Universitat Autònoma de Barcelona

IN VITRO EMBRYO PRODUCTION IN GOATS BY INTRACYTOPLASMIC SPERM INJECTION (ICSI): GAMETE TREATMENTS

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IN VITRO EMBRYO PRODUCTION IN GOATS BY INTRACYTOPLASMIC SPERM INJECTION (ICSI): GAMETE TREATMENTS

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La Dra. María Teresa Paramio Nieto, Catedrática del Departamento de Ciencia Animal y de los Alimentos de la Universidad Autónoma de Barcelona,

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Que la tesis titulada "**IN VITRO EMBRYO PRODUCTION IN GOATS BY INTRACYTOPLASMIC SPERM INJECTION (ICSI): GAMETE TREATMENTS**" presentada por Irene Menéndez Blanco para optar al grado de Doctora por la Universidad Autónoma de Barcelona, se realizó bajo mi dirección y con financiamiento del *Ministerio de Ciencia, Innovación y Universidades* (AGL2014-52408-R y AGL2017-85837-R) y una beca otorgada a Irene Menéndez Blanco por el *Ministerio de Educación y Formación Profesional* (FPU2015/00773).

Y para que así conste, firmo la presente:

Dra. María Teresa Paramio Nieto

Bellaterra a 09 de JULIO de 2020

A mis padres,

A Isaac,

A la Tata,

A mi familia,

2PN	Two pronuclei formation
3PN	Three pronuclei formation
6-DMAP	6-dymethylaminopurine
ART	Assisted reproductive technology
BCB	Brilliant Cresyl Blue (stain)
BSA	Bovine serum albumin
Ca^{2+}	Calcium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ED	Egg donation
EG	Ethylene glycol
ESHRE	European Society of Human Reproduction and Embryology
EU	European Union
FER	Frozen embryo replacement
FOR	Frozen oocyte replacement
FS	Fresh ejaculated semen
FTTS	Frozen-thawed testicular semen
GSH	Glutathione
GV	Germinal vesicle
HA	Hyaluronic acid
HIV	Human immunodeficiency viruses
HOST	Hypo-osmotic swelling test
HSA	Human serum albumin
ICSI	Intracytoplasmic sperm injection
ICSI-MGT	ICSI-mediated gene transfer
ID	Internal diameter
IMSI	Intracytoplasmic morphologically selected sperm injection
IVM	*In vitro* maturation
IVF	*In vitro* fertilization
IVC	*In vitro* embryo culture
IVEP	*In vitro* embryo production
JIVET	Juvenile *in vitro* embryo transfer
mDM	Modified defined medium
MII	Metaphase II
Min	Minute/s
MPF	Maturation promoting factor
MAPK	Mitogen activate protein kinasa
N_2	Nitrogen liquid
O_2	Oxygen
PA	Parthenogenic activation
PB	Polar body
PI	Propidium iodide
PICSI	Physiologic ICSI
PLCζ	Phospholipase C zeta
PVP	Polyvinylpyrrolidone
PZD	Partial zona dissection
ROS	Reactive oxygen species
SCNT	Somatic cell nuclear tranfer
SUZI	Subzonal insemination
TALP	Tyrode’s albumin lactate pyruvate

ABSTRACT

The development of intracytoplasmic sperm injection (ICSI) technique has been a great advance for human and animal reproductive laboratories. ICSI is a very useful technique in cases of male infertility, cryopreserved gametes and poor quality oocytes. Low quality oocytes are one of the main factors negatively affecting *in vitro* embryo production of humans and animals. Our previous studies using ovaries of prepubertal goats showed the low competence of their oocytes to develop up to blastocyst stage. Thus, we think that prepubertal goat oocytes could be considered as a good model for studying female poor quality oocytes and the strategies to improve their embryo development. For that reason the main objectives of this PhD thesis was 1) to test the cryopreservation of male (spermatozoa) and female (oocyte) gametes (Studies 1 and 2) on the gamete quality and embryo development after ICSI and 2) to assess the effect of crocetin, which is a natural antioxidant, and their supplementation during *in vitro* maturation (IVM) of prepubertal goat oocytes on the embryo development after ICSI.

In the first study we compared the use of fresh and frozen-thawed sperm samples on embryo development after ICSI and its relation to capacitation parameters of spermatozoa. Results of frozen-thawed spermatozoa showed significantly higher level of capacitation (65 vs. 35%, respectively) and dead acrosome reacted sperm (33 vs. 13%, respectively) than fresh spermatozoa. However these differences in capacitation and acrosome level of the sperm samples were not translated into differences on pronuclear formation and embryo development. At 17 hours post ICSI, no differences were found using frozen-thawed and fresh semen (37 vs. 39% respectively) in normal pronuclear formation zygotes (2PN). Moreover, no differences were obtained comparing the percentage of cleavage (65 vs. 60%) and blastocysts produced at day 9 post injection (12 vs. 18%, respectively).

The second study evaluated the effect of vitrification of IVM prepubertal goat oocytes on: 1) oocyte damage assessed by reactive oxygen species (ROS) level and apoptosis and 2) embryo development after ICSI and parthenogenic activation (PA). IVM-Oocytes were vitrified using an open cryoloop system in 15% ethylene glycol (EG), 15% dimethyl sulfoxide (DMSO) and 0.5 M sucrose. Vitrified oocytes

showed higher ROS levels (P<0.0001) assessed by 2'7'-dichlorodihydrofluorescein diacetate stain, lower live oocytes (44 vs. 66%) and higher dead non-apoptotic oocytes (33 vs. 13%) assessed by annexin V-propidium iodide (PI) staining compared to non-vitrified oocytes. Normal 2PN-zygotes (32 vs. 25%) and blastocyst rates development (0 vs. 4%) did not present differences in ICSI fertilized oocytes for vitrified and non-vitrified oocytes respectively. However, regarding PA results, cleavage (59 vs. 78%) and blastocyst formation (1 vs. 25%) were significantly decreased in vitrified oocytes compared to non-vitrified oocytes respectively. We concluded that vitrification process on prepubertal goat oocytes reduced oocyte competence by increasing dead oocytes and ROS levels given the high fragility of these oocytes.

Study 3 was aimed at improving the competence of prepubertal goat oocytes by supplementing the IVM medium with crocetin, a natural antioxidant. We evaluated the effect of crocetin on molecular and cellular parameters related to oocyte competence such as ROS, glutathione (GSH) and mitochondrial activity and the embryo development after ICSI, *in vitro* fertilization (IVF), and PA. In the first experiment of this study, we evaluated the effect of the IVM supplementation with 0 µM (control), 0.5 µM, 1 µM and 2 µM of crocetin on the blastocyst development after IVF. No significant differences were obtained on blastocyst formation among groups (12, 7, 10, 11%; respectively). We selected the 1 µM concentration of crocetin to perform the rest of the experiments. Thus, in experiment 2, we found out that 1 µM crocetin in the IVM medium reduced the ROS levels (P < 0.05) but did not modify the GSH concentration or mitochondrial activity in these oocytes. Finally, in experiment 3 we analyzed the effect of 1 µM crocetin on the blastocyst development of oocytes after ICSI and PA procedures. No statistical differences on oocyte cleavage, blastocyst rate and total cell number per blastocyst were observed among groups. However, the ICSI-crocetin group presented better cleavage (67 vs. 59%, respectively, P=0.09) and blastocyst rates (19 vs. 12%, respectively; P= 0.12) than prepubertal goat oocytes in control group.

In conclusion, we have demonstrated that fresh and frozen-thawed semen can be useful for ICSI of prepubertal goat oocytes without compromising the results. However, with the protocol used, prepubertal goat oocytes after vitrification were

not able to develop up to blastocyst after being fertilized by ICSI. The crocetin addition to IVM has not shown significant effects on embryo production from prepubertal goat oocytes in spite of the significant reduction on ROS level. However, ICSI is a very time consuming procedure which exposes the gametes to high oxygen (O_2) atmosphere, for that reason the use of antioxidants is highly recommended for this procedure.

RESUMEN

El desarrollo de la técnica de microinyección intracitoplasmática de espermatozoides (ICSI) ha supuesto un gran avance para los laboratorios de reproducción asistida humana y animal. La técnica de ICSI es de gran utilidad en casos de infertilidad masculina, uso de gametos criopreservados y para fertilizar ovocitos de baja calidad. Los ovocitos de baja calidad son precisamente uno de los principales factores que afectan negativamente la producción de embriones *in vitro* (PIVE) de humanos y animales. Estudios previos en nuestro laboratorio utilizando ovarios de cabras prepúberes demostraron la baja competencia de los ovocitos obtenidos para desarrollarse hasta la etapa de blastocisto. Por lo tanto, creemos que los ovocitos de cabras prepúberes podrían considerarse como un buen modelo para el estudio de los ovocitos de mujeres de baja calidad. Por esa razón, los objetivos principales de esta tesis doctoral fueron 1) Estudiar el efecto de la criopreservación de gametos masculinos (espermatozoides) y femeninos (ovocitos) (Estudios 1 y 2) sobre la calidad de los gametos y el desarrollo embrionario después de la ICSI y 2) estudiar el efecto de la suplementación con crocetina, que es un antioxidante natural, durante la maduración *in vitro* (MIV) de ovocitos de cabras prepúberes sobre su desarrollo embrionario después de la ICSI.

En el primer estudio comparamos la utilización de espermatozoides frescos y congelados para la técnica de ICSI evaluando su efecto en el desarrollo embrionario y su relación con los parámetros de capacitación de los espermatozoides. Los resultados mostraron un nivel significativamente más alto de capacitación (65 vs. 35%, respectivamente) y espermatozoides muertos con acrosoma reaccionado (33 vs. 13%, respectivamente) en los espermatozoides congelados comparado con los espermatozoides frescos. Sin embargo, estas diferencias en la capacitación y el nivel de reacción acrosomal de ambas muestras seminales no se tradujeron en diferencias en la formación pronuclear y el desarrollo embrionario. Así, 17 horas después de la ICSI, no se observaron diferencias usando semen congelado y fresco en los cigotos normales (cigotos con 2 pronúcleos) (37 vs. 39% respectivamente), ni en la división de los ovocitos

(65 vs. 60%) ni en los blastocistos producidos 9 días después de la inyección (12 vs. 18%, respectivamente).

El segundo estudio examinó el efecto de la vitrificación en ovocitos de cabras prepúberes madurados *in vitro* en: 1) el daño sobre los ovocitos estudiando el análisis de las especies reactivas de oxigeno (ROS) evaluadas mediante la tinción con 2'7' diacetato de diclorodihidrofluoresceína y la apoptosis evaluada por la tinción de Anexina V combinada con ioduro de propidio (IP) y 2) el desarrollo de embriones obtenidos mediante ICSI y activación partenogenética (AP). Los ovocitos se vitrificaron después de su MIV con 15% de etilenglicol (EG), 15% de dimetilsulfóxido (DMSO) y 0,5 M de sacarosa. Los resultados nos indicaron que el grupo de los ovocitos vitrificados tenían niveles de ROS más altos ($P<0.0001$), menos ovocitos vivos (44 vs. 66%) y más ovocitos no apoptóticos muertos (33 vs. 13%), en comparación con el grupo de los ovocitos no vitrificados. Después de la ICSI observamos que la formación normal de cigotos (32 vs. 25%) y de blastocistos (0 vs. 4%) no presentaron diferencias entre los ovocitos vitrificados y no vitrificados fertilizados mediante ICSI, respectivamente. Sin embargo, en los ovocitos activados partenogenéticamente, la división de los ovocitos (59 vs. 78%) y la formación de blastocistos (1 vs. 25%) disminuyeron significativamente después de la vitrificación. Por tanto, llegamos a la conclusión de que el proceso de vitrificación en los ovocitos de cabras prepúberes redujo la competencia de los ovocitos al aumentar los ovocitos muertos y los niveles de ROS dada la alta fragilidad de estos ovocitos.

El estudio 3 tuvo como objetivo mejorar la competencia de los ovocitos de cabras prepúberes al suplementar el medio de maduración con crocetina, un antioxidante natural. Para ello evaluamos el efecto de la crocetina sobre los parámetros moleculares y celulares relacionados con la competencia de los ovocitos como ROS, glutatión (GSH) y actividad mitocondrial y el desarrollo embrionario después de la ICSI, la fecundación *in vitro* (FIV) y la activación partenogenética. En el primer experimento de este estudio, evaluamos el efecto de la suplementación del medio de MIV con 0 µM (control), 0.5 µM, 1 µM y 2 µM de crocetina sobre el desarrollo embrionario hasta el estadio de blastocistos después de la FIV. En este experimento no se observaron diferencias significativas en la formación de

blastocistos entre los distintas concentraciones de crocetina (12, 7, 10, 11%; respectivamente). Según estos resultados, seleccionamos la concentración de 1 µM de crocetina para realizar el resto de los experimentos. En el experimento 2, demostramos que una concentración de 1 µM de crocetina en el medio de maduración redujo los niveles de ROS ($P < 0.05$) pero no modificó la concentración de GSH o la actividad mitocondrial en estos ovocitos madurados con el antioxidante. En el experimento 3 analizamos el efecto de la crocetina añadido al medio de MIV de los ovocitos sobre su desarrollo hasta blastocisto después de ser fecundados mediante ICSI y ser activados partenogenéticamente. En este experimento no observamos ninguna diferencia estadísticamente significativa en el porcentaje de división de los ovocitos, la formación de blastocistos y el número total de células por blastocisto entre los grupos. Sin embargo, en el grupo de ICSI con crocetina se observó una mayor división de los ovocitos (67 vs. 59%, respectivamente, $P=0.09$) y mayor formación de blastocistos (19 vs. 12%, respectivamente; $P=0.12$) que en los ovocitos madurados sin antioxidante.

En conclusión, en este trabajo hemos demostrado que el semen congelado puede ser tan eficiente como el semen fresco para producir embriones de ovocitos de cabras prepúberes mediante ICSI. Sin embargo, con el protocolo utilizado, los ovocitos de cabras prepúberes después de la vitrificación no pudieron desarrollarse hasta blastocisto después de ser fertilizados por ICSI. La adición de un antioxidante como la crocetina al medio de maduración no ha mostrado efectos significativos en la mejora de la producción de embriones a pesar de la significativa reducción en el nivel de ROS de los ovocitos de cabras prepúberes madurados *in vitro*. Sin embargo, la ICSI es una técnica que requiere mucho tiempo y expone a los gametos a una atmósfera con alto contenido de oxígeno (O_2), por lo que se recomienda el uso de antioxidantes para este procedimiento.

CHAPTER 1

General Introduction

The *in vitro* embryo production (IVEP) is one of the Assisted Reproductive Technologies (ARTs) used in animal breeding to improve the genetic background of livestock species by allowing the widespread of selected females. In other veterinary species, such as pets and horses, IVEP will help to overcome pathologic infertility.

In humans, this ART has become one of the most useful technologies for reproductive treatments of infertility from male or female origin which affect approximately 15% of couples in reproductive age worldwide [1]. The first baby born after *in vitro* fertilization (IVF) procedure was reported in 1978 [2]. Despite this early success, several clinics began to realize about the significant limitations of IVF. In fact, about 40% of IVF cycles were complicated by poor fertilization or complete fertilization failure even with good oocytes [3]. After testing different reproductive techniques such as partial zona dissection (PZD) and subzonal insemination (SUZI) the intracytoplasmic sperm injection (ICSI) was introduced as an alternative to the IVF in humans during the earliest 1990 thanks to their better results [4]. For that reason, after its introduction, ICSI has been rapidly incorporated into the routine of clinical practice.

The use of ICSI has increased from 40% of ART cycles in 1997 to 59% in 2004. To date, 1% of children have been born around the world as the result of ICSI [5]. ICSI was firstly performed in couples with severely impaired sperm characteristics [6]. Nowadays, ICSI is being increasingly utilized even in the presence of normal semen parameters. The increased utilization of ICSI corresponds to the rise in preimplantation genetic testing, use of cryopreserved oocytes, poor quality oocytes and *in vitro* matured oocytes [7].

In animals, ICSI has been used in different domestic animals such as cows [8], rabbits [9], sheep [10], horses [11], domestic cats and wild felids [12], pigs [13] and goats [14]. Salamone et al. concluded that ICSI has become a useful technique for clinical applications in the horse-breeding industry [15]. In farm animals, ICSI is an important tool for the reproduction of high-value specimens and a promising technique for genetic rescue of endangered and wild species. Moreover, for transgenic animal studies, the ICSI-mediated gene transfer (ICSI-MGT) seems to be helpful too. The results of ICSI in farm animals are poor. The most extreme

case is the cow, whose fertilization rates after ICSI are critically low [16]. In sheep and goats, although fertilization rates after ICSI can be improved by artificial activation treatments [17] development up to blastocyst stage continues to be low.

In general, results of ICSI in terms of production of good quality blastocysts, both in humans and animals, are disappointing and they need to be improved with more experimental research. In order to improve ICSI outputs by performing an important number of experiments, a source of easily available and cheap oocytes would be useful. Farm animals slaughtered in abattoirs can be a huge supply of these oocytes. Ruminant animals have been used as a good model for human reproductive techniques [18].

In our cultural conditions, goat meat is provided by 1 to 2-month-old animals. Ovaries from these prepubertal females are small, with follicles of small diameter and oocytes with a low competence to develop up to blastocyst after IVEP protocols [19]. This type of oocytes could be a good model to study low-quality oocytes, such as the oocytes of women requiring ARTs in humans.

This PhD thesis has been aimed at first evaluating the behavior of cryopreserved gametes in embryo development of prepubertal goat oocytes after ICSI. Given that ICSI is a time-consuming procedure, high levels of ROS are produced in cells during the injection, impairing oocyte development. Thus, according to promising results using different antioxidants for IVEP in our laboratory [20–22], in the second part of this thesis we have tried to avoid the negative effect of oxidation by adding the antioxidant crocetin to the *in vitro* maturation medium.

CHAPTER 2

Literature Review

2.1 ¿What is the Intracytoplasmic sperm injection (ICSI)?

Intracytoplasmic sperm injection is a powerful micromanipulation technique in the field of assisted reproduction technologies (ARTs) that provides new opportunities for humans and other animal species. It was firstly developed in hamster by Uehara and Yanagimachi in 1976 obtaining pronuclei [23]. Shortly after, Lanzerdorf et al. [24] obtained the first human pronuclei by this technique. The first successful pregnancy and birth using ICSI in human was achieved by Palermo et al. [6]. Since then, ICSI has gained popularity among human ARTs, being today the most widely technique used in clinical practice [25].

To understand ICSI success it is important to delve into its procedure. Basically, it consists in the injection of a single spermatozoon, previously selected, into the ooplasm of a MII-oocyte using a sophisticated microinjection system under an inverted microscope. One of the fundamental aspects for a successful ICSI is to be carried out by a trained and specialized technician. However, there are many other factors that should be taken into account to develop a successful ICSI procedure; some of them are: Sperm factor, such as selection, immobilization or quality of the sperm, and oocyte factors, such as correct cytoplasmic competence and activation. However, all these crucial steps of the ICSI technique have been mostly overwhelmed by the variety of protocols and strategies currently available. Thus, ICSI has been consolidated currently as one of the most important ART for human reproductive therapies and an interesting tool for livestock species and wild mammals [5,15].

2.2 ICSI in human ARTs

ICSI emerged as an alternative to *in vitro* fertilization (IVF) for those cases where there was a severe male factor as: oligozoospermia, asthenozoospermia, teratozoospermia, a combination of them, or even a complete absence of sperm cells in the ejaculate. It was preceded by other techniques such as partial zona dissection (PZD) and subzonal insemination (SUZI) that did not succeed given its low percentage of normal fertilization (20% after SUZI) [26,27]. Early attempts of ICSI in human resulted only in pronuclear formation without the establishment of pregnancy after embryo transfer [24]. However, shortly after, in 1992, the first live

new-born using ICSI technique was obtained by Gianpiero Palermo [6]. From this moment on, ICSI technique began to undergo a significant development and completely replaced SUZI and PZD in most laboratories, given their good rates of fertilization and more suitable embryos for transfer [28].

At first, ICSI was mainly indicated for couples with severe male factor and obstruction of the seminal excretory ducts, when sperm recovered from the epididymis and testis was used. ICSI produces rates of more than 80% fertilized oocytes that develop further to embryos of acceptable quality to be transferred. However, independently of the type of sperm used (e.g. fresh or frozen-thawed ejaculated, epididymal and testicular) the percentage of embryos transferred or stored is between 60 and 65% from normally fertilized oocytes (reviewed by Devroey and Van Steirteghem 2004) [4]. From the beginning, results derived from ICSI with fresh and frozen-thawed sperm were very similar [29]. Lately, the effect on the early embryo development of the use of fresh or frozen-thawed by ICSI has been evaluated by Eastick et al. [30] concluding that there were no differences in the morphokinetic parameters as early embryo development evaluated by time-lapse between groups.

Data from 1997 to 2015 collected by the European Society of Human Reproduction and Embryology (ESHRE) clearly pointed the gradual increase in the number of ICSI cycles, compared to IVF cycles as well as the increase in the total number of reproductive treatments undergone in the European Union (EU). Thus, in 2015, 849,811 cycles of reproductive treatments were reported, representing an increase of 9% compared to those reported in 2014 (776,556) and a much greater increase compared to 1997 (203,255 cycles). Regarding 2015 treatments, with data obtained from 1,343 institutions in 38 different countries of the EU, 155,960 treatments have been done by IVF, whereas 385,676 have been done by ICSI [25].

Therefore, nowadays ICSI has become the most widely used technique regardless of seminal quality. However, it has a number of limitations, which have been tried to be reduced over the years. Some of these limitations are the oocyte

degeneration after injection, which is highly related to ICSI technique, and the fertilization failure. Degeneration of oocytes does not seem to be related to the technician ability but to certain unexpected random technical tricks, such as oolema breakdown, injection of variable volume of sperm medium and volume of cytoplasm aspirated during the injection. To solve the problems derived from the technical part of ICSI, according to Rubino et al., new methodologies and protocols have been developed [5]. Currently there are some techniques that have been derived from a better sperm selection during the procedure, such as the intracytoplasmic morphologically selected sperm injection (IMSI) and physiologic ICSI (PICSI), further described below.

2.3 ICSI in animals

The development of the ICSI technique in animals has been delayed in time compared to human, but still has been significant. After the first successful ICSI in hamster gametes achieved by Uehara and Yanagimachi in 1976, the technique was further used in many other species: cows [8], rabbits [9], mice [31], sheep [10], horses [11], domestic cats and wild felids [12], pigs [13], hamsters [32] and goats [14]. ICSI technique has different applications in animals, depending on the species. ICSI can be applied to maintain the diversity especially from endangered species and the reproduction of high value specimens [33,34]. Moreover, ICSI could be useful to fertilize low quality gametes obtained from slaughtered animals, and allows us the use of sperm recovered from post-mortem males and cryopreserved gametes (reviewed by Garcia-Roselló et al., 2009) [35].

According to the review by Yanagimachi [36], ICSI efficiency is lower in animals than in humans. Moreover, it is difficult to calculate the efficiency in each animal species given that some studies failed to report the number of oocytes injected or the number of oocytes surviving ICSI. The efficiency of the technique has been low from the beginning in many species such as hamster (9%) and rabbit (4%), while in others, such as mouse the efficiency has been much higher (60-70%). According to Yanagimachi this variability may be due to the need to learn specific technical tricks for each species. For example, the removal of the sperm plasma membrane (more stable in some species than in others) and acrosome has been studied, concluding that it is necessary for hamster ICSI and could be very helpful

for larger species as well [32]. On *in vitro* conditions, especially during ICSI procedure, oocytes are exposed to non-physiological conditions such as different light sources or different oxygen tensions during the *in vitro* culture. Even visible light (<470–480nm) emitted from ordinary fluorescent light sources has a noticeable negative effect on ICSI success related to the generation of radical oxygen species (ROS) which harm oocytes [32].

Oocyte artificial activation treatments also seem to be helpful just in some species, such as cow, sheep, goat and pig, while in others, such as human, mice, horse and domestic cat, the injection itself correctly triggers oocyte activation and pronucleus formation [15,36]. Recently, several protocols have been developed and adapted for each species without obtaining better results [15,35].

ICSI has been developed in horse to a commercial level due to the problematic and low efficiency with standard *in vitro* fertilization that characterizes this specie [37]. On pigs, ICSI could be useful as well due to the high rates of polyspermy found during conventional IVF in this specie [38]. However, despite the multitude of applications in animals and the large number of groups studying on this technique, its performance in most species remains low.

Goat ICSI has had an even slower development compared to other domestic species, obtaining the first offspring in 2003 [14]. The reduced development of ICSI in goats could be related to its low *in vitro* and post-implantation embryo development rates. These lower developmental rates are accentuated when oocytes are recovered from prepubertal goats (reviewed by Roselló et al., 2009; Lopez-Saucedo et al., 2012) [35,39]. In our laboratory, Jimenez-Macedo et al. [40–42] performed different studies in prepubertal goat oocytes in order to improve ICSI protocols and spermatozoa treatments for injection, and to relate the size of oocytes and its implication in embryo development.

2.4 Sperm treatment and selection for ICSI

One of the critical steps for the success of ICSI is the treatment and selection of the spermatozoa. This is important because in contrast to IVF, ICSI bypass all the initial steps of natural fertilization, such as penetration of the cumulus cells, binding

to the zona pellucida and fusion with the oolemma, which naturally selects the best spermatozoa to fertilize the oocyte. The use of poor-quality sperm for ICSI, which in a normal IVF procedure will not fertilize the oocyte, has been widely discussed. Many authors have studied the relationship between the use of ICSI for fertilization and a greater risk of birth defects, such as major genetic malformations, neurological problems and an increased number of hospitalizations required by the offspring. The majority of these works do not observe significant difference between ICSI-derived and spontaneous-conceived children [43]. However, they have found an important relation between DNA quality of sperm and the outcomes of IVF and ICSI in terms of pregnancy rates (reviewed by Sakkas, 2003; Bach and Schlegel, 2016) [43,44].

Conventional sperm selection methods based on sperm motility, swim-up and density gradient centrifugation are the techniques usually followed in ART laboratories [45]. Depending on the origin (ejaculated, epididymal and testicular), quality of the sperm sample, and the species used, one or more methods for motile spermatozoa selection can be included on ICSI protocols [46]. Swim-up technique selects sperm according to motility, and density gradient centrifugation technique separates sperm according to nuclear sperm density [47]. However, there are other possibilities, like separation based on the migration ability of the sperm, or it could be forced to swim through a filter for separation purposes [48]. Regarding sperm selection treatment for ICSI in goats, fresh and frozen-thawed sperm samples are usually selected by density gradient [14]. Nevertheless, swim-up technique can be used as well for obtaining motile spermatozoa from fresh-ejaculated samples [40].

Capacitation of the sperm is the name given to the biochemical and biophysical changes that the sperm suffers before the acrosome reaction and the fusion with oolema to fertilize the oocyte [49]. Under *in vivo* condition it happens in the male epididymis and the female reproductive tract, whereas on *in vitro* procedures we need to imitate it before the fertilization technique, both for IVF or ICSI (reviewed by Stival et al., 2016) [50]. Human sperm capacitation is usually done by the incubation of the sperm sample with HAM's F-10 medium supplemented with human serum albumin (HSA) as described by the World Health Organization [46].

In animals, each species has its own requirement. As an example, in small ruminants the defined mDM medium [51] and heparin are the routine way of capacitation of semen. In our laboratory, using prepubertal goat oocytes, Jimenez-Macedo et al. in two consecutive studies [40,41] have demonstrated that the sperm capacitation prior to ICSI with heparin plus ionomycin significantly increased normal zygote formation (2PN) and embryo development compared to sperm capacitation alone with heparin.

There are as well important technical points regarding sperm and ICSI injection. Sperm tail breakage before injection should not be bypassed since it can improve the sperm nucleus decondensation [52]. Another important point is the sperm immobilization. Polyvinylpyrrolidone (PVP) is the common solution used in animal research due to its capacity to reduce the sperm motility, which allows the operator to easily immobilize the sperm. Other technical aspects as the inner diameter (ID) of the injection pipette or the position of the oocyte to fulfil the injection have been widely discussed too. The inner diameter of the injection pipette needs to be in accordance to the size of the sperm head. While human spermatozoa needs pipettes around 5.5 µm of ID, biggest such as 7 µm of ID are useful for cow, pig, sheep or goat [5,15].

Considering the importance of sperm study and selection for ICSI, multiple specific techniques have emerged to facilitate it prior to injection, such us:

- **Intracytoplasmic morphologically selected sperm injection (IMSI)**: IMSI is based on the selection of motile spermatozoa at 6000x optical magnification instead of 200-400x magnification used for conventional ICSI. IMSI have been shown to be very useful to detect subtle changes in sperm morphology parameters that cannot be detected under the conventional degree of magnification [53,54].
- **Birefringence selection of acrosome reacted sperm prior to ICSI:** It consists in carrying out the ICSI technique under a polarized light source that permits the analysis of the pattern of birefringence in the sperm head. With the help of this technology it is possible to selectively inject oocytes with acrosome reacted and acrosome non-reacted spermatozoa.

Spermatozoa undergoing the acrosome reaction seem to be more suitable to support the development of viable ICSI embryos [55].

- **Hypo-osmotic swelling test (HOST):** This test is useful when staining of spermatozoa should be avoided, e.g. when choosing them for ICSI. The test is mainly based on the response of the sperm after 30 minutes into a hypo-osmotic medium [46]. Swollen spermatozoa are identified by changes in the shape of the cell, as indicated by coiling of the tail [56]. Regarding the relation between sperm selection and their DNA damage, some techniques are focused on selecting spermatozoa with lower levels of DNA damage [43,44]. HOST can identify the individual spermatozoa with minimal DNA fragmentation and could be a good selection tool during ICSI [57]. Moreover HOST selection can also identify vitality of the sperm sample, spermatozoa with abnormal head morphology, nuclear immaturity, membrane damage and apoptosis [58].
- **Sperm binding to Hyaluronic Acid (HA):** The use of HA binding as a selection technique for ICSI has been widely studied and gave rise to the rise of the Physiological intracytoplasmic sperm injection (Physiological ICSI) which consists on the selection of sperm attached to HA for injection. Human sperm that binds to HA exhibit minimal DNA fragmentation, normal shape, and low frequency of chromosomal aneuploidies similar to the sperm that binds to zona pellucida [59]. Human studies also show the increase on implantation rates from 10% in normal ICSI to 17% in ICSI with sperm selected by HA binding [60]. Some commercial media for sperm selection are based on this HA binding ability, such as SpermSlow ™ (Origio), which emerged to simplify this process and demonstrates comparable clinical efficiency in sperm selection through HA-binding than PICSI ® (Sperm Selection Device. Origio) technique based on a culture dish with microdots of HA hydrogel attached to the bottom of the dish [61].

In conclusion, the current goal of sperm selection in humans is to resolve individual cases with more efficiency, giving a customized response to each couple with special issues [62]. In animals, the goal is similar and consists of

developing better protocols adapted for each species to improve the success of *in vitro* embryo production in terms of blastocyst rate and quality [15].

2.5 Oocyte treatment for ICSI

Oocyte treatment, prior and after ICSI, presents some differences between human and animal species. The use of *in vitro* matured oocytes for humans ARTs is quite unusual. First, because the majority of the oocytes recovered following ovarian stimulation protocols are at metaphase II (MII) stage and ready to be fertilized. Secondly, human *in vitro* matured oocytes have a lower developmental ability compared to *in vivo* matured oocytes [63]. Therefore, only *in vivo* mature oocytes are used for ICSI on a regular basis, whereas the immature oocytes are discarded.

The position of the polar body (PB) has been studied in relation to the sperm injection during ICSI. Based on the assumption that the MII spindle lies really close to the first PB, the spermatozoon is usually injected at the 3 o'clock position with the PB located at 6 or 12 o'clock position, in order to avoid any damage to the meiotic spindle. However, it could be useful to perform the injection at the 7 or 11 o'clock position since the proximity of the sperm to the metaphase plate seems to be beneficial [5]. Penetration of the oolema is one of the critical steps on animal ICSI procedure [64]. Some species present difficulties depending on the elastic properties of the oocyte membrane, which differs between them. Cow and human oocytes present the greatest resistance to injection. However, sheep and goat oocytes are more sensitive to handling and have higher rate of lysis after injection [15]. Very fragile oolemma leads to degeneration of oocytes resulting in sudden breakage of the membrane during ICSI [65].

Lastly, complete fertilization failure after ICSI is a very rare event, but that could happen even in the presence of apparently normal spermatozoa [66,67]. Low fertilization rates can be observed in repeated ICSI cycles for some couples [68]. Fertilization failure after ICSI still occurs in 3 to 5% of total ICSI cycles [69]. The causes of ICSI fertilization failure on human oocytes correctly injected are mainly related to an oocyte-activation deficiency [68], which is reported in approximately 40–70% of the ICSI unfertilized oocytes [66,70]. There are additional causes

including the failing of sperm head decondensation, spindle defects, premature sperm chromatin condensation and simply incorrect sperm injection [71].

It is known that ICSI bypass the steps for a conventional fertilization, for instance, the sperm-oocyte fusion that is closely related to the oocyte activation. For that reason, some species require oocyte activation protocols after ICSI (cows, sheep, goats and pigs) in order to improve pronuclear formation and oocyte activation. However, in other species (mice, humans, horses and domestic cats) the injection alone is enough to trigger oocyte activation [36]. Oocyte activation in mammals implies intracytoplasmic calcium (Ca^{2+}) oscillations evoked by the sperm union to the oolema and upon release of a soluble factor phospholipase C zeta (PLCζ) [72]. These calcium oscillations initiate a complex signaling cascade, which includes a decrease on maturation promoting factor (MPF) and mitogen activate protein kinasa (MAPK) levels, leading to the meiotic resumption [73]. Thus, deficiencies associated to the oocyte Ca^{2+} release or PLCz are likely to result in fertilization failures [74]. For that reason, assisted activation protocols for domestic species conventionally combine the use of physical or chemical Ca^{2+} inducer with an inhibitors of MPF and/or MPAK activities, such as 6-dymethylaminopurine (6-DMAP) as they trigger the resumption of meiosis [15]. However, many different oocyte activation techniques have been tested up to date in human and animal species.

- **Mechanical activation:** Tesarik et al. pointed out that the modification of ICSI technique could help to mechanically activate the oocyte and increase the fertilization rates. This modification consisted on a two times vigorous cytoplasmic aspiration from the central area of the ooplasm and its reinjection with the spermatozoon as close as possible to the opposite place of the injection in order to create an influx of Ca^{2+} [75].
- **Physical activation:** Some methods, as oocyte electrical activation, have been studied. Yanagida et al. described for the first time the use of combined ICSI with electrical stimulation of oocytes in two couples with previous total failed fertilization after ICSI and normal semen parameters [76].

- **Chemical activation:** Ionophores, such as ionomycin, are the most commonly used chemical artificial activating agents in human ARTs. However, protocols and exposure times could vary markedly between studies. Although ionophores are conventionally combined with MPF/MPAK inhibitors, according to Salamone et al. [15] the chemical activation protocol must include a window of 3 hours between Ca^{2+} ionophore and incubation with 6-DMAP in order to allow a correct extrusion of the second polar body.
- **Phospholipase C zeta (PLCζ):** the use of Phospholipase C z as the activating agent has been tested as well due to its physiologic activity during natural activation [77,78].

Regarding adults goat studies, different protocols have been used obtaining different results. Keskintepe et al. obtained 18% of blastocyst formation without chemical activation post injection [79]. On the other hand, Wang et al. obtained 35% of blastocyst using piezo-drill without subsequent activation protocol [14]. Some studies in our laboratory have been conducted for studying the oocyte activation in prepubertal goats. In the first one 5% of morulae were obtained using activation with ionomycin plus DMAP [40]. However, a high number of parthenogenic embryos were observed probably derived from the use of DMAP immediately after ionomycin. In a second study, 11% of blastocyst production was obtained after selecting oocytes larger than 125 µm of diameter to be injected [41]. In this second study, to avoid the activation of the injected oocytes, the sperm sample was capacitated using heparin and ionomycin according to the idea that higher sperm capacitation is related to a better male pronucleus formation.

A standardized protocol for human or animals is still missed but the develop of activation treatments that better mimic what occurs after regular fertilization is been very useful to avoid fertilization failures, and improving the embryo development and pregnancy outcomes.

2.6 Gamete cryopreservation

2.6.1 Applications of gamete cryopreservation

Cryopreservation describes techniques which permits freezing and subsequent warming of biological samples without loss of viability. Thus, gamete

cryopreservation has a broad of application on human and animal species. For examples, human oocyte cryopreservation could be very helpful for reducing the number of ovarian stimulation cycles, preserving the reproductive capacity in women who have the desire to delay reproduction plans for several reasons [80]. Cobo et al. have compared simultaneously vitrified oocytes, following the cryotop method previously described by Kuwayama et al. [81], with their fresh counterparts, in an oocyte donation program. They obtained similar laboratory results in terms of fertilization (76 vs. 82%, respectively) and blastocyst formation rates (49 vs. 47%, respectively) [82].

Some application of oocyte cryopreservation on animals are the genetic selection, facilitation of assisted reproduction procedures, international exchange of germplasm, conservation of livestock and animal endangered genetics or rescue of oocytes from died animals [83]. Regarding the vitrification of animal oocytes the efficiency differs between species. According to Mogas over the past two decades, many research efforts tried to overcome individual features of the bovine oocyte that make it notoriously difficult to cryopreserve [84]. In goats, Kharche et al. compared oocytes vitrified at germinal vesicle (GV) stage with propanediol to control oocytes non-vitrified finding important differences in maturation (27 vs. 74%, respectively) and fertilization rates (18 vs. 54%, respectively) [85]. Lately, Quan et al. vitrifying by Cryoloop method GV and MII oocytes, showed lower rates of normal morphology (60 vs. 83%, respectively), and cleavage rate (43 vs. 58%, respectively) [86]. However, blastocyst rates between GV and MII vitrified oocytes did not differ (8 vs. 12%, respectively). They conclude that although vitrification can greatly damage the structure and development capability of goat oocytes, MII oocytes are more tolerant to the vitrification process [86]. No studies have been done in prepubertal goat oocytes. Cryopreservation is still not well developed, and further improvements to the vitrification procedure should include specific *in vitro* maturation media formulated for the specific needs of the oocyte, to overcome their high sensitivity to cryopreservation.

Cryopreservation of sperm samples is helpful for management of male fertility before illness therapy, vasectomy and surgical infertility treatment, as well as for the sperm storage to synchronize with the oocyte recovery for the reproductive

treatment in humans [87]. The use of frozen sperm represents a major factor in the success of *in vitro* fertilization (IVF), with many millions of live births in human and veterinary applications. However, despite this progress made in gamete cryopreservation of several livestock species, there is no standardized universal procedure established [88]. This could be due to the different cryotolerance of animal spermatozoa which varies according to their specific features, such as size, shape, and lipid composition. According to Lv et al. the establishment of an efficient semen cryopreservation procedure will facilitate long-term conservation of small ruminant genetic resources and extension of artificial insemination in daily production [89].

ICSI is a useful technique for the fertilization of oocytes which are cryopreserved in contrast to conventional IVF [90]. Cryostress can lead to a premature exocytosis of cortical granules and zona hardening, hindering fertilization [91]. In fact, the increase in the use of ICSI for human reproductive therapies in recent years is related to the use of cryopreserved oocytes, as well as the improvement of preimplantation genetic testing and *in vitro* maturation protocols [7]. In some reproductive clinics [92], ICSI is used in all cases when cryopreserved sperm samples are used as a tool to compensate the poor survival of the cells upon thawing. Moreover, ICSI is the unique option when freeze-drying sperm is used for the fertilization [89]. Data collected in humans during 2015 by ESHRE demonstrated the large use of cryopreservation in the reproductive cycles: 218098 cases with frozen embryo replacement (FER), 64477 with egg donation (ED) and 4294 with frozen oocyte replacement (FOR) of the 849811 total cycles completed [25].

2.6.2 Techniques for oocyte cryopreservation

Although some protocols still rely on slow-freezing techniques, mainly for sperm samples, vitrification or ultra-rapid freezing are the most widely used protocols around the world for both oocytes and embryo cryopreservation [83]. A recent systematic review and meta-analysis of the literature provided further evidence of the efficacy of oocyte vitrification compared to the slow method. This study report a higher survival rate when vitrification rather than slow cooling was conducted

[93]. This is due to the decreased risk of damage caused by ice crystal formation [94]. Thus, vitrification transforms the water to a solidified but not crystallized state creating a glasslike system due to rapid cooling rates and the use of high concentration of cryoprotectants [95]. However, this generates such a high level of toxicity that the cells can only be exposed to the vitrification media for a very short period of time in a minimum solution volume (<1 µL) [94].

The most currently used protocol for oocyte vitrification has been described by Kuwayama et al. [81]. It consists of a two-step procedure which involves addition of cryoprotectants in different concentrations. The first step is an equilibration (5 min) of oocytes in a solution with 7.5% v/v ethylene glycol (EG) and 7.5% v/v dimethylsulphoxide (DMSO). Secondly, oocytes are transferred to a vitrification solution containing 15% v/v ethylene glycol and 15% v/v DMSO and 0.5 mol/L of sucrose during 1 min. Finally, oocytes are transferred to the cryopreservation device and plunged in liquid nitrogen (N_2) at −196°C, where the device is stored until use. Warming is done very rapidly to avoid the growth of ice crystals by immersing the device in pre-warmed warming solution which contains a high sucrose concentration (1 mol/L). Over the years, vitrification has been carried out on a variety of cryodevices [96].

2.6.3 Techniques for sperm cryopreservation

Although sperm cryopreservation has shown deleterious effects on morphology and function, the ability to use frozen sperm has been useful for human and animals. In human reproductive medicine, it is now standard practice to cryopreserve all donor semen until tests demonstrate that it is free of HIV and other pathogens [97]. Currently, cryopreservation in liquid nitrogen (N_2) and its vapors is the most common method for long-term preservation of mammalian sperm. However, there are three different approaches: slow equilibrium freezing, rapid non-equilibrium vitrification, and freeze-drying of sperm. The last procedure kill the cells but it is compensated by using intracytoplasmic sperm injection (ICSI) to achieve fertilization, full term pregnancies, and the birth of healthy offspring [97].

Slow freezing is characterized by the use of low concentrations of cryoprotectants compared to rapid freezing techniques. However, the ability of these

cryoprotectants, which are associated with chemical toxicity and osmotic shock, to prevent ice-crystal formation at these low concentrations is compromised [88]. Slow rates of cooling in both spermatozoa and oocyte have been done to maintain the correct balance between the ice crystal formation, osmotic injury, toxic effect of cryoprotectants, and chilling injury in order to prevent cell damages [98,99]. The techniques used in domestic mammal sperm preservation are slow cryopreservation methods which have freezing rate of 0.5–100 ◦C/min [88]. According to Arav et al. heat transfer in sperm cells is too slow to permit vitrification without the risks of solution effects or the crystallization [94]. Thus, slow freezing continues to be the most widely used technique of cryopreservation. All freezing media contains permeable (can enter the cell), and non-permeable (protect the cell extracellularly), cryoprotectants. The combination of both kinds of cryoprotectants minimizes ice formation and helps stabilize cell membranes and proteins. Glycerol, DMSO, EG, methanol and propylene glycol are some examples of permeable cryoprotectants [100]. The most-common non-permeable cryoprotectants are milk and egg yolk proteins, sugars, and high-molecular-weight compounds such as PVP, polyethylene glycols, and dextrans [74]. Different protocols and appropriate use of cryoprotectants during cryopreservation and sperm selection technologies after cryopreservation seem to have the greatest impact on preventing DNA fragmentation and the improving of sperm cryosurvival rates [87].

More recently, freeze-drying or lyophilization techniques for sperm preservation are taking the focus of research in the field of cryopreservation [101]. In fact, lyophilization has been documented as an alternative technology for storing sperm obtaining promising results in terms of blastocyst and live offspring on different animal species as: mice [102], bovine [103], equine [104] and ovine [105]. Although sperm viability and motility are totally compromised after freeze-drying, the sperm chromatin structure is not altered in comparison with fresh samples, which demonstrates that the freeze-drying technique does not affect the DNA integrity [106]. Although freeze-drying of sheep spermatozoa seems to be a very useful technique [105,107], no studies have been found using this technology in goats.

2.7 *In vitro* embryo production (IVEP) on small ruminant

In vitro embryo production (IVEP) of small ruminants: sheep and goats, is an important assisted reproductive technology (ART) aimed at increasing the genetic diffusion of females and improving their genetic qualities by promoting biotechnology studies such as cloning and transgenesis. This technology comprises the following procedures: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *In vitro* culture (IVC) of zygotes up to blastocyst stage [108].

The *in vitro* fertilization process is normally done by conventional IVF. However, the ICSI technique, since its introduction into animals research in the year 1976 [23], seems to be a great fertilization technique really useful on animal research [15]. In fact, this technique in humans ARTs has begun to routinely replace conventional IVF [5]. However, according to Harper et al. previous studies on animals could be really useful to evaluate the safety on the introduction of new techniques to human laboratories [109]. Therefore, the important role that animal research plays in the development of new techniques for its future application in humans seems to be clear.

The process of IVEP and its different steps (IVM, IVF and IVC) are well established in domestic species such as: Cattle, sheep and goat. However, there are some difficulties related to different aspects as the age of the donor, the follicular size or the *in vitro* maturation environments [110]. The juvenile *in vitro* embryo production (JIVET) is presented as a useful technique to reduce the generation interval and increase the rate of genetic gain in animal breeding programs [111]. Nevertheless, it is well known that oocytes from juvenile or prepubertal females, frequently obtained directly from the slaughterhouse and used for IVEP programs, are less capable of producing embryos and offspring to term compared to their adult counterparts. This has been widely demonstrated as well in other non-ruminant species such as the mouse [112]. We found humans on the opposite point: with advanced maternal age being one of the great problems of assisted reproductive techniques in this field [113].

The factors affecting the efficiency of IVEP and JIVET techniques have been widely studied [108,110], being oocyte quality also referred as competence, the

key factor in the success of *in vitro* embryo production programs [19]. Oocyte competence is the ability of the oocyte to resume meiosis, cleave after the fertilization process, develop to the blastocyst stage, induce pregnancy and bring healthy offspring to term [114].

In our group, some of these factors affecting small ruminant IVEP and specially JIVET have been assessed previously. Jimenez-Macedo et al. studied the effect of oocyte diameter on the *in vitro* embryo production of prepubertal goat oocytes using ICSI as fertilization technique [41]. They found a positive correlation between oocyte diameter and embryo development being oocytes with >125 µm more capable of develop up to blastocyst stage. Subsequently, this same group demonstrated that these differences were effectively related to the diameter of the oocytes and not to the effect of the ICSI technique or even another more invasive technique such as embryo biopsy [42].

Catalá et al. pointed out the BCB (Brilliant Cresyl Blue) selection method as an effective and feasible selection of larger and more competent oocytes in lamb. These selected oocytes were more competent to develop to the blastocyst stage than the no selected ones, due to more active mitochondria and MPF factor [115]. Moreover, Catalá et al. demonstrated that ICSI could eliminate these differences found between the embryo production of BCB^{+} (more competent oocytes) and BCB^{-} (less competent oocytes) observed using conventional *in vitro* fertilization in lamb oocytes [116].

Therefore, the study of the factors affecting IVEP can help us improve its rates and introducing the use of specialized techniques as ICSI that could help us to improve the development of ARTs in animals. Moreover, the evaluation of different criteria related to oocyte competence could be a useful tool to predict the oocyte quality and their expected embryo development.

CHAPTER 3

Objectives

The main aim of this work is delve into the *in vitro* production of prepubertal goat by ICSI technique focusing attention on gamete treatments. For that purpose, three specific objectives were addressed:

1. To determine the efficacy of fresh and frozen-thawed sperm samples on ICSI parameters.
2. To study the usefulness of vitrified prepubertal goat oocytes for ICSI procedure and its implications.
3. To evaluate the use of crocetin as antioxidant during IVM of prepubertal goat oocytes to improve their competence prior to ICSI procedure.

CHAPTER 4

Intracytoplasmic sperm injection (ICSI) of prepubertal goat oocytes using fresh and frozen-thawed semen

November 2018; Small Ruminant Research 170

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CHAPTER 5

Effect of vitrification of *in vitro* matured prepubertal goat oocytes on embryo development after parthenogenic activation and intracytoplasmic sperm injection

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CHAPTER 6

Effect of crocetin added to IVM medium for prepubertal goat oocytes on blastocyst outcomes after IVF, intracytoplasmic sperm injection and parthenogenetic activation

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CHAPTER 7

General Discussion

Since the development of the ICSI technique in 1992, its evolution has been very important for both human and animal reproduction laboratories. This technique has multiple advantages over conventional *in vitro* fertilization for some applications. In humans, its main application occurs in cases of infertility of male origin, but it is also very useful for cryopreserved gametes and poor quality oocytes, like aged oocytes.

The low quality of oocytes is one of the main factors affecting *in vitro* embryo production of humans and animals. It is widely known that age alone has a detrimental impact on fertility in women mostly due to the significant increase in aneuploidy and spontaneous abortion rates in line with advanced maternal age [117]. Advanced maternal age is a known cause for oocyte quality degradation and poor IVF outcomes [118]. In animals, both aged and prepubertal females have been related to poor quality oocytes [113]. The research in our laboratory is focused on *in vitro* embryo production of prepubertal goats using ovaries recovered from the slaughterhouse. Hence, oocytes from these ovaries mostly come from small follicles with a low embryo development competence. We consider that these oocytes are a good model for studying female poor quality oocytes and its strategies to improve their embryo development.

According to Farhi et al., [119] ICSI should be considered as the first option in advanced-age patient treatments with non-male factor infertility, specifically for those women aged 35 to 39, in order to avoid fertilization failure and to maximize embryo quality and development. In animals, the use of ICSI has been specially developed in some species where poor results are obtained with conventional IVF due to polyspermia, such as pig [38], or due to the impossibility to obtain repeatable results such as horses [120]. However, in animals like cows [121] and mice [122] it has also been suggested that the application of ICSI can be beneficial in oocytes of poor quality and advanced age. In our laboratory, Catalá et al. showed that ICSI was able to obtain similar blastocyst development (14 vs. 12%) and number of cells per embryo (71 vs. 54) in different quality oocytes selected by BCB: BCB^+ vs. BCB^- groups respectively [116]. These results reinforce the idea that ICSI could be a useful technique in case of poor quality oocytes.

The use of ICSI technique in goats was first reported by Keskintepe et al. [79] using frozen-thawed semen selected by swim up and capacitated with caffeine and heparin. Injected oocytes were artificially activated with calcium ionophore obtaining 62% of cleavage rate and 25% of blastocyst production. Wang et al. obtained the first goat offspring by using ICSI plus piezo pulse technique to break the oolema during the injection [14]. They perform the injection with fresh semen selected by Percoll ® gradient obtaining 89% of cleavage rate and 21% of blastocyst production. More recently, Kharche et al. using fresh semen selected by density gradient and capacitated during 1 hour in Tyrode's Albumin Lactate Pyruvate (TALP) medium with 4mg/ml of bovine serum albumin (BSA) has compared the piezo ICSI protocol with and without subsequent artificial activation of the oocyte by calcium ionophore. Data showed higher cleavage (61 vs. 18%) and blastocyst (16 vs. 4%) rates in the piezo activated group compared with the piezo non activated group [85].

The use of ICSI in prepubertal goats has been previously carried out in our laboratory by Jiménez-Macedo et al. in different studies using fresh semen and swim up technique for sperm selection [40,41]. In the first study, sperm was capacitated in mDM media containing heparin and injected oocytes were artificially activated with ionomicyn plus 6-DMAP obtaining 67% of cleavage rate and 5% of morulae formation but a high rate (53%) of parthenogenetic activated zygotes [40]. The second study demonstrated that the capacitation of the sperm with ionomycin and heparin prior to ICSI without subsequent oocyte activation of injected oocytes increased cleavage and blastocyst rate to 75% and 11% respectively and reduced parthenogenic embryos [41].

Several studies in different livestock species obtained diverse results on blastocyst formation after ICSI too. For example, in cattle, rates ranging from 8% [8] and 23% [123] were reported. The first data of blastocyst production in sheep were around 8% [124]. Later, Shirazi et al. compared the blastocyst production after ICSI of sheep oocytes testing no activation (3.0%), artificial activation with ionomycin (18%) and ionomycin plus DMAP (22%) [125]. There are fewer studies of ICSI in small ruminants, especially in goats, compared to other livestock species. However, this technique can be a good tool for improving results of *in vitro* embryo

production in goats. Moreover, according to Harper et al. previous studies on animals could be useful for the introduction of new techniques and protocols in human reproductive laboratories [109].

The aim of this experimental work has been to test the cryopreservation of gametes (spermatozoa and oocytes) on embryo development after ICSI procedures (Studies 1 and 2). During ICSI procedure, oocytes are exposed to high O_2 concentrations with the result of an increase of radical oxygen species (ROS), which are harmful for the cell. For that reason, the last experiment (Study 3) tested the effect of crocetin, a natural antioxidant, as a supplement of the *in vitro* maturation medium on the embryo development of these oocytes afterwards fertilized by ICSI.

For practical reasons, frozen-thawed semen has been conventionally used for *in vitro* embryo production in livestock species [88]. Thus, the aim of the first study (Study 1) was to test the effect of fresh and frozen-thawed semen on embryo development of prepubertal goat oocytes fertilized by ICSI.

The interest of ICSI for the utilization of cryopreserved gametes has been reported in humans [92]. Moreover, ICSI is the only possible technique to fertilize oocytes with freeze-drying sperm samples. Nagy et al. evaluated the use of different sperm human samples (fresh ejaculated, fresh epididymal, frozen-thawed epididymal and frozen-thawed testicular) for their use on ICSI in a retrospective study. They concluded that good fertilization rates after ICSI can be obtained with fresh or frozen-thawed epididymal and testicular spermatozoa (56, 56 and 48%, respectively) but rates were significantly higher for fresh ejaculated sperm (70%) [29]. Later, Ulug et al. compared the use of fresh ejaculated semen (FS) with frozen-thawed testicular semen (FTTS). They found significant differences on fertilization rates (72% vs. 66%, for FS and FTTS respectively), but no differences were found on the implantation and the pregnancy rates [126].

Fresh and frozen-thawed sperm samples in animals have been studied mainly for artificial insemination (AI). For example, Masoudi et al. compared in sheep the use of fresh and frozen-thawed sperm samples in vaginal, laparoscopic and trans-cervical AI. Regarding the results from laparoscopic insemination they obtained

66% of pregnancy rate with fresh and 42% with frozen-thawed semen [127]. Gomez et al. studied the effect of fresh and frozen-thawed samples and its capacitation on ICSI and IVF outcomes. Fertilization rates after ICSI were higher for fresh than for frozen-thawed sperm (35 vs. 28%, respectively). Less acrosome reacted sperm were found in fresh semen after Percoll centrifugation than in frozen-thawed (13 vs. 33%, respectively). However, they did not find any correlation between the percentages of acrosome-reacted spermatozoa and oocyte fertilization after ICSI concluding that the induction of the acrosome reaction in spermatozoa before ICSI is unnecessary [128]. However, Hashida et al. showed that the plasma and acrosomal membranes of the frozen-thawed sperm were expanded and the mitochondria became irregular in shape which could be responsible for the reduced sperm motility and subsequently decrease in IVF rate compared to fresh sperm [129]. In our study, we also found differences on capacitation and acrosome reaction between fresh and frozen-thawed spermatozoa.

Some authors have related the use of more capacitated and reacted spermatozoa with an improvement on the embryo development rates. In humans, Sathananthan et al. reported that acrosome reaction and deletion are possible prerequisites to allow the sperm incorporation into the oolema after injection [130]. Moreover, using sheep oocytes, Anzalone et al. [131] demonstrated that producing mechanical damage in the sperm plasma membrane and acrosome using piezo-pulsation before ICSI, improves the blastocyst development compared to intact spermatozoa (15 vs. 2%, respectively) contrary to the results obtained by Gomez et al. [128]. In our study, frozen-thawed semen, compared to fresh semen had significantly more reacted acrosome (39 vs. 22%, respectively) and capacitated sperm (65 vs. 35%, respectively). Urner and Sakkas stated that an increase on protein tyrosine phosphorylation is highly related to capacitation, hyper activated motility, zona pellucida binding, acrosome reaction and sperm-oocyte binding and fusion [132]. However, in our first study these differences on capacitation and acrosome reaction parameters were not related to differences on zygote pronuclear formation and embryo development.

In this study, we have obtained 39% and 37% 2PN zygotes for fresh and frozen-thawed groups, respectively. Similarly, in previous studies in our laboratory using prepubertal goat oocytes fertilized by ICSI, the percentages of normal pronuclei formation (2PN) obtained were 38% [40] and 40% [41]. This low percentage of normal zygotes from prepubertal goat oocytes could be explained by the poor quality of these oocytes given that in our laboratory we obtained rates around 43% of normal pronuclei formation after conventional IVF [22]. The high percentage of 3PN formation in both fresh and frozen-thawed sperm samples after ICSI could be due to an incorrect extrusion of the second PB induced by a deterioration of the meiotic spindle during the injection [133,134]. The presence of only one pronucleus could be explained by the incomplete sperm head decondesation or deficiencies on oocyte activation [135]. Moreover, we did not observe differences on embryo development between fresh and frozen-thawed sperm samples regarding cleavage (60 vs. 65% respectively) and blastocyst rates (18 vs. 12%, respectively). However, comparing these results with those previously obtained in our laboratory by Jimenez-Macedo et al. [41] using fresh semen samples, we observed an increase on blastocyst rates from 11 up to 18% that could be partly explained by improvements on maturation, sperm selection and culture media implemented in our laboratory since then. Moreover, in both experiments chemical activation was performed.

In our previous study [41] the fresh sperm samples were selected by swim-up and capacitated with ionomycin and heparin while in the study 1 sperm selection was done by HA-binding, prior to injection. HA-binding is a sperm selection method based on the attachment of the sperm to HA prior to ICSI. It has been proved that this selection reduces DNA fragmentation and chromosomal aberrations of offspring [136]. Thus, we hypothesized that this improvement of blastocyst formation can be also due to the HA binding sperm selection prior to ICSI. However, this accurate selection method has probably hindered our ability to observe significant differences between fresh and frozen semen. Therefore, we concluded that selecting the spermatozoon by HA-binding prior to ICSI has been successful in producing embryos without oocyte activation after injection of prepubertal goat oocytes.

In humans, oocyte cryopreservation and ICSI are important and useful techniques used in reproductive treatments. Vitrification is the conventional technique used nowadays to cryopreserve oocytes [83]. Oocyte cryopreservation is conventionally used in case of oocyte donors, in patients suffering from cancer and, nowadays, it has become a tool that allows the elective fertility preservation of young women [137].

The aim of the second study (Study 2) presented in this thesis was to test the effect of vitrification of *in vitro* matured (IVM) prepubertal goat oocytes fertilized by ICSI on their embryo development. We tried to connect these results with oocyte damage assessed by reactive oxygen species (ROS) level and apoptosis and with embryo development after parthenogenic activation (PA). In this study we have observed impaired oocyte parameters of *in vitro* matured prepubertal goat oocytes after vitrification as it has been observed in other animal species as cattle [84]. It was also proved in mice by Chang et al. [138], concluding that vitrification of *in vivo* matured oocytes induced abnormal ROS generation, increased the apoptotic rate and disrupted early embryonic development.

In our study, vitrified oocytes showed significantly higher levels of ROS compared with non-vitrified IVM oocytes. It is widely known that vitrification increases ROS level in oocytes. For example, in pig oocytes, ROS generation in vitrified oocytes was higher than in not vitrified ones (79 vs. 32%, respectively) with a subsequent decrease of blastocyst development after IVF (8 vs. 23%, respectively) [139]. Zhao et al. comparing vitrified and fresh cattle oocytes, observed that, in addition to high ROS levels, there was an increase of apoptotic parameters such as caspase-3 proteins, more DNA fragmentation and higher percentage of early apoptotic oocytes (assessed by Annexin V) [140]. In our study, we did not observe any direct effect on the percentage of early apoptotic oocytes, but we observed significant lower alive oocytes in vitrified group compared to non-vitrified oocytes (45 vs. 67%, respectively). We concluded that the high fragility of prepubertal goat oocytes lead to a loss of viability on vitrified oocytes causing their death without showing previous apoptotic parameters.

After ICSI, differences in normal pronuclear formation (2 PN) were not found between vitrified and control group (25 and 32%; respectively) and neither in

cleavage rate (56 and 46%; respectively). This high rate of oocyte cleavage compared to normal zygote formation that was observed in this study suggests a strong oocyte activation after the injection and artificial activation with ionomycin procedures during ICSI on prepubertal oocytes. Succu et al. showed that, in sheep, a significant decrease on cleavage and (42 vs. 72%) and embryo formation rates (6 vs. 68%) took place in vitrified oocytes compared to fresh ones [141]. Despite not having observed significant differences in the cleavage rate, we observed that the embryo development outcome was really compromised by the vitrification process, as we did not obtain any blastocyst from vitrified oocytes.

However, using parthenogenic activation, we obtained embryo development but still with significant lower cleavage (59 vs. 78%) and blastocyst (1 vs. 25%) rates in vitrified oocytes comparing to non-vitrified control group. Those results obtained in the control group were comparable to results obtained by PA in other recent studies conducted in our laboratory where cleavage ranged from 75 to 90% and blastocyst formation from 11 to 21% [142,143].

In line with previous publications, we have demonstrated that the vitrification process performed before PA has led to an important reduction of cleavage rates and blastocyst development. Thus, using adult goat oocytes, Begin et al. observed a cleavage rate of 58% followed by 0% of blastocyst development [144]. Srirattana et al. observed the same decrease on cleavage rate (28 vs. 91%) and blastocyst formation (3 vs. 12%) on vitrified oocytes compared to fresh ones [145]. This decrease on blastocyst formation (10 vs. 37%) of vitrified adult goat oocytes has been observed after IVF too [146]. Using sheep model, Hosseini et al. demonstrated that vitrification affects negatively the developmental competence of different groups of oocytes (young and aging) regardless of the procedure used (IVF, ICSI. SCNT, PA) [147]. We have finally concluded that the vitrification process clearly increased ROS on prepubertal IVM goat oocytes but there was not a clear relation between that and the apoptotic level of the oocytes. Prepubertal goat oocytes are fragile and did not survive to the vitrification process leading to no blastocyst formation after ICSI.

The second part of this PhD thesis was focused on improving oocyte competence prior to ICSI through the use of crocetin as antioxidant during the IVM of

prepubertal goat oocytes (Study 3). Different antioxidants have been tested in our lab during IVM with the objective of improving the embryo developmental competence of oocytes from small follicles of prepubertal goats. These were: cysteamine [20], melatonin [22] and resveratrol [21]. These antioxidants have shown to play an important role in protecting oocytes during maturation by different effects such as reducing ROS, increasing GSH, modifying mitochondrial activity or increasing IVEP outcomes. Recently, Aitken has reviewed the importance of antioxidant for the ICSI protocol concluding that the use of antioxidants should be considered to reduce the problems related to high levels of oxidation on sperm and oocytes during the procedure [148]. Crocetin, which is an antioxidant compound obtained from saffron [149], has been recently tested as antioxidant in the culture media of cattle embryos obtaining promising results [150,151].

The aim of the third study was to test the effect of crocetin added to the *in vitro* maturation (IVM) of prepubertal goat oocytes on the embryo development after *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and parthenogenetic activation (PA). Moreover, we assessed molecular and cellular parameters such as ROS, GSH and mitochondrial activity to understand the effect of crocetin as antioxidant on oocytes from prepubertal goat.

First, we evaluated the effect of the IVM supplementation with 0 µM (control), 0.5 µM, 1 µM and 2 µM of crocetin on the blastocyst development after IVF. No significant differences were obtained on blastocyst formation among groups (12, 7, 10, 11%; respectively) therefore, we selected the 1 µM concentration of crocetin to perform the rest of the experiments. Crocetin decreased ROS levels of prepubertal goat oocytes after IVM compared to control group. However, this decrease on ROS level produced by crocetin was not directly related to an increase of GSH levels. According to Chen et al. [152] crocetin protects somatic cells from ROS damage by acting as a direct scavenger of hydroxyl and superoxide anion radicals. The effects of crocetin have been also tested on sperm cells obtaining a decrease of ROS level and lipid peroxidation [153] and on blastocyst where decrease on ROS level was observed too [151]. Mitochondrial activity was not affected by crocetin supplementation during IVM of prepubertal goat oocytes.

However, in our laboratory we have observed that the addition of resveratrol did not affect mitochondrial activity either [21]. In opposition, we observed that the supplementation during IVM with melatonin increases the mitochondrial activity of prepubertal-goat oocytes [154].

In the second part of this study, we compared ICSI and PA on oocyte development after the addition of 1 µM crocetin to the IVM medium. Although this concentration of crocetin during IVM prior to ICSI of prepubertal goat oocytes did not show significant differences on embryo development, it slightly increased the rate of blastocyst (19 vs. 12% P=0.12; crocetin vs. control). No differences were found on embryo development after conventional IVF or PA, or number of cells of the blastocysts obtained, in any of the three procedures.

However, Zullo et al. reported higher blastocyst (grade 1 and 2) rate in cattle between control and 1 µM crocetin group (34 vs. 46%, respectively) using crocetin during *in vitro* culture [150]. Dos Santos et al. confirmed these results obtaining an increased embryo development rate on crocetin (1 µM) group compared to control (39% vs. 26%, respectively). They also found differences in total cell number of the blastocyst obtained from crocetin group compared to control one (115 vs. 95, respectively) [151]. Our study demonstrated that crocetin used during IVM was unable to improve oocyte competence of prepubertal goat oocytes and its only positive effect was to reduce ROS levels. However, we observed interesting results from this study. Control groups of the three different treatments, although not directly compared, obtained similar rates. This suggests that the IVEP outcome with prepubertal goat oocytes depends mainly on the initial oocyte competence instead of the sperm selection or protocol used for fertilization or activation.

In conclusion, using two different sperm procedures during study 1 (HA binding selection without activation) and studies 2 and 3 (PVP selection plus ionomycin activation) we can confirm that the use of frozen-thawed semen selected by PVP and the subsequently artificial activation of the oocytes with ionomycin alone seems to be the most accurate protocol for prepubertal goat obtaining good results of blastocyst formation (12-19%) and any parthenogenetic activated blastocyst derived from ICSI procedure. We have demonstrated that fresh or frozen-thawed

semen can be useful for IVEP without compromising the results. However, oocyte vitrification impairs fertilization by ICSI of prepubertal female oocytes. Therefore, it is not advisable to include the vitrification protocol used in our study with MII-oocytes among the routine techniques used for IVEP in oocytes of prepubertal goats.

The use of antioxidants during IVM in our laboratory has helped us to improve IVEP outcomes. However, they were not tested in combination with ICSI procedure. In our last study (Study 3), although crocetin did not show statistically significant positive effect on embryo development, we agree with the possibility of further use of antioxidants during ICSI in order to improve its outcomes.

CHAPTER 8

General conclusions

1. Fresh semen did not show differences with frozen semen samples regarding their use for ICSI procedure.
2. Vitrification impaired ICSI fertilization of poor-quality oocytes.
3. Crocetin used as antioxidant during IVM of prepubertal goat oocytes slightly improves ICSI outcomes.

CHAPTER 9

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