin, may regulate oocyte maturation.

Various studies reported that supplementing IVM media with exogenous activin-A improves oocyte developmental potential. The increase of oocyte maturation has been reported in prepubertal rats (Itoh *et al*., 1990; Sadatsuki *et al*., 1993), monkeys (Alak *et al*., 1996), cows (Stock *et al*., 1997) and humans (Alak *et al*., 1998). In bovine, it has been observed a dose dependent effect of activin-A. Thus, Silva and Knight (1998) showed an enhance in the blastocyst rate with a dose of 500 ng/mL activin-A in the IVM medium, whereas Stock *et al*., (1997) obtained the best blastocyst rate after adding 10 ng/mL to the IVM media. While other studies performed in bovine (Van Tol *et al*.,

1994) and porcine (Coskun and Lin, 1994) oocytes reported no effect of activin-A on oocyte maturation. Also in bovine, IVM in the presence of 10 ng/mL activin-A did not change the proportion of cleaved oocytes that reached the blastocyst stage (Izadyar *et al*., 1996). The reasons for these differences are likely related to a fundamental difference between culture conditions and size and maturation stage of COCs.

2.1.3.3.5. Serum and other substances

In vitro oocyte maturation (IVM) procedures frequently include supplements of animal origin, such as serum and follicular fluid, and it is considered as non-defined culture medium because of its 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, the supplementation of maturation media with serum is practiced widely. In goat, maturation media are generally supplemented with 10–20% heat-treated serum, among them: fetal bovine or calf serum (FCS) (Rho *et al*., 2001; Cognié *et al*., 2004; Katska-Ksiazkiewicz *et al*., 2007; Khatun *et al*., 2011), steer serum (SS) (Jiménez-Macedo *et al*., 2006; Romaguera *et al*., 2010b), estrous goat serum (EGS) (Keskintepe *et al*., 1994; Teotia *et al*., 2001; Baldassarre *et al*., 2002; Izquierdo *et al*., 2002), estrous sheep serum (ESS) (Tajik and Esfandabadi, 2003), serum of peritoneal fluid from rabbit and goat (Malik and Lohan, 1999), and bovine serum albumin (BSA)+EGS (Rajikin *et al.*, 1994). The follicular fluid is also used as a supplement in the IVM media in goats and sheep (Cognié *et al*., 2004). The supplementation of IVM media with follicular fluid from non-atretic or gonadotropin stimulated large follicles (>4 mm) resulted in beneficial effects in both sheep and goat oocytes (reviewed by Tibary *et al*., 2005). In goat, Mogas *et al*., (1997a) tested EGS (at different times of estrus), FCS and SS and did not find any significant differences on maturation and embryo production. However, the presence of serum or follicular fluid in culture media introduces a variation from batch to batch thus contributing to the lack of reproducibility often observed in IVEP laboratories. Furthermore, serum and follicular fluid contain many components including hormones, trace elements, and growth factors.

Culture media supplemented with different growth factors have been used to study their potential role on maturation and embryonic development in a growing number of studies. Among the latter, epidermal growth factor (EGF) has been implicated in oocyte maturation and subsequent development in several species. In sheep, *in vitro* matured oocytes in the presence of EGF had greater cumulus cell expansion and higher fertilization rates (Guler *et al.,* 2000) and increases blastocyst formation in FSH-treated ewes (Grazul-Bilska *et al*., 2003). In goat, the expression of EGF-R both in oocytes and in follicular cells in goat cumulus-oocyte complexes (Gall *et al.,* 2004), suggested that EGF may regulate the oocyte growth and may be involved in nuclear and cytoplasmic maturation. In prepubertal gilt, addition of 10 ng/mL EGF to IVM medium increased the percentage of meiotically matured oocytes (88% vs. 70%) and also the embryos presented a higher number of cells per blastocyst compared with those of control blastocysts (51.1±5.1 % vs 36.0±3.1 %) (Grupen *et al.,* 1997), showing that EGF plays an important role in both the meiotic and cytoplasmic *in vitro* maturation of porcine oocytes. Another factor to consider is insulin-like growth factor-I (IGF-I) that has also been known to stimulate oocyte maturation and promoting blastocyst development in several species, such as bovine (Matsui *et al.,* 1995), ovine (Guler *et al.,* 2000), porcine (Grupen *et al*., 1997) and caprine (Magalhães-Padilha *et al*., 2012).

Also the positive influence of growth hormone (GH) on oocyte maturation has been well reported. In ovine, Shirazi *et al.,* (2010) showed a positive effect of GH added to *in vitro* maturation medium that resulted in 73% of hatched blastocyst. In bovine, it was observed that GH induced cumulus expansion and promoted subsequent embryonic development in terms of enhancement of the number of cleaved embryos and blastocysts (Izadyar *et al.,* 1996).

A part of hormones and growth factors that are found in the follicular fluid, polyunsaturated fatty acids (PUFAs) constitute the major portion of the fatty acid content of the follicular fluid in small and large follicles (Homa and Brown, 1992). PUFAs concentration in the follicular fluid is associated with the content of the diet and may alter the oocyte maturation process and affect its further development. Actually, studies performed in cattle have shown that *in vitro* supplementation of bovine oocytes with physiological concentrations of α-linolenic acid (ALA; 18:3n-3) or linoleic acid (LA; 18:2n-6) during *in vitro* maturation had diverse effects on maturation and

subsequent embryo development; stimulatory effects were achieved by ALA supplementation (an increased maturation rate and a higher blastocyst yield and production of better quality blastocysts) whereas LA was inhibitory compared with untreated controls (Marei *et al*., 2009; 2010). However, to our knowledge, no studies of the effect of PUFAs on *in vitro* embryo development in goats have been reported.

2.2. *In vitro* fertilization (IVF)

In vitro fertilization is a complex procedure whose success requires appropriate oocyte maturation, sperm selection, sperm capacitation, and IVF media. In goat, freshly ejaculated semen is usually used for IVF (Cox *et al*., 1994; Keskintepe *et al*., 1994; Crozet *et al*., 1995; Mogas *et al*., 1997a; Izquierdo *et al*., 1998; Anguita *et al*., 2007; Romaguera *et al*., 2011). Few trials have been described where IVF was carried out using frozen–thawed sperm (Keskintepe *et al*., 1998; Rho *et al*., 2001; Bormann *et al*., 2003).

Under *in vivo* conditions, potentially fertile spermatozoa are separated from immotile spermatozoa, debris and seminal plasma in the female genital tract by active migration through the cervical mucus. So, prior to *in vitro* fertilization, spermatozoa need to be selected and prepared to inseminate the oocytes. The ejaculate comprises of a mixture of seminal plasma, mature and immature spermatozoa, non-reproductive cells, various microorganisms and non-specific debris. In bucks, the most common methods used for separating the sample into motile and non-motile fractions from fresh ejaculate or from the frozen-thawed sperm are the swim-up (Keskintepe *et al*., 1994; Izquierdo *et al*., 1998; Katska-Ksiazkiewicz *et al*., 2004; Jiménez-Macedo *et al*., 2005; Anguita *et al*., 2007; Romaguera *et al*., 2010b) and discontinuous density gradient centrifugation (Pawshe *et al*., 1996; Rho *et al*., 2001; Wang *et al*., 2002a; Bormann *et al*., 2003), or sephadex filtration (Rho *et al*., 2001) among others. Greater yields of highly motile spermatozoa can be obtained by swim-up, compared to density gradient centrifugation, but no differences were observed in terms of oocyte penetration and cleavage rate after IVF with fresh goat semen (Palomo *et al*., 1999).

Once the most viable and motile spermatozoa are selected, sperm capacitation is carried out *in vitro* and using defined media. Capacitation is a crucial process that mammalian

sperm must undergo in order to achieve fertilizing ability. It is defined as the phenomenon leads to "acrosome reaction" causing the release of proteolytic enzymes that may assist sperm penetration into the oocyte. In goat, several capacitating agents have been used to capacitate spermatozoa and to yield good fertilization and cleavage rates, such as heat-inactivated estrous from sheep (De Smedt *et al.*, 1992), goat serum (Koeman *et al*., 2003; Katska-Ksiazkiewicz *et al*., 2004), heparin using fresh semen (Izquierdo *et al*., 1998; Jiménez-Macedo *et al*., 2005) or frozen-thawed sperm (De Souza *et al*., 2013), heparin and ionomycin (Wang *et al*., 2002a; Urdaneta *et al*., 2004), calcium ionophore (Pereira *et al*., 2000) and heparin and caffeine (Younis *et al*., 1991).

Regarding the fertilization media used in goats, these include: modified Defined Media (mDM) (Crozet *et al*., 1995) Tyrode's Albumin Lactate Pyruvate (TALP) medium (Parrish *et al*., 1986) supplemented with hypotaurine (Mogas *et al*., 1997b; Izquierdo *et al*., 1998), and Synthetic Oviductal Fluid (SOF) medium (Rho *et al*., 2001). Incubation of sperm selected by swim-up in TALP with heparin (50 mg/mL) for 45 min resulted in excellent fertilization rates (Palomo *et al*., 1999; Katska *et al*., 2002; Katska-Ksiazkiewicz *et al*., 2004). Similarly, mDM plus heparin for sperm capacitation and TALP medium with hypotaurine for oocyte fertilization provided the highest proportion of penetrated oocytes (Izquierdo *et al*., 1998).

2.3. *In vitro* culture (IVC)

The last stage of *in vitro* embryo production is the culture of the presumptive zygotes in culture media where they undergo a number of divisions until the blastocyst stage 6-7 days after *in vitro* fertilization in ruminant species (Gardner *et al*., 1994). This period of postfertilization culture is the period having the greatest impact on the blastocyst quality (Rizos *et al.*, 2002a). Several major developmental events take place, including the first cleavage division; the switching on of the embryonic genome; the compaction of the morula and the blastocyst formation. The blastocyst stage involves the differentiation of two types of cells, the inner cell mass (ICM), which after further differentiation gives rise to the fetus, and the trophectoderm (TE), which ultimately contributes to the placenta formation (Watson, 1992). Clearly, any modifications of the culture environment, which could affect any or all of these processes, could have a major effect

on the quality of the embryo. Some of these factors that could influence on the rate of embryo development and the quality of the embryo are described below.

The *in vitro* oxygen concentration under which embryo development occurs has been found to modify the development up to the blastocyst stage. The oxygen tension that most mammalian embryos encounter in the reproductive tract, range from 3.5 to 8% (Fischer and Bavister, 1993). The positive effect of low oxygen tension on embryo culture has been reported for many species. For instance, the culture of *in vitro* fertilized pig embryos in low oxygen increased the number of cells (Booth *et al.,* 2005) and embryo production rate (Karja *et al.,* 2004) to greater than those cultured in 20% oxygen. In bovine, embryo development and survival rates after cryopreservation were higher when 5% oxygen was applied during IVC compared to 20% oxygen in air (Rizos *et al.*, 2001). Those results suggested that greater O_2 tension during culture is detrimental for embryo development, probably due to the accumulation of reactive oxygen species (ROS).

Different culture systems have been studied to support pre-implantation development of embryos up to the blastocyst stage. Given that female reproductive tract secretions have several amino acids that can be used as energetic substrate by the embryo, the use of amino acids in culture media improves embryo development probably through an antioxidant action and reducing the stress and cell fragmentation caused by *in vitro* embryo culture. On the other hand, in the undefined culture systems, serum is one of the main components. It can provide many beneficial factors to the embryo such as amino acids, vitamins, growth factors, and energetic substrates; however, it may also contaminate the culture media with embryotoxic factors (Bavister, 1995).

Diverse culture media have been successfully used for small ruminant embryo development such as TCM199 (Wani *et al*., 2012), B2 (Katska-Ksianzkiewicz *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 among 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 with some modification (Takahashi and First, 1992). Some laboratories routinely supplement SOF medium with serum. Studies in bovine have shown that serum has a biphasic effect; the

presence of serum can inhibit the early cleavage divisions, while it can have an accelerating effect later in development, resulting in the appearance of blastocysts earlier in culture (Lonergan *et al*., 1999; Gutiérrez-Adán *et al*., 2001). Thus, some researchers supplement IVC medium with 5-10% FCS (Cognié *et al*., 2003; Jiménez-Macedo *et al*., 2005; 2006; Anguita *et al*., 2007) at 2-3 days post-insemination to promote a higher viability after transfer of such IVP embryos (Cognié, 1999). Other studies only add BSA to SOF media (Leoni *et al.*, 2007; Shabankareh and Akhondi, 2012; Wang *et al.*, 2013b).

Currently, several animal and human studies attest a beneficial effect of addition of growth factors to culture media. However, there is still ambiguity regarding the exact role of growth factors in embryonic development, the optimal dose of growth factors to be added to the culture media, and the combinatorial effect of growth factors in embryonic development (Hegde and Behr, 2012).

Previous research has shown that growth factors such as EGF and IGF-I induced a positive effect on preimplantation development by stimulating metabolism and growth of embryos. In bovine, Palma *et al*., (1997) demonstrated that culture media containing high concentrations of IGF-I improved the development of embryos produced *in vitro*, and significantly reduced apoptosis (Makarevich and Markkula, 2002). Furthermore, combination of EGF and IGF-I showed an additive effect, and higher rates of embryos developed into blastocysts (Sakagami *et al*., 2012). In murine embryos, it has been reported that a high dose of EGF resulted in an improvement on blastocyst rate and cell number (Głabowski *et al*., 2005). Similarly, Mtango *et al*., (2003) demonstrated that the addition of growth factors and growth hormone (GH) to the culture media of bovine embryos had favorable effects on *in vitro* embryo development, freezing sensitivity and post-thawing survival, hatching rate and total cell number of blastocysts. Other growth factors may also have beneficial effects on embryo development. Regarding the use of hormones, mouse blastocysts that have been cultured with growth hormone showed a higher chance for implantation after transfer to the recipient (Fukaya *et al*., 1998). Moreover, ghrelin is a widespread hormone that several studies have linked with reproductive physiology (Garcia-Garcia *et al*., 2007; Tena-Sempere, 2008). In sheep (Wang e*t al*., 2013b), 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.

Since activin-A is produced by oviduct epithelial cells (Gandolfi *et al*., 1995), the addition of activin-A to embryo *in vitro* culture may reproduce the environment of the cleavage-stage embryos in the oviduct *in vivo*. Activin-A and activin receptors have been detected in mouse (Lu *et al*., 1993) and bovine (Yoshioka *et al*., 1998b) embryos from zygote to the morula stage, suggesting that the protein might play a role in embryogenesis. According to studies performed *in vitro,* activin-A has been shown to have an effect in regulation of the development of bovine embryos (Yoshioka and Kamomae, 1996; Yoshioka *et al*., 1998a). However, the effect of activin-A on *in vitro* embryo culture is still controversial. That depending on the timing of its addition to the culture medium, exogenous activin-A may promote the morula and blastocyst development when the protein was added at the earliest stages of bovine (Yoshioka and Kamomae, 1996; Yoshioka *et al*., 1998a; Lee *et al*., 2009) embryo culture. Similar beneficial effects of activin, when added to the second half of the embryo culture, were reported as increased expanding and hatching rates (Park *et al*., 2010; Trigal *et al*., 2011). As described by Park *et al*., (2010), activin-A, during the later stage of *in vitro* bovine embryo development, can enhance *in vitro* development of embryos by affecting expression levels of genes related to hatching and implantation in defined culture medium, and the development-enhancing effect of activin-A may be associated with timing of activation of appropriate genes within the embryonic genome (Trigal *et al*., 2011). Also, maternal levels of activin protein in the mouse embryo decline during early cleavage stages but increase, presumably due to embryo transcription, in the compacted morula (Albano *et al.,* 1993). It has been also shown a dose effect when activin-A was supplemented to *in vitro* culture media of IVM/IVF bovine oocytes. Yoshioka *et al*., (1998a) observed that the percentages of zygotes that developed to the morula and blastocyst stages increased dose-dependently with addition of activin-A, similar to the percentages of expanded blastocysts. Therefore, it may be advisable to test what culture system or culture medium will provide better results for a given lab.

2.4. Assessment of embryo quality

A proper evaluation of blastocyst quality remains an important challenge for every researcher involved in embryology to select the best embryos for transfer, chance to obtain pregnancies and to define its sanitary condition.

The noninvasive methods used for evaluating the embryo quality are the timing of early cleavage (Van Soom *et al*., 1992), understanding that good quality embryos must exhibit appropriate kinetics and synchrony of division, the evaluation of the timing of blastocyst formation (Van Soom *et al*., 1997) and the blastocyst morphology (Van Soom *et al*., 2003). All of those methods are practical to perform and without any consequence for the embryo's viability.

The cryopreservation of embryos is an essential method in assisted reproduction technology (ART) for world-wide embryo transfer (ET) technology that also allows to select the most robust and highest quality embryos will be able to support the freeze-thaw process. Therefore, the ability of embryos to survive cryopreservation is commonly used as an indicator of embryo quality and viability (Rizos *et al*., 2008). Studies performed in several species reported differences on survival rates and hatching percentages after the cryopreservation of blastocysts depending on the origin of embryos (*in vivo* versus *in vitro*), the stage of embryo development, the age of the donor female, and media for IVEP.

Additionally, research on animal models has demonstrated that stress, and the resultant adaptation to conditions during pre-implantation stages, can affect pregnancy loss and fetal growth. It is therefore important to understand the role of each medium component and to identify possible sources of cellular stress to the embryo that will ultimately affect the function and viability of the fetus.

Serum supplementation has been associated with accumulation of cytoplasmic lipid droplets in IVP embryos (Abe *et al.,* 1999) and can lead to poor cryosurvival (Leibo and Loskutoff, 1993). In ovine, Shirazi *et al*., (2012) showed that a significantly lower survival rate of vitrified-warmed blastocysts derived from oocytes matured in presence of FCS, might be due to the excess accumulation of cytoplasmic lipid droplets in resulting blastocysts compared to the other group. In bovine, Moore *et al*., (2007) used a serum replacer (SR) for maturation of oocytes and results showed an improved embryo development *in vitro* as well as better cryotolerance and survival when replacing serum in culture. In the same way, the presence of somatic cells during the IVC was shown to affect the freezability of *in vitro* produced bovine embryos (Gómez *et al.,* 2008). Shehab-El-Deen *et al*., (2009) demonstrated that the addition of elevated concentrations of saturated fatty acids (palmitic and stearic acid) during IVM of bovine oocyte have

deleterious on embryo quality, featured by a significantly reduced post-warming survival of vitrified blastocysts.

In research, the quality of embryos obtained can be also assessed with invasive techniques. Invasive assessment of embryo quality mostly involves a kind of fixation and staining of the embryo. The assessment of embryo quality is carried out under a stereomicroscope on grounds of morphological criteria. Many measures can be investigated to determine embryo quality such as total cell number, the ICM/TE ratio of blastocysts, the degree of cell fragmentation, and determination of genes related with embryo quality and ability to achieve pregnancy after transfer. A simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts (Thouas *et al*., 2001) permits the differentiation of the two cell populations as the blue ICM cell nuclei (stained only with Hoechst) that can be clearly distinguished from the pink TE cell nuclei (stained both with propidium iodide and Hoechst). This technique has been applied to embryos from different species, including bovine (Iwasaki *et al*., 1990; Fouladi-Nashta *et al*., 2005), ovine (Catalá *et al*., 2011), porcine (Dang-Nguyen *et al*., 2011) and buffalo (Selokar *et al*., 2012) among others. Several factors have been reported to affect the proportion of cell number in each blastocyst compartment. If compare *in vivo* versus *in vitro* produced embryos, it has been reported that the *in vitro* production systems affect the blastocyst quality in terms of total cell number, incidence of cell apoptosis, oxidative stress and up and down regulation of gene expression. In bovine, Knijn *et al*., (2003) reported a significantly lower total cell number for those embryos produced completely *in vitro* compared to that of embryos developed entirely *in vivo,* as well as a higher incidence of DNA fragmentation.

Until the blastocyst stage of development is attained, the embryo is somewhat autonomous (*i.e.*, does not need contact with the maternal reproductive tract) as evidenced by the fact that blastocysts can be successfully developed *in vitro* in large number using IVF technology and transferred to reproductive synchronized recipients (Mamo *et al*., 2012). However, the ability to undergo implantation and pregnancies depends on blastocyst quality. Thus, cell number and apoptosis levels are also important parameters that are emerging as useful indicators of embryo development and health (Brison and Schultz, 1997; De la Fuente and King, 1997). This was further supported by Fouladi-Nashta *et al*., (2005) who demonstrated that counting the total number of blastomeres and the percentage of apoptotic nuclei provides more detailed information

on embryo quality and developmental potential. These authors investigated the effect of serum supplementation on total cell number, ICM/TE ratio and apoptosis index after *in vitro* production of bovine embryos. Results of this study showed that supplementation of serum will possibly increase the number of TE cells leading a reduced ratio of ICM/TE. The high number of TE cells in the serum treated embryos may cause the increased placenta size in the large offspring syndrome.

Another parameter used to assess the quality of embryos is the ratio of programmed cell death or DNA fragmentation. Cell death is present in mammalian embryos, which might be involved in the elimination of abnormal cells and its normal pattern is crucial for further development necessary to maintain the morphogenesis and to allow cell renovation. Cell death was confirmed in goat blastocysts using the terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) assay (Jiménez-Macedo *et al.*, 2007). TUNEL allows in situ assessment of DNA breaks in the nuclei (reviewed by Neuber *et al*., 2002) and it has been observed a negative relation with the implantation rates.

Likewise, oxygen free radicals can cause DNA strand breaks and other forms of cellular damage (lipid peroxidation) which are known to result in cell cycle arrest or death. Although apoptosis can be considered to be a normal process in preimplantation embryos to eliminate deviating cells, a high incidence of apoptotic cells is correlated with abnormal morphology of the embryo. Moreover, embryonic cell apoptosis has received increasing attention, mostly due to its suggested role in the cellular response to suboptimal developmental conditions (Betts and King, 2001). Thus, increased apoptosis is an important indicator of inadequate *in vitro* conditions for eggs.

Ending, environmentally induced changes in oocyte and embryo biology have both immediate effects on the viability and development of these structures, but can also have pronounced and often persistent effects on the resultant offspring. For all above mentioned and keeping on mind that the ultimate test of the quality of an embryo is its ability to produce live and healthy offspring after transfer to a recipient, it is really important consider and optimize the conditions of the *in vitro* culture systems in order to get embryos able to undergo pregnancies.

OBJECTIVES

Objectives of the study

The overall aim of this thesis work was to improve the *in vitro* embryo production from oocytes of prepubertal goats. More specifically, the aims were:

I: To examine the effect of supplementing *in vitro* maturation medium with low level of hormones and/or insulin-transferrin-selenium plus ascorbic acid in the developmental competence and embryo quality of small oocytes from prepubertal goats.

II: To evaluate the effect of adding activin-A at the *in vitro* oocytes maturation and *in vitro* embryo culture media on meiotic maturation, embryo development and embryo quality of *in vitro*-produced blastocysts.

Developmental competence and embryo quality of small oocytes from pre-pubertal goats cultured in IVM medium supplemented with low level of hormones, insulin-transferrin-selenium and ascorbic acid

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Developmental Competence and Embryo Quality of Small Oocytes from Pre-pubertal Goats Cultured in IVM Medium Supplemented with Low Level of Hormones, Insulin–Transferrin–Selenium and Ascorbic Acid

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Contents

The aim of this study was to test the effect of insulin–transferrin–selenium (ITS) and L-ascorbic acid (AA) supplementation and the hormonal level during *in vitro* maturation (IVM) of small oocytes from pre-pubertal goat on the blastocyst yield and quality. Concretely, we used four maturation media: conventional IVM medium (CM), growth medium (GM: CM + ITS + AA and low level of hormones), modified CM (mCM: CM with low level of hormones) and modified GM (mGM: CM + ITS + AA and normal level of hormones). Cumulus–oocyte complexes (COCs) were classified into two categories according to oocyte diameter: < 125 μm and ≥125 μm. Large oocytes were matured 24 h in CM (Treatment A). Small oocytes were matured randomly in six experimental groups: Treatment B: 24 h in CM; Treatment C: 12 h in GM and 12 h in CM; Treatment D: 24 h in mGM; Treatment E: 12 h in mGM and 12 h in CM; Treatment F: 12 h in mCM and 12 h in CM; and Treatment G: 12 h in GM and 12 h in mGM. After IVM, oocytes were fertilized and cultured for 8 days. The blastocyst quality was assessed by the survival following vitrification/warming and the mean cell number. When different maturation media were combined, the blastocyst rate did not improve. The large oocytes produced the highest blastocysts yield. However, the culture of small oocytes in GM (53.3%) enhanced the post-warming survival of blastocysts compared to large oocytes matured in CM (35.7%). In conclusion, IVM of pre-pubertal goat small oocytes in GM would be useful to improve the quality of in vitro-produced blastocysts.

Introduction

In vitro embryo production using oocytes from pre-pubertal females has been studied for years and has reported a low embryo development (reviewed by Armstrong 2001). In pre-pubertal goats, oocytes smaller than 125 μm were unable to develop into blastocysts after *in vitro* fertilization (IVF) (Anguita et al. 2007) and ICSI (Jimenez-Macedo et al. 2006) procedures. This poor development of *in vitro*-derived zygotes suggests that culture conditions during *in vitro* maturation (IVM) of small oocytes do not allow for a correct cytoplasmic maturation and normal embryo development after fertilization. Thus, over the last few years, several systems supporting IVM of oocytes have been studied to improve the ability of the culture system to stimulate embryonic development and/or blastocyst quality [bovine (Gomez et al. 2008); ovine (Shirazi et al. 2010)]. In pig, Wu et al. (2006) have defined a growth medium (GM) for culturing oocytes derived from small follicles of pre-pubertal gilt ovaries. This medium has been considered as a 'growth-supporting and less maturation-promoting environment', and it is used before the culture in conventional IVM medium, achieving a blastocyst yield comparable to full-growth oocytes. Comparing this to the conventional IVM medium, the growth medium has a low level of hormones and is supplemented with the combination of insulin–transferrin–selenium (ITS) and L-ascorbic acid. In mice, some studies show that oocytes from pre-antral follicles can complete their growth *in vitro* with low concentrations of hormones (Peluso 1988; Eppig and Schroeder 1989). Moreover, insulin, a polypeptide hormone that promotes the uptake of glucose and amino acids (Lee et al. 2005), transferrin and selenium are essential trace elements and have antioxidant activity (Tatemoto et al. 2004). Thus, ITS supplementation has been routinely used in several IVM systems, including mouse (De la Fuente et al. 1999), bovine (Shamsuddin et al. 1993) and porcine (Wu et al. 2006; Jeong et al. 2008). Finally, ascorbic acid has a vital role as an antioxidant, and Tatemoto et al. (2001) concluded that a critical intracellular concentration of ascorbic acid inside the oocytes would be necessary for their normal cytoplasmic maturation and their embryo developmental competence.

Thus, to improve the *in vitro* embryo production from small pre-pubertal goat oocytes, the objectives of the present study are the following: (i) to examine the effect of GM previous maturation in conventional IVM medium to increase blastocyst rate, (ii) to investigate the effect of GM on blastocyst quality and (iii) to test the effect of adding to the maturation medium a low level of hormones and/or insulin–transferrin–selenium (ITS) and ascorbic acid in a sequential maturation process on embryo development to the blastocyst stage.

Material and Methods

Reagents

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

Oocyte collection, classification and IVM

Pre-pubertal goat (1–2 months old) ovaries were obtained from a slaughterhouse and transported to the laboratory in phosphate-buffered saline (PBS) at 38°C. Cumulus–oocyte complexes (COCs) were recovered by the slicing technique. The COCs with a compact cumulus and homogeneous cytoplasm were selected for IVM and classified into two categories according to their oocyte diameter: < 125 μm and ≥125 μm.

The COCs were washed three times in maturation medium. Groups of 25–30 COCs were transferred into 100-μl drops of IVM medium covered with mineral oil and incubated at 38.5°C and 5% CO_2 in humidified air. The basic medium used for IVM was TCM199 supplemented with 275 μg/ml sodium pyruvate, 146 μg/ml L-glutamine, 50 μg/ml gentamycin, 10% (v/v) donor bovine serum (CanSera; Ontario, Canada) and 100 μM cysteamine. Four types of IVM media were used: (i) basic medium supplemented with 10 μg/ml follicle stimulating hormone (FSH), 10 μg/ml LH, 1 μg/ml and 17 β-oestradiol (conventional IVM medium, CM), (ii) basic medium supplemented with 0.04 μg/ml FSH, 0.04 μg/ml LH, 0.004 μg/ml 17β-oestradiol, 100 μg/ml ascorbic acid and 5 μl/ml ITS (growth medium, GM), (iii) basic medium supplemented with 0.04 μg/ml FSH, 0.04 μg/ml LH and 0.004 μg/ml 17β-oestradiol (modified conventional IVM medium, mCM) and (iv) basic medium supplemented with 10 μg/ml FSH, 10 μg/ml LH, 1 μg/ml 17β-oestradiol, 100 μg/ml ascorbic acid and 5 μl/ml ITS (modified growth medium, mGM).

In vitro fertilization (IVF) and *in vitro* embryo culture (IVC)

After IVM, 25–30 oocytes were transferred to 100-μl microdrops of modified Tyrode's medium (TALP) covered with mineral oil and cocultured with fresh goat sperm in a final concentration of 4×10^6 motile sperm/ml at 38.5°C and 5% CO_2 in humidified air, as described by Romaguera et al. (2010). At 24 h post-insemination (pi), groups of 10–15 presumptive embryos were placed into 20-μl droplets of synthetic oviductal fluid medium (Takahashi and First 1992) [SOF medium supplemented with 10% foetal calf serum (FCS)] under mineral oil in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2 for 8 days. Cleavage rate was evaluated at 48 h pi, and blastocyst rates were recorded at Days 7, 8 and 9 pi.

Evaluation of blastocyst quality

Blastocyst vitrification and warming

Blastocyst vitrification was carried out using the cryotop method described by Kuwayama et al. (2005). All warming steps were performed as described by Morato et al. (2010). Briefly, the holding medium (HM) for formulating all vitrification-warming solutions consisted of TCM 199 Hepes buffered with 20% FCS. The blastocysts were transferred into equilibration solution (ES) consisting of 7.5% ethylene glycol (EG) and 7.5% dimethylsulphoxide (DMSO) in HM for 10–15 min. Then, the blastocysts were transferred to the vitrification solution (VS) containing 15% DMSO, 15% EG and 0.5 M sucrose dissolved in HM. After incubating for 30–40 s, the blastocysts were loaded onto the cryotop and the sample was quickly plunged into liquid nitrogen. The entire process from exposure to VS to plunging was completed within 1 min. For warming, the cryotop was directly immersed into the warming solution containing 1 M sucrose dissolved in HM. After 1 min, the blastocysts were transferred to the dilution solution, which contained 0.5 M sucrose dissolved in HM for 3 min. Next, the blastocysts were incubated in HM for 5 min. Finally, the blastocysts were transferred to the SOF medium and incubated at 38.5°C in a 5% CO_2, 5% O_2 and 90% N_2. After vitrification and warming, survival rates were assessed according to the extent of re-expansion of the blastocysts after 3, 24 and 72 h of recovery.

Hoechst staining

To account the cell number, the blastocysts were stained with 1 mg/ml Hoechst 33342 (H3570; Invitrogen) for 5 min at 38°C. Cell count was conducted under a fluorescence microscope.

Experimental design

Effect of growth medium during IVM on subsequent embryo development of pre-pubertal goat small oocytes

The objective was to test the effect of culturing small oocytes from pre-pubertal goat on GM previous to maturation in CM on blastocyst yield. The COCs were classified into two categories based on oocyte diameter: <125 μm and ≥ 125 μm. The ≥ 125-μm oocytes were matured in CM for 24 h (Treatment A). The <125-μm oocytes were distributed into two experimental groups: COCs matured in CM (Treatment B) and COCs cultured in GM for 12 h followed by another 12 h in CM (Treatment C). After IVM, all COCs were fertilized and cultured *in vitro*. The cleavage and blastocyst rates were assessed at Days 2, 7, 8 and 9 post-insemination.

Effect of growth medium during IVM on the tolerance to blastocyst vitrification and blastocyst cell number

The objective of this study was to examine the effect of GM during IVM on blastocyst quality measured by survival following vitrification/warming and blastocyst cell number. The experimental groups were the same as those in Experiment 1 (Treatments A, B and C). The blastocysts were vitrified at Day 8 post-insemination and cultured for 72 h after warming to examine their viability *in vitro*. Survival rate was defined as post-warming blastocyst re-expansion and its maintenance in SOF medium for 3, 24 and 72 h. The blastocyst cell number was evaluated at the end of the culture period (Day 9 pi).

Effect of different supplements in the maturation medium on pre-pubertal goat embryo development according to oocyte diameter

In this experiment, the effect of ITS, ascorbic acid and hormonal levels was evaluated to optimize embryo development of small oocytes from pre-pubertal goats. The experimental groups are described in Table 1. The cleavage rate was assessed at 48 h pi. At Days 7, 8 and 9, blastocysts were evaluated and classified according to the extent of blastocoele expansion into one of four groups as follows: (i) non-expanded or early, (ii) expanded, (iii) hatching and (iv) hatched blastocysts.

Table 1. Description of different IVM treatments in terms of oocyte diameter, level of hormones and ITS and ascorbic acid supplementation

		Time of IVM (24 h)	
Oocyte diameter	Maturation treatments	First 12 h	Following 12 h
≥125 μm	A (CM)	Normal level of hormones	Normal level of hormones
<125 μm	B (CM)	Normal level of hormones	Normal level of hormones
<125 μm	C (GM/CM)	Low level of hormones ITS and ascorbic acid	Normal level of hormones
<125 μm	D (mGM)	Normal level of hormones ITS and ascorbic acid	Normal level of hormones ITS and ascorbic acid
<125 μm	E (mGM/CM)	Normal level of hormones ITS and ascorbic acid	Normal level of hormones
<125 μm	F (mCM/CM)	Low level of hormones	Normal level of hormones
<125 μm	G (GM/mGM)	Low level of hormones ITS and ascorbic acid	Normal level of hormones ITS and ascorbic acid

CM, Conventional Medium; GM, Growth Medium; mCM, modified Conventional Medium; mGM, modified Growth Medium; ITS, insulin–transferrin–selenium; IVM, *in vitro* maturation.

Table 2. Effect of Growth Medium on cleavage and blastocyst development of pre-pubertal goats according to oocyte diameter

				Blastocysts, n (%)		
Oocyte diameter	Maturation treatments	Oocytes, n	Cleavage, n (%)	Day 7	Day 8	Day 9
≥125 μm	A (CM)	305	171 (56.06)	36 (11.80)	53 (17.37)[a]	63 (20.65)[a]
<125 μm	B (CM)	298	114 (38.25)	20 (06.71)	28 (09.39)[b]	31 (10.40)[b]
<125 μm	C (GM/CM)	309	128 (41.42)	24 (07.76)	28 (09.06)[b]	30 (09.70)[b]
	p value		0.052	0.1826	0.0057	0.0002

Values with different letters within each column differ significantly ($p < 0.05$).
CM, Conventional Medium; GM, Growth Medium.

Statistical analysis

Data were analysed using the Statistical Analysis Systems package (SAS, v9.1: NC, USA). Cleavage rate, blastocysts rate, blastocysts stage and blastocyst cell number were analysed by the Turkey's test. Blastocysts survival rates were analysed by chi-square analysis. For all the statistical analyses, differences were considered significant when $p < 0.05$.

Results

Experiment 1

The results of cleavage rate and embryo development to blastocyst stage of pre-pubertal goat oocytes in relation to their oocyte diameter and maturation medium are presented in Table 2. At 48 h pi, although the oocytes with a diameter ≥125 μm presented a cleavage rate higher than did small oocytes (<125 μm), no statistical differences were observed among the three treatments (56.06%, 38.25% and 41.42%; Treatments A, B and C, respectively). The yield of blastocysts was significantly higher ($p < 0.05$) in the group of large oocytes than in those of small oocytes. There was no difference between blastocyst rate derived from Treatment C and Treatment B of small oocytes.

Experiment 2

The post-warming viability of the vitrified blastocysts and the total cell number of Day 9 blastocysts are shown in Table 3. At 3 h post-warming, a significant increase in the survival rate was observed on the blastocysts derived from small oocytes matured in CM (76.5%), as compared to those obtained from the large oocytes (64.1%). There were no differences in survival rates at 24 h after warming. The post-warming viability at 72 h was significantly higher in the blastocysts derived from small oocytes matured in GM than in the blastocysts produced in CM, regardless of the size of the oocyte (35.7%, 30.8% and 53.3%; Treatments A, B and C, respectively). The cell number of blastocysts obtained from ≥125-μm oocytes was significantly higher than that obtained from <125-μm oocytes.

Experiment 3

Table 4 shows the effect of different maturation culture conditions on embryo development of pre-pubertal goat oocyte. No significant differences were observed on cleavage rate among the seven treatments. When blastocyst yield was determined on Day 7 and Day 8, there were no significant differences among the large oocytes (Treatment A) and the small oocytes from Treatments B (CM) and F (mCM/CM), but on Day 9, the blastocyst rate from ≥125-μm oocytes was significantly higher than that obtained with the six treatments of <125-μm oocytes. With respect to the small oocytes, on Days 8 and 9, the blastocyst rates in Treatment D (mGM) (6.22% and 7.05%, respectively) and E (mGM/CM) (7.23% and 7.66%, respectively) were significantly lower than those of Treatment B (11.20% and 13.13%, respectively) and Treatment F (12.80% and 14.40%, respectively).

When data were analysed by day and blastocyst stage (Table 5), the proportions of early, expanded, hatching and hatched blastocysts on Days 7 and 9 were not

Table 3. Cryotolerance assessed by blastocoele re-expansion at 3, 24 and 72 h post-warming and mean cell number of pre-pubertal goat blastocysts according to IVM treatment and oocyte diameter

		Cryotolerance				Mean cell number	
			% Re-expansion after warming				
Oocyte diameter	Maturation treatments	Blastocysts, n	3 h	24 h	72 h	Blastocysts, n	Blastocyst mean cell number (Mean ± SEM)
≥125 μm	A (CM)	39	64.1[a]	56.4	35.7[a]	21	315.71 ± 24.14[a]
<125 μm	B (CM)	17	76.5[b]	58.8	30.8[a]	20	243.45 ± 24.38[b]
<125 μm	C (GM/CM)	19	68.4[ab]	57.9	53.3[b]	19	207.53 ± 26.91[b]

Values in the same column with different letters differ significantly ($p < 0.05$).
CM, Conventional Medium; GM, Growth Medium.

Table 4. Effect of different supplements in the maturation medium on pre-pubertal goat embryo development according to oocyte diameter

				Blastocyst, n (%)		
Oocyte diameter	Maturation treatments	Oocytes, n	Cleavage, n (%)	Day 7	Day 8	Day 9
≥125 μm	A (CM)	294	159 (54.08)	41 (13.95)[a]	51 (17.35)[a]	62 (21.09)[a]
<125 μm	B (CM)	259	105 (40.54)	24 (09.27)[ab]	29 (11.20)[abc]	34 (13.13)[b]
<125 μm	C (GM/CM)	254	105 (41.34)	21 (8.27)[b]	26 (10.24)[bcd]	29 (11.42)[bc]
<125 μm	D (mGM)	241	88 (36.51)	12 (04.98)[cd]	15 (06.22)[d]	17 (07.05)[c]
<125 μm	E (mGM/CM)	235	102 (43.40)	9 (03.83)[d]	17 (07.23)[cd]	18 (07.66)[c]
<125 μm	F (mCM/CM)	250	108 (43.20)	23 (09.20)[abc]	32 (12.80)[ab]	36 (14.40)[b]
<125 μm	G (GM/mGM)	182	67 (36.81)	10 (05.49)[b]	14 (07.69)[bcd]	18 (09.89)[bc]
	p value		0.0701	0.0011	0.010	0.003

Values in the same column with different letters differ significantly ($p < 0.05$).
CM, Conventional Medium; GM, Growth Medium; mCM, modified Conventional Medium; mGM, modified Growth Medium.

significantly different among treatments. On Day 8, significant differences were found in expanded blastocyst rates between large oocytes (13.73%), which attained the maximum of expanded blastocysts 1 day before, and the small oocytes from Treatments B (CM), C (GM/CM), F (mCM/CM) and G (GM/mGM) (51.72%, 50.00%, 43.75% and 42.86%, respectively). The expanded blastocyst rate derived from Treatment D (mGM) (26.67%) was significantly lower than that of Treatment B and Treatment C.

Discussion

We hypothesized that the presence of low hormone levels, ascorbic acid and ITS (GM) during the first 12 h of IVM of pre-pubertal goat small oocytes could positively influence the *in vitro* embryo development. However, our results showed that the use of GM did not improve the blastocyst percentage, when compared to those matured in conventional IVM medium. In ovine, Catala et al. (2010) also observed that GM failed to significantly increase embryo production of lamb oocytes. These discrepancies with the findings of Wu et al. (2006) on gilt oocytes may be related to variability among biological requirements of immature oocytes depending on the species used.

In relation to blastocyst quality, the ability of blastocysts derived from ≥125-μm oocytes to survive the vitrification/warming process at 3 h is lower than that of blastocysts obtained from small oocytes. At Day 8, the decrease in the cryosurvival rate may be explained by the developmental stage of blastocysts derived from large oocytes (hatching and hatched), as compared to the stage of development of the blastocysts obtained from small oocytes groups (early and expanded). Our findings are in agreement with previous data in bovine (Morato et al. 2010), suggesting that these differences in survival rates of *in vitro*-produced blastocysts depend on their developmental stage. In this study, we could not avoid the heterogeneity of blastocyst stages because of the small number of blastocysts obtained in each one of the replicates. At 72 h post-warming, the re-expansion rate was significantly higher in the blastocysts obtained from small oocytes matured during the first 12 h in GM (Treatment C) than in the blastocysts from oocytes IVM in conventional medium, reflecting a higher oocyte quality. Previous studies in bovine showed that the culture medium supplementation (Rizos et al. 2001; Gomez et al. 2008) affects intrinsic embryo quality in terms of cryosurvival. On the other hand, with respect to the blastocyst total cell number on Day 8, it was found that blastocysts produced from the large oocytes had the highest cell number. As reported by Jiang et al. (1992), the cell number of bovine blastocysts varied depending on the morphological grade and that the later developing blastocysts were of poor quality as judged by the cell number. The total blastocyst cell number did not vary significantly between the small oocytes treatments. Thus, growth medium did not significantly influence blastocyst cell number of small pre-pubertal goat oocytes.

Based on the previous results, we studied the effects of different growth medium components' combinations (low level of hormones, insulin–transferrin–selenium and ascorbic acid) on the cleavage and developmental capacity of pre-pubertal goat oocytes. We found that embryo development of these oocytes is more related

Table 5. Effect of different supplements in the maturation medium on pre-pubertal goat blastocyst stage according to oocyte diameter

Oocyte diameter	Maturation treatments	Day 7				Day 8				Day 9			
		Early n (%)	Expanded n (%)	Hatching n (%)	Hatched n (%)	Early n (%)	Expanded n (%)	Hatching n (%)	Hatched n (%)	Early n (%)	Expanded n (%)	Hatching n (%)	Hatched n (%)
≥125 µm	A (CM)	11/41 (26.83)	18/41 (43.90)	3/41 (07.32)	9/41 (21.95)	7/51 (13.73)	7/51 (13.73)^{c}	8/51 (15.69)	29/51 (56.86)	2/62 (03.23)	5/62 (08.06)	4/62 (06.45)	51/62 (82.26)
< 125 µm	B (CM)	10/24 (41.67)	7/24 (29.17)	5/24 (20.83)	2/24 (08.33)	2/29 (06.90)	15/29 (51.72)^{a}	6/29 (20.69)	6/29 (20.69)	0/34 (00.00)	3/34 (08.82)	3/34 (08.82)	28/34 (82.35)
< 125 µm	C (GM/CM)	10/21 (47.62)	6/21 (28.57)	3/21 (14.29)	2/21 (09.52)	2/26 (07.69)	13/26 (50.00)^{a}	5/26 (19.23)	5/26 (19.23)	0/29 (00.00)	4/29 (13.79)	3/29 (10.34)	22/29 (75.86)
< 125 µm	D (mGM)	3/12 (25.00)	5/12 (41.66)	3/12 (25.00)	1/12 (08.33)	2/15 (13.33)	4/15 (26.67)^{bc}	2/15 (13.33)	7/15 (46.67)	0/17 (00.00)	1/17 (05.88)	1/17 (05.88)	15/17 (88.24)
< 125 µm	E (mGM/CM)	3/10 (30.00)	4/10 (40.00)	1/10 (10.00)	2/10 (20.00)	1/17 (05.88)	7/17 (41.18)^{abc}	3/17 (17.65)	6/17 (35.29)	0/18 (00.00)	2/18 (11.11)	3/18 (16.67)	13/13 (72.22)
< 125 µm	F (mCM/CM)	7/23 (30.43)	9/23 (39.13)	5/23 (21.74)	2/23 (08.70)	3/32 (09.37)	14/32 (43.75)^{ab}	7/32 (21.87)	8/32 (25.00)	1/36 (02.78)	4/36 (11.11)	7/37 (19.44)	24/36 (66.67)
< 125 µm	G (GM/mGM)	4/10 (40.00)	4/40 (40.00)	1/10 (10.00)	1/10 (10.00)	1/14 (07.14)	6/14 (42.86)^{ab}	2/14 (14.29)	5/14 (35.71)	0/18 (00.00)	2/18 (11.11)	4/18 (22.22)	12/18 (66.67)

Values in the same column with different letters differ significantly ($p < 0.05$).
CM, Conventional Medium; GM, Growth Medium; mCM, modified Conventional Medium; mGM, modified Growth Medium.

to oocyte diameter than to the conditions of IVM medium culture, which confirms our previous results (Jimenez-Macedo et al. 2006; Anguita et al. 2007). In addition, in oocytes < 125 µm, the supplementation of conventional medium with ascorbic acid and ITS for 12 and 24 h in the presence of the normal level of hormones (Treatments E and D) significantly decreased the percentage of blastocysts, when compared to the conventional IVM medium (7.66% and 7.05% vs 13.13%, respectively). This result indicates a detrimental effect of ITS and ascorbic acid added to conventional medium. In calf oocytes, Cordova et al. (2010) observed that the presence of ascorbic acid significantly reduced the proportion of embryos developing to the blastocyst stage. In contrast, other authors have shown the beneficial effects of ITS on the nuclear and cytoplasmic maturation of pig oocytes (Jeong et al. 2008) and on the yield of blastocysts in buffalo (Raghu et al. 2002). On the other hand, in the absence of supplementation with ITS and ascorbic acid, no differences were observed in cleavage and blastocyst rates using low or normal levels of hormones during the first 12 h of IVM. The growth of mouse oocytes in a medium without FSH has been demonstrated, and these oocytes acquire competence to undergo maturation, fertilization and embryonic development (Peluso 1988; Eppig and Schroeder 1989). Also, the addition of LH to the medium has been shown to have no effect on the growth of mouse follicles (Murray et al. 2008). These studies suggest that gonadotrophins may not be required for the acquisition of full development competence by growing oocytes.

With respect to the developmental stages (early, expanded, hatching and hatched) of blastocysts on Days 7, 8 and 9, only significant differences were found in the expanded blastocyst rate on Day 8 between the large oocytes and small oocytes from Treatments B (CM), C (GM/CM), F (mCM/CM) and G (GM/mGM). This result can be related to the process of IVM of each oocyte diameter in the patterns of gene expression involved in the expansion and hatching of embryos. In goat, Tan et al. (2009) demonstrated that the oocytes from larger follicles achieve cytoplasmic maturity earlier than those from small follicles to support embryo development. Thus, the ability of oocytes to develop after fertilization is related to the capacity to store the appropriate set of mRNA and proteins that will be required to support early embryo development and to initiate embryonic genome transcriptional activity. Finally, on Day 8, it was observed that expanded blastocysts from small oocytes matured with a high level of hormones and in the presence of ITS and ascorbic acid 12 h or 24 h (Treatments D and E) were not significantly different, when compared to the blastocysts from the large oocytes (Treatment A). This result could be related to the modest number of blastocysts obtained from the small oocytes.

We can conclude that the addition of ascorbic acid, insulin–transferrin–selenium and a low level of hormones during the first 12 h of IVM of pre-pubertal goat oocytes would be useful to comply with the requirements of the intrinsic quality of blastocysts produced *in vitro*, thus enhancing their cryosurvival during the process of cryopreservation. On the other

hand, the capacity of those oocytes to develop to the blastocyst stage *in vitro* seems to be more related to oocyte diameter than to the conditions of the IVM system.

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Conflict of interest

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

Author contributions

The work presented here was carried out in collaboration between all authors. D. Izquierdo, R. Morató, T. Mogas and MT. Paramio defined the research theme, designed methods and experiments. S. Hammami, R. Morató, R. Romaguera, M. Roura and M. Catalá carried out the laboratory experiments. D. Izquierdo, S. Hammami and R. Morató wrote the paper. All authors have contributed to and approved the manuscript.

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In vitro developmental competence of prepubertal goat oocytes cultured with recombinant activin-A

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The present study was designed to evaluate the effect of activin-A during the in vitro *oocyte maturation (IVM) and* in vitro *embryo culture (IVC) on nuclear maturation, blastocyst yield and blastocyst quality of prepubertal goat oocytes. In Experiment 1, three groups of oocytes were used during the IVM of prepubertal goat oocytes to determine the optimal concentration of recombinant human activin-A added to the maturation medium. Cumulus–oocyte complexes were matured in an IVM medium containing 0, 10 and 100 ng/ml (groups A0, A10 and A100), fertilized and* in vitro *cultured using standard procedures. In Experiment 2, the addition of 10 ng/ml activin-A at IVM (A10A0), IVC (A0A10) or IVM + IVC (A10A10) was studied and compared with the control group (A0A0). Results of the first experiment demonstrated that the addition of activin-A yielded similar percentages of maturation (⩽71.0%) and blastocyst formation rates (⩽24.9%) than the control group (A0). Experiment 2 showed that exposure of prepubertal goat oocytes to an IVC medium containing 10 ng/ml activin-A (A0A10) significantly increased the rates of development to the blastocyst stage, as compared with the control group (A0A0) (19.5 ± 2.21% v. 13.1 ± 2.37%, respectively;* $P < 0.05$*). With regard to the blastocyst quality, total number of cells, inner cell mass (ICM) and trophectoderm of prepubertal goat embryos produced in the presence of activin-A did not differ significantly among experimental groups. In summary, these results indicate that supplementation of the IVC medium with activin-A enhances embryo development of prepubertal goat oocytes.*

Keywords: *in vitro* maturation, nuclear stage, *in vitro* culture, blastocysts, goat

Implications

In vitro embryo production in small ruminants provides an excellent source of low-cost embryos for basic research on developmental biology and for commercial application of the emerging biotechnologies such as nuclear transfer, transgenesis, embryo sexing and stem cells. Moreover, this technology would allow a fast multiplication of unique and expensive animals as endangered species. The use of oocytes from prepubertal female goats into breeding programs is advantageous because it can reduce the generation interval and increase the rate of genetic gain. Thus, a challenge is to establish a system that could potentially provide an unlimited source of competent oocytes for biomedical application.

Introduction

The use of prepubertal females as oocyte donors in *in vitro* embryo production allows for an acceleration in genetic progress by shortening the generation interval. However, reduced developmental competence in oocytes of juvenile animals was addressed in numerous studies on ruminant species, including bovine (Khatir *et al.*, 1998), ovine (Ledda *et al.*, 1997) and caprine (Romaguera *et al.*, 2011). The low blastocyst formation rates obtained *in vitro* is mainly because of insufficient cytoplasmic maturation in the oocyte or inability to maintain embryo development beyond the transition of embryonic genome activation (Sirard *et al.*, 2006).

Oocyte growth and maturation are strictly dependent on establishing functional communications with the surrounding cumulus cells through gap junctions and reciprocal interactions mediated by paracrine and endocrine signals. These processes are accurately regulated by numerous growth factors and hormones. Moreover, during mammalian development, some cytokines, including activin-A, play a functional role in the process of cellular proliferation, differentiation and morphogenesis. In early pre-implantation embryos, cytokines produced by both the female genital tract and the embryo itself act on embryonic cells as paracrine/autocrine factors (reviewed by Heyner *et al.*, 1993).

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Activin is an important member of the transforming growth factor β (TGFβ) superfamily, and its roles in gonadal functions have been extensively studied in mammals (Mather *et al*., 1997). Activins are homodimers or heterodimers of the βA or βB subunits of inhibin linked to one another by a single disulfide bond (Hata *et al*., 1988). Dimerization of these β-subunits gives rise to three forms of activin referred to as activin-A (βAβA), activin-AB (βAβB) and activin-B (βBβB) (Ying, 1988). The expression of protein and mRNA for activin-A and activin receptors in the ovary has been localized in both oocyte and granulosa cells of follicles at various developmental stages (primates: Sidis *et al*., 1998; swine: Van den Hurk and Van de Pavert, 2001; bovine: Hulshof *et al*., 1997, Izadyar *et al*., 1998; ovine: Thomas *et al*., 2003; and caprine: Silva *et al*., 2004). In addition to being a local regulator of folliculogenesis, activin-A is able to directly stimulate FSH synthesis and secretion, and to promote the release of the gonadotrophin-releasing hormone (GnRH) (Childs and Unabia, 1997). Activin-A can also stimulate the increase of FSH and LH receptors in granulosa cells, and it plays a role in progesterone production (Alak *et al*., 1998; Tsuchiya *et al*., 1999). Thus, the granulosa cells are likely to be the main source of paracrine factors and are crucial for oocyte maturation.

Activin-A and activin receptors have been detected in mouse (Lu *et al*., 1993) and bovine (Yoshioka *et al*., 1998b) embryos from zygote to the morula stage, suggesting that the protein might play a role in embryogenesis. As activin-A is produced by oviduct epithelial cells (Gandolfi *et al*., 1995), the addition of activin-A to embryo culture *in vitro* may reproduce the environment of the cleavage-stage embryos in the oviduct *in vivo*. However, the effect of activin-A on *in vitro* embryo culture (IVC) is still controversial. Positive effects of activin-A on embryo development were shown when the protein was added at the earliest stages of bovine (Yoshioka and Kamomae, 1996; Yoshioka *et al*., 1998a, Lee *et al*., 2009) embryo culture, whereas no effects on blastocyst formation rates, depending on the timing of its addition to the culture medium, were reported in the literature either (Park *et al*., 2010; Trigal *et al*., 2011).

Summarizing, although prepubertal oocytes give rise to similar rates of fertilization and cleavage to those achieved using adult oocytes, the capacity of prepubertal oocytes to develop in the blastocyst stage is relatively lower and it would seem that embryos from prepubertal oocytes are less capable of establishing pregnancies (reviewed in Armstrong, 2001). Therefore, to improve the developmental competence of prepubertal oocytes after their recovery from the follicle, two approaches have been described. The first has been the addition of putative growth promoting substances to the culture medium (gonadotrophins, steroids, growth factors). The second approach has attempted to mimic the intrafollicular conditions of the oocytes. However, none of these approaches have managed to improve developmental competence from prepubertal goat oocytes. Keeping in mind that protein and mRNA for activin-A and activin receptors have been localized in both oocyte and granulosa cells of follicles at various developmental stages in a variety of species including caprine (Silva *et al*., 2004 and 2006), and the potential role of activin-A in the regulation of oocyte maturation has been demonstrated to promote *in vitro* oocyte maturation (IVM) in the rat (Itoh *et al*., 1990), cow (Stock *et al*., 1997), rhesus monkey (Alak *et al*., 1996) and human (Alak *et al*., 1998), we hypothesized that the addition of activin-A to the maturation and/or embryo culture medium could improve the *in vitro* potential of prepubertal goat oocytes for nuclear and cytoplasmic maturation and embryo development. Such a system could potentially provide an unlimited source of competent oocytes for biomedical application.

To the best of our knowledge, there is no study that reports the effects of activin-A on *in vitro* developmental potential of prepubertal goat oocytes. Therefore, the present study was conducted to evaluate the effect of adding activin-A at the IVM and IVC media on meiotic maturation, embryo development and embryo quality of *in vitro*-produced blastocysts.

Material and methods

Reagents

Unless specified, all chemicals, reagents and hormones were purchased from Sigma (St Louis, MO, USA). Activin-A was purchased from R&D Systems (Minneapolis, MN, USA), and it was reconstituted at 50 μg/ml in sterile phosphate-buffered saline (PBS) containing at least 0.1% bovine serum albumin (BSA) and stored in aliquots at −20°C until use.

Oocyte collection and in vitro *maturation (IVM)*

Prepubertal goat (1 to 2 months old) ovaries were obtained from a slaughterhouse and transported to the laboratory in PBS at 38°C within 1 h of collection. Cumulus–oocyte complexes (COCs) were recovered by the slicing technique. Only COCs with a compact cumulus and homogeneous cytoplasm were selected for IVM. The COCs were washed three times in the IVM medium (TCM199 supplemented with 275 μg/ml sodium pyruvate, 146 μg/ml L-glutamine, 50 μg/ml gentamycin, 10% (v/v) donor bovine serum (CanSera, Ontario, Canada), 10 μg/ml FSH, 10 μg/ml LH, 1 μg/ml 17 β-estradiol and 100 μM cysteamine. Groups of 25 to 30 COCs were transferred into drops of 100 μl of the IVM medium, covered with mineral oil, and incubated for 24 h at 38.5°C and 5% CO_2 in humidified air.

In vitro *fertilization (IVF)*

Fresh semen was collected with an artificial vagina from four Murciano-Granadina bucks of proven fertility and transported to the laboratory at 35°C within 30 min. The motile sperm fraction was selected by the swim-up method (Parrish *et al*., 1986). Briefly, 90 μl of ejaculate mixture was deposited at the bottom of a conical tube containing 2 ml mDM (defined medium modified by Younis *et al*., 1991). After 1 h of incubation at 38.5°C, the upper layer of the tubes was recovered in a tube of 15 ml and centrifuged at 160 × **g**

for 3 min. The sperm pellet was resuspended in a 1 : 1 proportion (v/v) with mDM containing heparin sodium salt (final concentration of 50 μg/ml), and it was incubated for 45 min at 38.5°C and 5% CO_2 in humidified air. After maturation, oocytes were washed three times in the fertilization medium (Tyrode's medium (TALP) supplemented with 1 μg/ml hypotaurine and 0.3 mg/ml glutathione) and groups of 25 to 30 oocytes were transferred to microdrops of 100 μl IVF medium, under mineral oil and co-cultured with sperm in a final concentration of 4×10^6 motile sperm/ml at 38.5°C and 5% CO_2 in humidified air.

In vitro *culture (IVC)*
At 24-h post-insemination (hpi), presumptive zygotes were denuded of surrounding cumulus cells and attached spermatozoa, washed three times in synthetic oviductal fluid medium (SOF (Takahashi and First, 1992)), and groups of 20 presumptive embryos were placed into droplets of 20 μl of SOF medium supplemented with 10% fetal calf serum (FCS) under mineral oil and cultured in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2 for 8 days. The cleavage rate was evaluated at 48 hpi and blastocyst formation rates were recorded at Days 7, 8 and 9 post-insemination (pi).

Evaluation of nuclear maturation stage
After the *in vitro* maturation, oocytes were freed from their surrounding cumulus cells by vortexing and then washed three times in PBS. To evaluate meiotic progression, the oocytes were fixed in ethanol : acetic acid (3 : 1) overnight. The oocytes were mounted on microscope slides with vaseline, covered with a glass coverslip and stained with 1% lacmoid in 45% acetic acid solution. The stage of nuclear maturation was assessed by phase-contrast microscopy (magnification, ×40). The criteria used to determine oocyte maturation stage have been described elsewhere (Rodriguez-Gonzalez *et al*., 2002). Oocytes were scored as: germinal vesicle (GV) stage, when their chromatin, either filamentous or slightly condensed, was enclosed in a nuclear membrane; germinal vesicle breakdown (GVBD) stage, when there was no visible nuclear membrane and chromatin was condensed; metaphase I (MI) stage, when the chromatin was distributed at the equator of a large spindle; anaphase I stage, when homologous chromosomes could be seen to move away from each other and there was a visible spindle; telophase I stage, when homologous chromosomes were apart, and metaphase II (MII) stage, when the chromatin was located at the spindle and the first polar body extrusion was detected.

Evaluation of blastocyst quality
Hoechst staining. At the end of IVC, blastocysts were stained with 1 mg/ml Hoechst 33342 (H3570; Invitrogen, Life Technologies, Burlington, Ontario, Canada) for 5 min at 38°C. Cell count was carried out under a fluorescence microscope (Olympus BX53; Olympus, Germany).

Blastocyst differential staining. The cellular composition of Day 8 pi blastocysts was assessed by differential staining of inner cell mass (ICM) and trophectoderm (TE) cells, as described by Thouas *et al.* (2001), with some modifications. Briefly, embryos that reached the blastocyst stage were first incubated in PBS with 1% (v/v) Triton X-100 and 100 μg/ml propidium iodide for up to 15 s. Blastocysts were then immediately transferred into a fixative solution of 100% ethanol supplemented with 25 μg/ml Hoechst 33342 for 2 h. Fixed and stained whole blastocysts were mounted on a slide in a drop of 3 μl of glycerol and flattened with a coverslip. Cells were counted under UV illumination (Olympus BX53) and a 460 nm excitation filter. The images showed ICM and TE nuclei as blue and red/pink, respectively.

Experimental design
Two experiments were conducted in this study. Each experiment was replicated at least five times.

Experiment 1. Effect of activin-A during IVM on the nuclear stage, embryo development and blastocyst quality of prepubertal goat oocytes. The objective was to assess the effects of recombinant human activin-A at concentrations of 10 ng/ml and 100 ng/ml added at the IVM of prepubertal goat oocytes on nuclear maturation, cleavage and blastocyst formation rates, and embryo quality as compared with the control group, without activin-A. COCs were randomly assigned into one of the three IVM treatment groups: (1) A0: IVM medium without activin-A (control group); (2) A10: supplemented with 10 ng/ml activin-A and; (3) A100: supplemented with 100 ng/ml activin-A. After IVM, COCs were fertilized and the cleavage rate and blastocyst yield were assessed at Day 2 pi and Days 7, 8 and 9 pi, respectively. At the end of the culture period (Day 9 pi), and to determine the effects on embryo quality, *in vitro*-produced blastocysts were fixed and stained to determine the total cell number. In each repetition, a sample of COCs was obtained after IVM, denuded, washed in PBS, fixed overnight, lacmoid-stained and observed under a phase-contrast microscope to determine their nuclear status.

Experiment 2. In vitro *embryo development in the presence of 10 ng/ml activin-A during IVM and IVC of prepubertal goat oocytes*. The aim was to evaluate the effect of recombinant human activin-A added to the IVM and IVC media on the cleavage rate, embryo development and blastocyst quality of oocytes from prepubertal goat. COCs were randomly allocated into four groups: (1) A0A0: COCs matured in the IVM medium and cultured in the SOF medium; (2) A0A10: COCs matured in the IVM medium and cultured in the SOF medium supplemented with 10 ng/ml activin-A; (3) A10A0: COCs matured in the IVM medium plus 10 ng/ml activin-A and cultured in the SOF medium; and (4) A10A10: COCs matured in the IVM medium plus 10 ng/ml activin-A and cultured in the SOF medium plus 10 ng/ml activin-A. The cleavage rate was recorded at 48 hpi and blastocysts yield at Days 7, 8 and 9 pi, respectively. To assess the quality of blastocysts obtained from the treatment groups, cell number and the cellular composition of Day 8 pi blastocysts were evaluated by differential staining of ICM and TE.

Statistical analysis

Data were analyzed using the Statistical Analysis Systems package (SAS, v9.1; Cary, NC, USA). At least five replicates were performed in all experimental groups. χ^2 analysis was used to determine which concentration groups differed in the nuclear stage after *in vitro* maturation. Cleavage rates, blastocyst yield and blastocyst cell number were analyzed using ANOVA. All data were checked for normality and homogeneity of variances using the Levene and Kolmogorov–Smirnov tests. The most suitable activin-A concentration for conducting the experiments depicted in Table 1 was determined after evaluating the effects of supplementing IVM with three different concentrations of activin-A (0, 10 and 100) on cleavage and blastocyst development rates by running a one-way ANOVA (facto r = activin-A concentration in IVM) and a post-hoc Sidak test for pairwise comparisons). The effects of supplementing IVM and IVC media with 10 ng/ml activin-A on cleavage rate, number of cells per blastocyst, and accumulated number and percentages of blastocysts at Day 9 were evaluated through a two-way ANOVA where the factors were the composition of IVM (A0 *v.* A10) and IVC (A0 *v.* A10). On the other hand, the effects of 10 ng/ml activin-A on the blastocyst development parameters, evaluated as numbers and percentages of blastocysts at Days 7, 8 and 9, were tested by a three-way ANOVA for repeated measures, where the two inter-subjects factors were IVM and IVC, and the intra-subject factor was the culturing time (Days 7, 8 and 9). A post-hoc Sidak's test was also used for multiple comparisons. In all the cases, the interaction between factors within the model was also evaluated, and the significance level was set a $P < 0.05$.

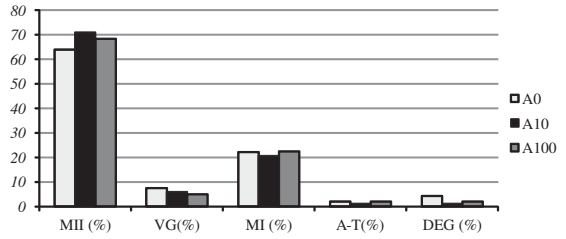

Figure 1 Meiotic status of prepubertal goat oocytes matured in TCM 199 supplemented with various concentrations of activin-A during IVM. Treatments A0, A10 and A100: oocytes matured in TCM199 supplemented with 0, 10 and 100 ng/ml of activin-A for 24 h. GV = germinal vesicle; MI = metaphase I; A–T = anaphase–telophase; MII = metphase II; DEG = degenerated oocytes.

Results

Experiment 1. Effect of activin-A during IVM on the nuclear stage, embryo development and blastocyst quality of prepubertal goat oocytes

At the end of the IVM period, nuclear maturation stages were established and results are shown in Figure 1. Oocytes cultured in the presence of activin-A showed similar rates of oocytes at GV, MI, ATI and MII stage to those of the control group (A0). Indeed, the percentages of MII oocytes recorded after maturation for the 10 ng/ml and 100 ng/ml concentrations were not significantly different from those observed for the control oocytes ($P > 0.05$). No differences according to activin-A concentration were observed ($P > 0.05$).

Table 1 shows the embryo development rates of oocytes cultured in the maturation medium alone or in the medium supplemented with activin-A (10 and 100 ng/ml). No differences in cleavage rates were detected among the different experimental groups ($P > 0.05$). Similarly, no significant differences ($P > 0.05$) were observed in the percentage of embryos developing to the blastocyst stage on Days 7, 8 and 9 pi. No interaction ($P > 0.05$) between activin-A concentration and culture period were either observed.

An amount of 54 blastocysts were stained to assess the embryo quality. The total cell number of Day 8 blastocysts obtained from oocytes cultured in the maturation medium alone or in the medium supplemented with activin-A is

Table 1 *Effects of supplementing IVM medium with three different concentrations of activin-A (0, 10 and 100 ng/ml) on cleavage and blastocyst development rates from prepubertal goat oocytes*

	Activin-A treatments[a]				Contrasts (*P*-values)		
Items	A0	A10	A100	RMSE	A0 *v.* A10	A0 *v.* A100	A10 *v.* A100
No. of oocytes	398	406	406				
% of cleaved oocytes	41.67	46.47	45.90	7.88	0.50	0.58	0.99
% Blastocysts (Day 7)*	11.70	16.95	12.50	4.50	0.10	0.94	0.18
% Blastocysts (Day 8)*	16.69	21.97	18.52	5.65	0.21	0.82	0.50
% Blastocysts (Day 9)*	21.74	24.93	21.89	5.65	0.55	0.99	0.58
% Blastocysts (Day 9)**	9.03	11.43	9.85	2.62	0.23	0.83	0.51

RMSE = root mean squared error.
Data are given as means. Treatments A0, A10 and A100 were: oocytes matured in TCM199 supplemented with 0, 10 and 100 ng/ml of activin-A, respectively.
[a]Overall treatment effect not significant for any of the measured parameters ($P > 0.05$). Data analyzed by ANOVA with activin-A concentration in *in vitro* maturation medium as a treatment factor.
*Rate of blastocyst is calculated from cleaved embryos.
**Rate of blastocyst is calculated from *in vitro* matured oocytes.

Table 2 *Effect of supplementing IVM medium with three different concentrations of activin-A (0, 10 and 100 ng/ml) on mean cell number from prepubertal goat blastocysts*

	Activin-A treatments[a]				Contrasts (*P*-values)		
Items	A0	A10	A100	RMSE	A0 *v.* A10	A0 *v.* A100	A10 *v.* A100
No. of blastocysts	18	18	18				
Blastocyst mean cell number	176.72	192.83	187.94	75.77	0.80	0.90	0.98

RMSE = root mean squared error.
Data are given as means. Treatments A0, A10 and A100 were: oocytes matured in TCM199 supplemented with 0, 10 and 100 ng/ml of activin-A, respectively.
[a]Overall treatment effect not significant for any of the measured parameters ($P > 0.05$). Data analyzed by ANOVA with activin-A concentration in *in vitro* maturation medium as a treatment factor.

Table 3 *Effects of supplementing IVM and IVC media with 10 ng/ml activin-A on cleavage and blastocyst development rates from prepubertal goat oocytes*

	Activin-A supplementation					
Items	A0A0	A10A0	A0A10	A10A10[c]	RMSE	*P*-value
No. of oocytes	296	267	283	287		
% of cleaved oocytes	50.87	50.79	54.96	51.68	10.76	0.89
% Blastocysts (Day 7)*	7.32	8.14	11.27	8.91	3.39	0.24
% Blastocysts (Day 8)*	10.51	13.54	14.64	11.25	3.95	0.26
% Blastocysts (Day 9)*	13.13[a]	15.77[ab]	19.51[b]	13.69[ab]	4.81	0.12
% Blastocysts (Day 9)**	6.23[a]	7.74[a]	10.52[b]	6.91[a]	2.09	0.01

IVM = *in vitro* maturation; IVC = *in vitro* embryo culture; RMSE = root mean squared error.
Data are given as means. Treatment A0A0 and A0A10 were: oocytes matured in TCM199 and cultured in SOF medium supplemented with 0 and 10 ng/ml of activin-A, respectively. Treatments A10A0 and A10A10 were: oocytes matured in TCM199 supplemented with 10 ng/ml and cultured in SOF medium with 0 and 10 ng/ml of activin-A, respectively.
[a,b]Values in the same row with different superscript letters differ significantly ($P < 0.05$).
[c]Interaction effects were observed only between addition of activin-A at IVM medium and IVC medium ($P = 0.001$). Data analyzed by ANOVA where the factors were the composition of IVM (A0 *v.* A10) and IVC (A0 *v.* A10).
*Rate of blastocyst is calculated from cleaved embryos.
**Rate of blastocyst is calculated from *in vitro* matured oocytes.

summarized in Table 2. There were no differences among groups, irrespective of the maturation medium ($P > 0.05$). All blastocysts showed a similar number of cells.

Experiment 2. In vitro *embryo development in the presence of 10 ng/ml activin-A during IVM and IVC of prepubertal goat oocytes*

There were no significant differences in the cleavage rate among treatments. No significant differences were either observed when activin-A was added at the *in vitro* maturation medium (A10A0; A10A10) ($P > 0.05$). However, embryo development, in terms of total number of blastocysts obtained at Day 9, improved significantly ($P < 0.05$) with the addition of activin-A at the *in vitro* culture medium (A0A10). The highest blastocyst formation rate (19.5%) was obtained when activin-A was added at the *in vitro* culture medium (A0A10) but not at the *in vitro* maturation medium. Moreover, interaction effects between addition of activin-A at IVM medium and IVC medium were observed ($P = 0.001$) (Table 3).

Blastocyst quality was evaluated in terms of total number of cells per blastocyst and the proportion of ICM cells and TE cells assessed by the differential staining (Table 4). No differences in any of these variables were detected among groups ($P > 0.05$).

Discussion

The present study was designed to evaluate the effect of activin-A during the IVM and IVC on maturation rates, embryo development and embryo quality of prepubertal goat oocytes. In general, prepubertal oocytes show reduced developmental competence, when compared with results obtained with adult oocytes (Armstrong, 2001). Indeed, the defective capacity of these oocytes to develop up to blastocyst at a rate equivalent to that of adult oocytes has been reported by several other studies in cattle (Presicce *et al.*, 1997), in sheep (Ledda *et al.*, 1997), in pig (Marchal *et al.*, 2001) and more recently in goats stimulated hormonally (Leoni *et al.*, 2009).

In the present work, the presence of activin-A during IVM of cumulus-enclosed prepubertal goat oocytes did not affect nuclear maturation rates. Thus, the results obtained in Experiment 1 indicate that activin-A added to maturation media at concentrations of 10 and 100 ng/ml resulted in

Table 4 *Number of TE and ICM cells in blastocysts developed from goat oocytes after IVM and IVC with or without 10 ng/ml activin-A*

	Activin-A supplementation[a]					
Items	A0A0	A10A0	A0A10	A10A10	RMSE	*P*-value
Total blastocysts	9	8	14	10		
Total number of cells	72.44	76.75	79.00	73.80	14.12	0.69
TE cells	50.78	54.62	56.50	52.00	10.30	0.56
ICM cells	21.67	22.12	22.50	21.80	4.72	0.97
% ICM : TE	0.43	0.41	0.40	0.42	0.06	0.70

TE = trophectoderm; ICM = inner cell mass; IVM = *in vitro* maturation; IVC = *in vitro* embryo culture; RMSE = root mean squared error.
Data are given as means. Treatment A0A0 and A0A10 were: oocytes matured in TCM199 and cultured in presence of 0 and 10 ng/ml of activin-A, respectively. Treatments A10A0 and A10A10 were: oocytes matured in TCM199 supplemented with 10 ng/ml and cultured SOF medium supplemented with 0 and 10 ng/ml of activin-A, respectively.
[a]Overall treatment effect not significant for any of the measured parameters ($P > 0.05$). Data analyzed by ANOVA where the factors were the composition of IVM (A0 *v.* A10) and IVC (A0 *v.* A10).

similar rates of oocytes in the MII stage, as compared with those oocytes that matured without activin-A. Similar results have already been reported in bovine (Vantol *et al.*, 1994; Izadyar *et al.*, 1996) and porcine (Coskun and Lin, 1994) oocytes, suggesting no effect of activin-A on oocyte maturation. Considering that activin-A is able to directly stimulate FSH synthesis and secretion, promote the release of the GnRH, and also stimulate the increase of FSH and LH receptors in the granulosa cells (Alak *et al.*, 1998; Norwitz *et al.*, 2002), the ineffectiveness of exogenous activin-A may be either because of activin-A production by the COCs and/or the presence of FSH and LH in our culture medium. However, this does not exclude the possibility that activin-A, secreted by the granulosa cells and present in the follicular fluid, has an effect on the cytoplasmic maturation of goat oocytes, which would affect subsequent pre-implantation embryo development. Nevertheless, our results demonstrate that activin-A added to IVM has no effect on the percentage of blastocysts obtained after IVF. This observation is consistent with other reports in bovine (Vantol *et al.*, 1994; Izadyar *et al.*, 1996) and pig (Coskun and Lin, 1994), which showed that adding activin-A during IVM did not result in an improvement of blastocyst development.

Regarding the effect of activin-A during the whole embryo culture period, our data clearly show that activin-A promotes embryonic development, showing an improving effect on the blastocyst yield of prepubertal goat oocytes. Given that activin-A is produced by the oviduct epithelial cells (Gandolfi *et al.*, 1995), addition of activin-A to embryo culture *in vitro* could help reproduce the environment of the cleavage-stage embryos in the oviduct *in vivo*. Controversial results have been reported about the effects of activin-A on embryo development depending on the timing of its addition to the culture medium. Thus, Yoshioka *et al.* (1998a) observed that activin-A had an effect on blastocyst yield when it was added to IVC medium of bovine embryos before the 9- to 16-cell stage. However, a recent study on bovine (Trigal *et al.*, 2011) has demonstrated a better effect of activin-A when it is added later in culture media. The development-enhancing effect of activin-A may be associated with the timing of the activation of appropriate genes within the embryonic genome. Studies in mouse (Lu *et al.*, 1993) and bovine (Yoshioka *et al.*, 1998b) showed that activin-A and activin receptors are detected in embryos from the zygote to the morula stage, suggesting that the protein might play a role in embryogenesis during the early stage of embryo development. Our data show an evident improving effect on Day 9 blastocyst yield, suggesting an effect during the late stages of embryo development in prepubertal goats. Otherwise, we should also consider the possibility of an accumulative effect of activin-A added during *in vitro* maturation and culture medium. Consequently, greater effects are observed as the *in vitro* culture is longer. Thus, further studies will be required to determine the functions of activin-A during these stages of development.

Finally, the quality of blastocysts, as determined by the evaluation of their cell number, seemed to be similar among all of the groups. The number of cells of the embryo may be informative of embryonic viability (VanSoom *et al.*, 1997). The lower quality of embryos produced *in vitro* could be attributed to a lower number of cells. Cell counts were unaffected by the presence of activin-A, similarly to other authors showing no differences in total cell counts between bovine blastocysts produced in the presence or absence of activin-A (Yoshioka *et al.*, 1998a, Trigal *et al.*, 2010). In addition, in our study, blastocyst cell counts were similar to those reported in adult and prepubertal goats (Koeman *et al.*, 2003; Romaguera *et al.*, 2010).

Several studies have demonstrated that the expression of protein and mRNA for activin-A and activin receptors in the ovary is present in both oocytes and granulosa cells of follicles at various stages of development (pigs: (Van den Hurk and Van de Pavert, 2001); and ruminants: (Hulshof *et al.*, 1997; Izadyar *et al.*, 1998)), suggesting a physiological role of activin in the growth and development of follicles and/or oocytes. Although there is convincing evidence that activin signaling is important for ovarian function, information on its localization and function is mainly obtained from rodents and cows. Few studies on goats (Silva *et al.*, 2004 and 2006) have demonstrated that activin-A, its binding protein

follistatin and its receptors are formed in all types of goat follicles, in all compartments of antral follicles and in ovarian surface epithelium. The widespread distribution pattern of the follistatin–activin–activin receptor system in goat ovaries points to a crucial role of this system in various reproductive processes, including follicle growth and differentiation, and luteal activity. However, there are limited data on goats and on prepubertal animals (Brawtal, 1994; Zhao *et al*., 2001; Patel *et al*., 2007) about the expression of protein and mRNA for activin-A and activin receptors. Therefore, it is necessary to determine whether activin-A and its receptors are present in the embryos from the zygote to the morula stage to improve the results on *in vitro* embryo development of prepubertal goat oocytes.

In conclusion, the addition of 10 ng/ml of activin-A throughout the whole embryo culture improves blastocyst yield. This is the first study that provides data of the effects of activin-A on *in vitro* developmental potential of prepubertal goat oocytes. Nevertheless, to the best of our knowledge, there is no study reporting the gene expression and protein localization for activin-A and activin receptors in prepubertal goats. A detailed profiling of the activin-A effects on embryonic development of prepubertal goat oocytes and determination of gene expression and protein synthesis for activin-A require further experiments.

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Chapter 6

GENERAL DISCUSSION

General Discussion

This thesis is focused on improvement of the *in vitro* embryo production from oocytes of prepubertal goats. For several years, *in vitro* embryo production from prepubertal goats has been considered a promising option for providing an excellent source of embryos for basic research on development biology and for commercial applications of transgenic and cloning technologies. However, despite successful results and reports of numerous attempts, the effectiveness of *in vitro* embryo production protocols for prepubertal goat oocytes has never reached a satisfactory level. Oocyte quality is the main factor for embryos reaching blastocyst stage from IVM/IVF/IVC oocytes. Until now, attention has been focused on selection of criteria to assess the most competent oocytes to develop up to blastocyst stage. In our laboratory, several studies have been reported describing the best criteria to select the larger and more competent oocytes for *in vitro* blastocyst production: Brilliant Cresyl Blue (BCB) stain (Rodriguez-Gonzalez *et al.,* 2002; Catalá *et al*., 2011), oocyte (Jiménez-Macedo *et al*., 2006; Anguita *et al*., 2007) and follicle (Romaguera *et al*., 2010a; 2011) diameters.

People working in this field has to combine the most adequate oocyte selection criteria and the *in vitro* culture protocol to became high rates of blastocyst development. For this reason, the two studies included in the present thesis work were designed to optimize *in vitro* culture system of prepubertal goat oocytes collected from ovaries of slaughtered goat females, using supplements added during IVM and/or IVC, in order to increase both rates of development and quality of *in vitro*-produced blastocysts at a rate equivalent to that of adult oocytes.

The first study was conducted to examine the effect of supplementing *in vitro* maturation medium with low level of hormones and/or insulin-transferrin-selenium plus ascorbic acid in the developmental competence and embryo quality of small oocytes from prepubertal goats.

Previous studies in our laboratory using prepubertal goat oocytes showed that not only the age of the oocyte donor is crucial for embryo production, but also the size of oocyte plays an important role in embryo production. Firstly, we hypothesized that

supplementing the IVM medium of small oocytes with low hormone levels, ITS and L-ascorbic acid called growth medium, could significantly improve maturation along with developmental competency compared to the conventional goat oocyte maturation system. However, our results have showed that the culture of small prepubertal goat oocytes in the growth medium during IVM did not improve the blastocyst percentage when compared to those matured in conventional IVM medium (Hammami *et al*., 2013). Similarly, in ovine, Catalá *et al*., (2013) also observed that growth medium failed to increase embryo production of lamb oocytes. These discrepancies with the findings of Wu *et al*., (2006) on gilt oocytes and Córdova *et al*., (2010) on calf oocytes may be related to variability among biological requirements of immature oocytes depending on species used.

In these initial studies it was possible to duplicate the performance of blastocyst production with the use of oocyte diameter as selection criteria. The rate of blastocysts production from prepubertal goat oocytes ≥ 125 μm was 20.65% compared to 10.40 % from oocytes < 125 μm diameter. Thus, the capacity of those oocytes, once fertilized, to develop to the blastocyst stage *in vitro* seems to be more related to oocyte diameter than to the conditions of *in vitro* maturation system. Several studies concluded that oocyte diameter is correlated to follicle diameter and as both increase the developmental capability of the oocytes improves (revised by Gandolfi *et al*., 2005). It is noteworthy that COCs coming from a wide range of follicle diameters and selected based on their morphology alone produced higher cleavage and blastocyst rates than oocytes from small follicles. One possibility could be, as Romaguera *et al*., (2010a) discussed, that oocytes from follicles of ≥ 3 mm could have enough time to be submitted to prematuration processes and to acquire a complete developmental competence to blastocyst stage. Anguita *et al*., (2007) confirmed this hypothesis previously in our laboratory showing that prepubertal goat oocyte diameter and, therefore, oocyte developmental competence seems to be related to the amount of cyclin B, cdc2 kinase protein and MPF activity. Maturation promoting factor (MPF) is a heterodimer composed of a p34cdc2 catalytic subunit, with serine-threonine kinase activity, and a cyclin B1 regulatory subunit. MPF activity appears just before germinal vesicle break down (GVBD) increasing until metaphase I; its activity is decreased in anaphase-telophase while its maximum level is reached at metaphase II (MII). In prepubertal goats, Anguita *et al*., (2007) showed a higher MPF activity in oocytes with a diameter

larger than 135 μm and, after IVM, p34cdc2 protein expression increased with the oocyte diameter. Possibly, the p34cdc2 RNA accumulation in the oocyte takes place during the last growth stage and the translation begins when the oocyte reaches its maximum size. MPF activity seems to confer higher developmental competence to oocytes through promotion of cytoplasmic maturation. Hence, enough time to be submitted to prematuration processes and to acquire a complete oocyte competence is important for successful embryo development.

When analysing the effects of different growth medium components combinations (low level of hormones, ITS and ascorbic acid with the conventional medium during IVM, on the cleavage and developmental capacity of prepubertal goat oocytes, our findings indicated that the supplementation of conventional medium with ascorbic acid and ITS during 12h and 24h in presence of normal level of hormones provided a significant decrease in the percentage of blastocysts obtained from <125 μm oocytes diameter, compared to the conventional IVM medium (7.65% and 7.05% vs 13.12%, respectively). According to our data, in calf oocytes, Córdova *et al*., (2010) observed that the presence of ascorbic acid significantly reduced the proportion of embryos developing to the blastocyst stage. These data are in disagreement with observations in pig and buffalo oocytes in that the presence of ITS during *in vitro* maturation had a beneficial effect on the nuclear and cytoplasmic maturation (Jeong *et al*., 2008) and on the yield of blastocysts (Raghu *et al*., 2002a). On the other hand, in absence of supplementation with ITS and ascorbic acid, no differences were observed on cleavage and blastocyst rates using low or normal level of hormones during the first 12h of IVM. The growth of mouse oocytes in a medium without FSH has been demonstrated, and these oocytes acquire competence to undergo maturation, fertilization and embryonic development (Peluso, 1988; Eppig and Schroeder, 1989; Wu *et al*., 2000; Murray *et al*., 2008). Also, when mouse follicles have been cultured in presence of LH, their growth was not affected (Murray *et al*., 2008). These results suggest that gonadotrophins may not be required for the acquisition of full development competence by growing oocytes.

Aside from adding antioxidants, vitamins and/or different hormone levels during IVM, in the second study we investigated whether activin-A supplementation to the IVM medium of prepubertal goat oocytes, could affect their nuclear maturation and competence to develop up to blastocyst stage. Results of this study (Hammami *et al*.,

2014) showed no effect of activin-A on oocyte maturation and subsequent embryo development, similarly to results reported in bovine (Van Tol *et al*., 1994; Izadyar *et al*., 1996) and porcine (Coskun and Lin, 1994) oocytes. Considering that activin-A is able to directly stimulate FSH synthesis and secretion, promote the release of the gonadotrophin-releasing hormone (GnRH), and also stimulate the increase of FSH and LH receptors in granulosa cells (Alak *et al*., 1998; Norwitz *et al*., 2002), the ineffectiveness of exogenous activin-A may be either due to activin-A production by the COCs and/or the presence of FSH and LH in our culture medium.

The two studies suggest that in our conditions no effectiveness on the subsequent development to the blastocyst stage of prepubertal goat oocytes is obtained, neither when different maturation media are combined (ascorbic acid, insulin–transferrin–selenium, and a low level of hormones) with the conventional IVM medium using small oocytes diameter, nor when activin-A is added to the conventional IVM medium.

Given that activin-A is produced by oviduct epithelial cells (Gandolfi *et al*., 1995), addition of activin-A to *in vitro* embryo culture could help to reproduce the environment of the *in vivo* embryonic development in the oviduct. In bovine, activin-A was reported a benefit effect depending on the timing of its addition to the culture medium (Yoshioka *et al*., 1998a; Trigal *et al*., 2011). Our study also sought to determine the suitability of a culture medium supplemented with activin-A for the *in vitro* culture of *in vitro* matured and fertilized prepubertal goat oocytes. The main finding of this study was that the addition of 10 ng/mL of activin-A throughout the whole embryo culture promotes embryonic development, improving the blastocyst yield (Hammami *et al*., 2014). Otherwise, we should also consider the possibility of an accumulative effect of activin-A added during *in vitro* maturation and culture medium. Consequently, greater effects are observed as the *in vitro* culture is longer. Thus, further studies will be required to determine the functions of activin-A during these stages of development. The development-enhancing effect of activin-A may be associated with the timing of the activation of appropriate genes within the embryonic genome. Studies in mouse (Lu *et al.*, 1993) and bovine (Yoshioka *et al*., 1998b) showed that activin-A and activin receptors are detected in embryos from the zygote to the morula stage, suggesting that the protein might play a role in embryogenesis during the early stage of embryo development. As far as we know, no studies on detection of the activin receptors in goat oocytes and embryos have been previously reported, though studies using other species

confirm the presence of them in different stages of development. A detailed profiling of the activin-A effects on embryonic development of prepubertal goat oocytes and determination of gene expression and protein synthesis for activin-A require further experiments.

Finally, the effect of growth medium during IVM on blastocyst quality was measured by post-warming survival following vitrification method and blastocyst cell count. The ability of blastocysts derived from big oocytes to survive the vitrification/warming process at 3h was lower than that of blastocysts obtained from small oocytes. According to previous data in bovine (Morató *et al*., 2010), these differences in survival rates of *in vitro*-produced blastocysts depend on their developmental stage. So, the decrease in the cryosurvival rate may be explained by the developmental stage of blastocysts derived from large oocytes (hatching and hatched) as compared to the stage of development of the blastocysts obtained from small oocytes (early and expanded). Otherwise, at 72h the re-expansion rate was significantly higher in the blastocysts obtained from small oocytes matured in growth medium than in those blastocysts from oocytes matured in conventional medium, reflecting a higher oocyte quality. Previous studies in bovine showed that the culture medium supplementation (Rizos *et al*., 2001; Gómez *et al*., 2008) affects intrinsic embryo quality in terms of cryosurvival. Thus, a higher oocyte quality was showed after the oocyte culture in growth medium. However, our results showed that the use of growth medium did not improve the blastocyst cell counts. Similarly, cell number was unaffected by the presence of activin-A in the IVM media.

So, in summary, we can conclude that the addition of ascorbic acid, insulin-transferrin-selenium and a low level of hormones during the first 12h of IVM of prepubertal goat oocytes would be useful to comply with the requirements of the intrinsic quality of blastocysts produced *in vitro*. Notwithstanding, the capacity of those oocytes to develop to the blastocyst stage *in vitro* seems to be more related to oocyte diameter than to the conditions of the IVM system. Finally, according to the results obtained in the second study, the addition of 10 ng/mL of activin-A throughout the whole embryo culture improves blastocyst yield.

GENERAL CONCLUSIONS

General Conclusions

The results of this PhD thesis let conclude that:

1. The addition of ascorbic acid, insulin-transferrin-selenium and a low level of hormones during the first 12h of IVM of prepubertal goat oocytes would be useful to comply with the requirements of the intrinsic quality of blastocysts produced *in vitro*.

2. The capacity of prepubertal goat oocytes to develop to the blastocyst stage *in vitro* seems to be more related to oocyte diameter than to the conditions of the IVM system.

3. Activin-A added to IVM did not result in an improvement of blastocyst development.

4. The addition of 10 ng/mL of activin-A throughout the whole embryo culture period promotes embryonic development, showing an improving effect on the blastocyst yield of prepubertal goat oocytes.

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