SPERM PREPARATION AND EGG ACTIVATION FOR BOVINE INTRA-CYTOPLASMIC SPERM INJECTION (ICSI)

by

Judit Gómez Martínez

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LIST OF ABBREVIATIONS

AI	Artificial insemination
AMH	Anti-Müllerian hormone
AOA	Artificial oocyte activation
AR	Acrosome reaction
ART	Advanced reproductive technologies
ATP	Adenosine triphosphate
Ba^{2+}	Barium
BSA	Bovine Serum Albumin
BWW	Biggers-Whitten-Whittingham
cAMP	Cyclic adenosine monophosphate
cRNA	complementary RNA
Ca^{2+}	Calcium
Ca^{2+}_{i}	Intracellular calcium ions
CaIo	Calcium ionophore A23187
CAMKII	Calmodulin-dependent protein kinase II
CB	Cytochalasin B
CHX	Cycloheximide
COCs	Cumulus-oocyte-complexes
CSF	Cytostatic factor
D2	Day 2
D8	Day 8
6-DMAP	6-Dimethylaminopurine
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ER	Endoplasmic reticulum
Eth	Ethanol

ET	Embryo transfer
ExdB	Expanded-blastocyst
ExpB	Expanding-blastocyst
Expt	Experiment
FAO	Food and Agriculture Organization
FAR	Fully acrosome reacted
FCS	Fetal calf serum
Fert	Fertilisation
Fig	Figure
FSH	Follicle stimulating hormone
GAG	Glycosaminoglycan
GSH	L-Glutathione
GV	Germinal vesicle
Hep	Heparin
HB	Hatched-blastocyst
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
Hg^0	Mercury
hpa	hours post-activation
hpi	hours post-insemination
hpm	hours post-maturation
HS	Heparan sulfate
ICM	Inner cell mass
ICMART	International Committee for Monitoring Assisted Reproductive Technologies
ICSI	Intra-Cytoplasmic Sperm Injection
IETS	International embryo transfer society
IGF-1	Insuline-like growth factor 1
IP_3 or $InsP_3$	Inositol 1,4,5-trisphophate

Io	Ionomycin
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
IVP	*In vitro* production
LH	Luteinizing hormone
LPC	Lysophosphatidylcholine
LSD	Least significant differences
MAPK	Mitogen-activated protein kinase
MB	Mid-blastocyst
MβCD	Metyl-β-cyclodextrin
Mg^{2+}	Magnesium
MII	Metaphase II
MPF	M-phase promoting factor
mRNA	Messenger RNA
mSOFaaci	Modified synthetic oviduct fluid medium
N-linked	A nitrogen within an asparagine or arginine side group
NA	Not available
O-linked	An oxygen on a hydroxyl group to a serine, threonine or tyrosine
OPU	Ovum pick-up
P1	Protamine type 1
P2	Protamine type 2
Parth-Diploid	Diploid parthenogenetic group
Parth-Haploid	Haploid parthenogenetic group
Parth-H	Haploid parthenogenetic group
PB	Polar body
PBS	Phosphate buffered saline
Pen/Strep	Penicillin and streptomycin
PFA	Paraformaldehyde

PI	Propidium iodide
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PLCζ	Phospholipase C zeta
PN	Pronuclei
PNA-FITC	Lectin from Peanut Agglutinin-Fluorescein isothiocyanate
PVP	Polyvinylpyrrolidone
R	Receptor
RNA	Ribonucleic acid
S	Slaughterhouse
SACY	Soluble adenyl cylase
SCNT	Somatic cell nuclear transfer
SNAREs	Soluble NSF Apptachment Protein Receptors
Sr^{2+}	Strontium
$SrCl_2$	Strontium chloride
TALP	Tyrode's albumin lactate pyruvate
TE	Trophectoderm
TK	Tyrosine Kinase
ZP	Zona pellucida

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INTRODUCTION

Intra-Cytoplasmic Sperm Injection (ICSI), which consists in the microinjection of a single spermatozoon across the membrane of a metaphase II (MII) oocyte, has been a crucial advanced reproductive technology (ART) in humans to combat the male factor infertility. This accounts for between 25-50% infertility cases which in turn has a global prevalence of around 15%. To date around two million babies have been born worldwide as a consequence of ART (Palermo *et al.*, 2014; Kumar and Singh, 2015; Dyer *et al.*, 2016; Palermo *et al.*, 2017). ICSI bypasses the normal critical step of gamete interaction that occurs during natural conception and thereby avoids sperm anomalies such as motility, concentration or immunity to sperm, thereby increasing the chances of fertilisation (Palermo *et al.*, 1996; Rosenwaks and Pereira, 2017).

Since its accidental discovery by Palermo *et al.* (1992), ICSI has become the first choice for ART in cases of human male infertility, sperm infection with sexually transmitted diseases (HIV, HBV and HCV), low sperm counts or cryopreserved oocytes (Palermo *et al.*, 2017). ICSI now accounts for 67% of total aspiration cycles reported globally by the International Committee for Monitoring Assisted Reproductive Technologies (ICMART) in 2010 with a pregnancy and delivery rate of 26.8% and 20.0% respectively (Dyer *et al.*, 2016; Calhaz-Jorge *et al.*, 2017).

ICSI, however, has not been restricted to humans. In fact, it was performed previously in invertebrates (Hiramoto, 1962) and other mammalian species (Uehara and Yanagimachi, 1976; Iritani *et al.*, 1988; Goto, 1990), with the first ICSI offspring reported in rabbits (Iritani *et al.*, 1988). The main interests to use ICSI in livestock species have been to generate high genetic-value animals, in transgenic livestock production, biodiversity conservation and to solve fertilisation problems in IVF systems (Morozumi and Yanagimachi, 2005; Jo *et al.*, 2014; Arias *et al.*, 2015).

In particular, the cattle industry is interested in increasing the selection of genetic superior females due to their high economic value (Moore and Thatcher, 2006; Espinosa-Cervantes and Córdova-Izquierdo, 2013; Rath *et al.*, 2013). Therefore, sex-sorted semen plays a key role in the dairy industry (Kaimio *et al.*, 2013; Butler *et al.*, 2014). However, as a result of sperm exposure to mechanical and chemical stresses during the flow-cytometric sorting process (Sartori *et al.*, 2004; Garner and Seidel, 2008), sperm concentrations are reduced significantly, together with several detrimental effects on spermatozoa which reduce its life span, alter motility pattern and accelerate the acrosome reaction (Bermejo-Álvarez *et al.*, 2008; García-Roselló *et al.*, 2009; Carvalho *et al.*, 2010). This in turn, lead to ineffective sperm preparation for artificial insemination (AI) or *in vitro* fertilisation (IVF) (Mocé *et al.*, 2006; Xu *et al.*, 2009; Mikkola *et al.*, 2015). ICSI could overcome many of these problems and improve success with the use of sex-sorted semen.

Unlike successful fertilisation achieved by ICSI in humans, the application of this technique has enjoyed limited success in domestic species, including the cow, pig and sheep due to low efficiency for *in vitro* production (IVP) after insemination (Suttner *et al.*, 2000; Oikawa *et al.*, 2005; Galli *et al.*, 2014; Jo *et al.*, 2014; Salamone *et al.*, 2017). There are just few reports of the birth of calves following ICSI in cattle (Goto, 1990; Hamano *et al.*, 1999; Horiuchi *et al.*, 2002; Wei and Fukui, 2002; Galli *et al.*, 2003; Oikawa *et al.*, 2005).

A problem commonly found in the cow is low fertilisation rates and limited embryo development (Jo *et al.*, 2014; Oikawa *et al.*, 2005). It is thought that the main causes of these poor results are related to sperm factors leading to suboptimal egg activation (García-Roselló *et al.*, 2009), delayed sperm nuclear decondensation (Vanden-Meerschaut *et al.*, 2014) and the introduction of hazardous acrosomal content (Katayama *et al.*, 2005; Morozumi and Yanagimachi, 2005; Anzalone *et al.*, 2016). However, lack of an appropriate oocyte activation stimulus and, consequently, a failure to induce appropriate calcium fluctuations following bovine ICSI could interact with oocyte factors, including the initial quality, cytoskeletal damage as a result of micromanipulation process or, indirectly, metabolic competency as a

consequence of maternal nutrition (Leroy *et al.*, 2008a and 2008b; Merton *et al.*, 2009; Hara *et al.*, 2011; Leroy *et al.*, 2011; Galli *et al.*, 2014).

Recent investigations in the field have focused on the development of sperm treatments prior to ICSI based on promoting the availability of paternal genetic material to the oocyte through different mechanisms, together with the application of suitable egg activation protocols in order to enhance pronuclear formation and subsequent, embryo development in cattle (Salamone *et al.*, 2017). Accordingly, the main purpose of the current research was to increase bovine ICSI efficiency by improving sperm pre-treatment and oocyte activation in order to promote the use of sex-sorted semen as an efficient and high valuable breeding technique for the dairy cattle industry.

CHAPTER 1:

Literature review

The current research project investigated the influence of treating bull sperm prior to Intra-Cytoplasmic Sperm Injection (ICSI) on pronuclear formation and subsequent embryo development in cattle. At the same time, it also studied the effect of a novel exogenous stimulus of egg activation and its implications for *in vitro* culture (IVC). For this reason, the current chapter summarises the main processes that take place during fertilisation and early embryo development, including sperm capacitation, acrosome reaction (AR), gamete fusion, egg activation and first mitotic cell cycle. It then briefly describes the current status of ART in breeding livestock, the potential of ICSI in the dairy cattle industry and principal limitations of this technique in the cow compared to other mammalian species.

1.1 FERTILISATION IN MAMMALS

During fertilisation, a sequence of consecutive and complex processes allows sperm-egg fusion that enables the restoration of chromosome number and transmission of recombined genetic material to offspring. These events are highly conserved across many species, including gamete contact and recognition, fusion of the sperm and egg membranes followed by polyspermy block, recombination of the genetic material, and activation of egg metabolism to initiate embryo development (Gadella, 2013; Gilbert and Barresi, 2016).

1.1.1 Capacitation and hyperactivation

Mammalian spermatozoa do not mature completely during the transit from the epididymis. A period of contact and time in the oviduct is required to acquire the ability to recognise and fertilise the egg (Vadnais *et al.*, 2007).

1.1.1.1 Oviduct-sperm interactions

Once ejaculated spermatozoa are deposited in the vagina or directly into the uterine cavity depending on the species (Suarez and Pacey, 2006), initiate a progressive and sequential process of maturation through their physical and molecular interactions with the oviduct; a process known as capacitation (Suarez, 2016).

During the first phase of migration in the female reproductive tract, spermatozoa must deal with several chemical, biological and physical barriers which are designed to select more competent spermatozoa and to reduce the incidence of polyspermy and the risk of microbial infections. These female barriers include acidic pH and high immune responses in the vagina, cervix and uterus (Suarez and Pacey, 2006). Spermatozoa can overcome these barriers in part due to the presence of pH buffers and immune-inhibitors in the seminal plasma together with the sperm surface coating of protective components acquired during epididymal maturation (Suarez and Oliphant, 1982; Dostál *et al.*, 1997). Several studies have confirmed that sperm exposure to low pH provokes a loss of motility in human and bull spermatozoa (Acott and Carr, 1984; Carr *et al.*, 1985; Amaral *et al.*, 2004) and the antibody-antigen reaction at the surface of ejaculated spermatozoa leads to infertility (Menge and Edwards, 1993). Furthermore, viscoelastic cervical mucus and oviduct fluid in some species result in a modification of the swimming speed and bending pattern of sperm due to the significant penetration resistance of these fluids (Jansen, 1978; Jansen and Bajpai, 1982; Suarez *et al.*, 1997; Tung *et al.*, 2014). The narrowness and lumenal complexity of the uterotubal junction also interferes with sperm motility and limits the number of sperm entering the tubal isthmus region where spermatozoa will be held until ovulation (Suarez and Pacey, 2006; Suarez, 2016). The level of complexity varies among species being more complicated in cows, pigs and rabbits due to the presence of mucosal folds compared to humans and rodents (Hook and Hafez, 1968; Wrobel *et al.*, 1993; Suarez and Pacey, 2006).

The lack of synchronisation between sperm arrival and the time of ovulation requires the oviduct to develop a strategy to facilitate fertilisation. This strategy, which involves the formation of a sperm reservoir characterised by the binding of sperm heads with the oviductal epithelial cells in the isthmus, is observed in a number of species including the hamster (Yanagimachi and Chang, 1963), mouse (Suarez, 1987), rabbit (Harper, 1973), cow (Hunter and Wilmut, 1984), pig (Hunter, 1981), sheep (Hunter and Nichol, 1983) and human (Baillie *et al.*, 1997).

According to the literature, physiological function of sperm reservoir has been linked to prolong the sperm life until mating time which may vary between a few hours (human, mice) or more (cattle, sheep and pig) to several days (equine) (Hunter, 2012), maintaining at the same time its ability to fertilise the egg. This would be achieved by keeping the spermatozoa like in quiescent state which included suppression of sperm motility and intact surface membrane preservation, thereby interrupting the completion of capacitation (Suarez and Pacey, 2006; Coy *et al.*, 2012; Hunter, 2012; Suarez, 2016).

Although the sperm reservoir has been observed as a common preserved feature in several mammalian species, several studies have found species-specific differences in the nature of molecules expressed on the sperm surface which are responsible for establishing binding with carbohydrate residues present on the oviductal epithelial cells (Suarez and Pacey, 2006; Talevi and Gualtieri 2010; Coy *et al.*, 2012; Suarez, 2016). For example, sialic acid has been reported as the main mediator of these interactions in hamsters (DeMott *et al.*, 1995), galactose in horses (Dobrinski *et al.*, 1996), fucose in cattle (Ignotz *et al.*, 2001), the simultaneous recognition of galactosyl residues with mannose in pigs (Ekhlasi-Hundrieser *et al.*, 2005) or with N-acetylgalactosamine in llama (Apichela *et al.*, 2010).

Furthermore, it has shown that the sperm reservoir may contribute to the reduction in incidence of polyspermy as a result of the sequential release of a reduced number of spermatozoa (Hunter, 1973; Coy *et al.*, 2012; Gilbert and Barresi, 2016). The mechanisms implicated in this detachment are poorly

understood, however, it is widely accepted that molecular modifications induced in sperm membrane surfaces during capacitation, and hyperactive motility acquired at the end of this process, lead the spermatozoa to decrease its binding affinity for oviductal epithelial cells and its subsequent release (Suarez and Pacey, 2006; Coy *et al.*, 2012). It is also important to highlight the key role that the oviduct plays in sperm detachment directly through the secretion of factors that contribute to modify sperm binding-affinity or, indirectly, by the formation of a suitable environment (pH, oxygen, temperature, ion and hormone levels) which promotes capacitation and hyperactivation at ovulation time (De Lamirande *et al.*, 1997; Fraser, 1998; Suarez and Pacey, 2006; Vadnais *et al.*, 2007).

1.1.1.2 Biochemical changes and motility

Capacitation therefore involves a series of molecular and biochemical changes in the sperm head in parallel with the acquisition of a specific motility pattern (hyperactivation), thereby enabling sperm detachment from the reservoir, resumption of their journey to the ampulla and, finally, binding to the oocyte. This ability to establish bindings and subsequent penetration through zona pellucida appears to be representative of capacitated spermatozoa exclusively (Signorelli *et al.*, 2012; Stival *et al.*, 2016).

Physiological changes associated with this process include an increase in sperm membrane fluidity and display of key lipid-raft domains responsible for zona pellucida binding. This arises as a consequence of cholesterol efflux mediated by albumin proteins from the female reproductive tract (Signorelli *et al.*, 2012; Gilbert and Barresi, 2016). Also variations of certain intracellular ion levels (calcium and bicarbonate, mainly) and the subsequent stimulation of key proteins such as the soluble adenylyl cyclase (SACY) and Protein Kinase A (PKA), increased levels of cAMP, cytoplasmic alkalization, hyperpolarization of the sperm plasma membrane and protein tyrosine phosphorylation (Fig. 1.1) (Parrish *et al.*, 1989; Galantino-Homer *et al.*, 1997; Visconti *et al.*, 1997; Gonçalves *et al.*, 2014; Gilbert and Barresi, 2016; Stival *et al.*, 2016).

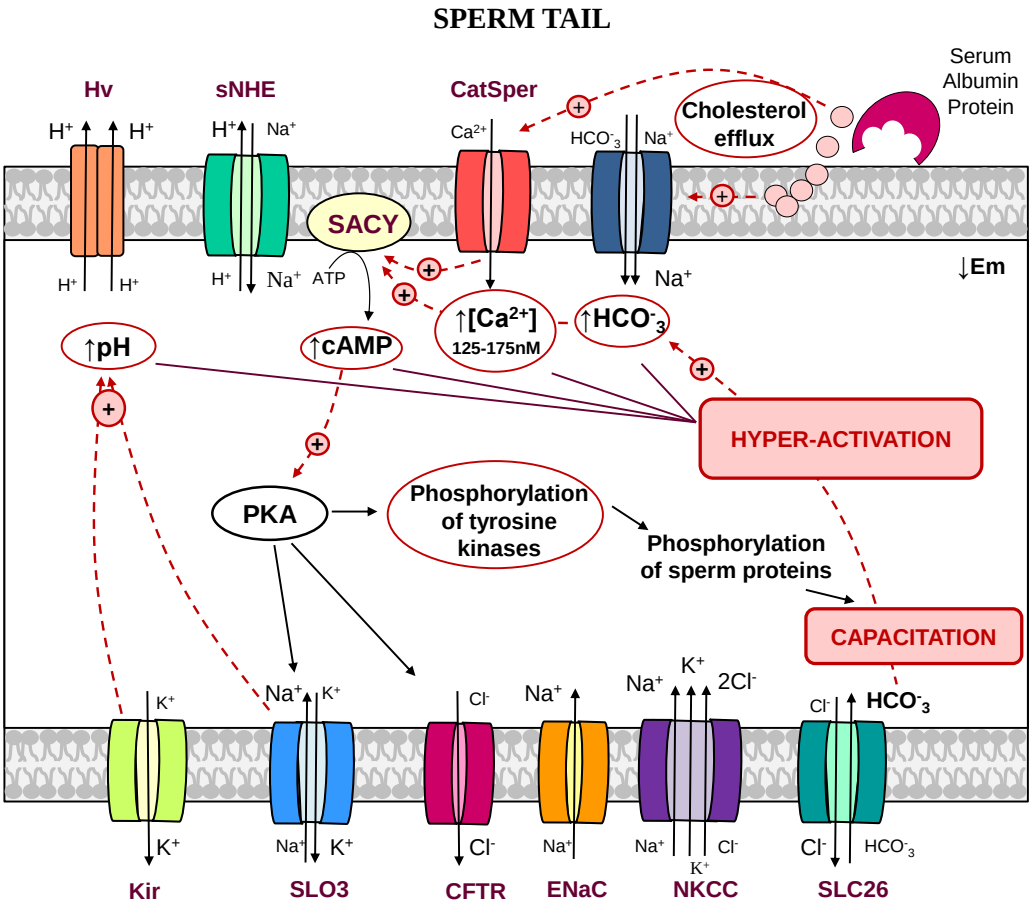


Figure 1.1 Capacitation process in mammalian spermatozoa. Main molecular events that take place into sperm lumen during their transit across oviduct until reach the oocyte in the ampulla.

Hyperactive motility has been characterised as a vigorous swimming with high amplitude and asymmetrical flagellar beating, which sperm acquire by the end of capacitation, and provide the spermatozoa with extraordinary abilities to swim against oviductal barriers, such as binding to epithelium that forms the reservoir (detachment), and mucus observed in tubal lumen and in the vicinity of the oocyte. However, this hyperactive pattern also allows the penetration of egg extracellular matrix (Suarez and Dai, 1992; Quill *et al.*, 2003; Suarez and Pacey, 2006; Coy *et al.*, 2012; Gilbert and Barresi, 2016).

Hyperactivation, therefore, has been included as a part of the final steps of capacitation. However, its regulation seems to be mediated by a different

pathway from that controlling the acrosomal responsiveness (Marquez and Suarez, 2004). It is known that activation of a specific ion channel, named as CatSper, which allows an increase in intracellular calcium levels, is one of the main regulators in the acquisition of this hyperactive motility. In fact, several studies have reported that suppression of CatSper activity was associated with infertility in mice and humans (Darszon *et al.*, 2011; Coy *et al.*, 2012; Signorelli *et al.*, 2012).

Other factors that may also participate in the capacitation process include progesterone (chemotaxis), extracellular pH in the oviductal fluid during estrus, and the presence of an ascendant thermal gradient (thermotaxis) from the isthmus to the ampulla (Darszon *et al.*, 2011; Bahat *et al.*, 2012; Coy *et al.*, 2012; Signorelli *et al.*, 2012; Gilbert and Barresi, 2016). Chemotaxis and thermotaxis are important in mammalian sperm guidance across the oviduct. However, the ability to discriminate between minimal differences in temperature and hormone levels is limited exclusively to capacitated spermatozoa (Ralt *et al.*, 1991; Cohen-Dayag *et al.*, 1995; Eisenbach and Tur-Kaspa, 1999).

1.1.1.3 Capacitation *in vitro*

Sperm capacitation can also be induced *in vitro* by incubating sperm under physiological conditions (temperature, pH, oxygen and carbon dioxide) with nutrients such as bicarbonate, albumin, calcium and energy sources (glucose, pyruvate, lactate) that are present in oviductal fluid for a period of time that varies among mammalian species. For example, while murine, cat and ram ejaculated spermatozoa only need one or two hours to complete all the events involved in the capacitation; bull, rabbit, boar, stallion and human require more than 4 hours (Vadnais *et al.*, 2007).

1.1.2 Sperm-egg interactions

Once capacitated and hyperactive spermatozoa arrive in the vicinity of the oocyte, it must undergo a series of processes to penetrate the zona pellucida. It

achieves this by the extrusion of acrosomal content (acrosome reaction, AR) and expression of new protein markers that enable sperm-oocyte membrane fusion. The sperm factor is then released into the oolemma and this activates the egg, leading to exocytosis of cortical granules which blocks directly the entrance of additional spermatozoa, preventing then polyspermy. Calcium oscillations are induced and these drive the resumption of meiosis and subsequent embryo development (Bi *et al*., 2003; Gadella, 2013; Gonçalves *et al.*, 2014).

Mammalian oocytes are coated by a singular extracellular network of interconnected filaments composed of a few repeated glycoproteins, called zona pellucida (ZP), which is not positioned directly above the oocyte plasma membrane, leading to the presence of perivitelline space (Wassarman *et al.*, 1987; Wassarman and Litscher, 2008; Gadella, 2010).

ZP is highly conserved across mammalian species, indicating it may have an essential function before, during and following fertilisation. In fact, empirical data have reported cases of infertility as a result of female failure in ZP production during oogenesis (Wassarman and Litscher, 2008; Gupta *et al.*, 2012; Gadella, 2013). Nevertheless, the proportion and type of glycoproteins vary among species which contribute to structural differences found in its thickness (7-20 μm), porosity and elastic properties, together with variations in establishment of sperm-egg interactions and binding affinities (Phillip and Shalgi, 1980; Wassarman, 1988; Wassarman, 2008). It is noteworthy to mention, however, the role of the oviduct which appears to have modulating effect on the biochemical and biological properties of the ZP; an effect which seems to be reversible (Coy *et al.*, 2008).

Principal roles associated with the ZP when interacts with spermatozoa, including species-specific sperm selection, induction of the AR and maintenance of acrosome reacted spermatozoa within the matrix in order to enable sperm-oocyte plasma membrane binding. The ZP also minimises polyspermy by acting as a physical barrier. Once cortical reaction has been triggered after the fertilisation of the egg, this extracellular matrix becomes

resistant to proteolytic digestion (ZP hardening), reducing its binding-affinity for other spermatozoa. Lastly, it provides support and protection for the oocytes and embryos during early development (Wassarman, 1999; Gupta *et al.*, 2012).

1.1.2.1 Recognition and penetration of zona pellucida (primary and secondary zona pellucida binding)-Acrosome Reaction (AR)

All mammalian ZP glycoproteins (ZP1-4) possess a common polypeptide (ZP domain), conserved most likely because of its functional relevance in the polymerization and assembly of glycoproteins which constitute ZP (Bork and Sander, 1992; Jovine *et al.*, 2002). These glycoproteins are exposed to heterogeneous glycosylation where oligosaccharide attachment is mediated by a nitrogen within an asparagine or arginine side group (N-linked), or through an oxygen on a hydroxyl group to a serine, threonine or tyrosine (O-linked). These glycoprotein modifications attribute unique features to the ZP, leading to inter-species differences in the sperm-egg binding. It is interesting to highlight the fundamental roles that ZP2 and ZP3 appear to play in gamete recognition and interaction in mammals based on its high degree of preservation (~65-98% identity) (Wassarman and Litscher, 2008).

The murine ZP has been widely studied, and three different glycoproteins designed as ZP1, ZP2 and ZP3 have been identified (Bleil and Wassarman, 1980a). It is used as a referent model to describe functional and structural characteristics of ZP despite that the lack of a fourth ZP (ZP4) identified in humans, other non-human primates, pigs, ruminants, dogs, rabbits and other rodents such as the hamster and rat (Hedrick and Wardrip, 1987; Noguchi *et al.*, 1994; Lefievre *et al.*, 2004; Hoodbhoy *et al.*, 2005; Ganguly *et al.*, 2008; Goudet *et al.*, 2008; Izquierdo-Rico *et al.*, 2009). However, the ZP of pig, cow and dog does not express ZP1, being only composed of three glycoproteins (Z2, ZP3 and ZP4) (Kanai *et al.*, 2008; Gadella, 2010).

In the mouse, heterodimers of ZP2 and ZP3 form long filaments which are interconnected by ZP1 cross-links (Bleil and Wassarman, 1980a). Functional

and descriptive approaches have revealed that the mouse ZP1 seems to be involved in the stabilisation and integrity of ZP to provide the necessary support and protection required during fertilisation and preimplantation embryo development (Gupta *et al*., 2012), while human ZP1 has been linked with the establishment of initial interactions with capacitated spermatozoa and the subsequent AR induction (Ganguly *et al.*, 2010; Gadella, 2013). Nevertheless, the absence of ZP1 in the pig, cow and dog does not obviate the three dimensional formation of ZP from these species. Phylogenetic studies have proposed that ZP1 and ZP4 maybe share a common genetic origin as a result of its sequence similarity that could be reflected in their biological properties too (Goudet *et al.*, 2008).

Concerning mouse ZP3, it has been identified as the putative primary sperm receptor that binds with intact-acrosome capacitated spermatozoa and, in particular, between certain oligosaccharides attached to ZP3 and specific proteins located in the lipid-raft domains of spermatozoa. These domains are exposed in the apical region of its head at some point during capacitation and mediate the induction of the AR following sperm-egg binding (Bleil and Wassarman 1980b, 1983; Beebe *et al*., 1992; Wassarman and Litscher, 2008; Buffone *et al.*, 2009; Gupta *et al.,* 2012). O-linked glycosylation of mannose and galactose seems particularly important for sperm receptor activity, inducing AR in the mouse (Bleil and Wassarman, 1988; Cornwall *et al.*, 1991). In contrast, N-linked glycosylation appears to be particularly important when linked with α-mannosyl residues in the cow (Amari *et al.*, 2001), β-galactosyl residues in the pig (Yonezawa *et al.*, 2005) and several oligosaccharides such as mannose, N-acetyl glucosamine or glycoconjugates in humans (Miranda *et al.*, 1997). Apart from these species-specific differences, primary zona pellucida binding and induction of AR is associated with the simultaneous actions of ZP3 and ZP4 (Chiu *et al.*, 2008; Gupta *et al.*, 2009).

The AR refers to the process of multipoint fusions between outer acrosomal membrane and plasma membrane at the anterior region of sperm head, which leads to extensive generation of vesicles, containing a mix of these two membranes, followed by exposure of inner acrosomal membrane

simultaneously with the progressive release of acrosomal content (hydrolytic enzymes) (Wassarman *et al.*, 2001; Pérez-Aguirreburualde, *et al.*, 2012; Gadella, 2013; Caballero *et al.*, 2014). Evidence suggests that there are also species-specific differences in the type of components present in the acrosome and in the kinetics of its release (Gadella, 2010).

From a biochemical perspective (Fig. 1.2), induction of AR has be linked to a rapid and sustainable intracellular increase in calcium ($Ca^{2+}{}_i$) which varies from 125-175 nM in capacitated spermatozoa to 300-500 nM in AR sperm (Florman, 1994). Apparently, this $Ca^{2+}{}_i$ elevation is mediated by the activation of certain Ca^{2+}-permeable channels as a result of sperm binding to its ZP receptors (Florman, 1994; Fraser, 1998; Wassarman and Litscher, 2008; Darszon *et al.*, 2011). The $Ca^{2+}{}_i$ rise is also accompanied by an increase in pH regulated through G proteins in mammalian spermatozoa (Arnoult *et al.*, 1996), leading to rise cAMP levels and protein activation (phosphatases, kinases (A and C), tyrosine kinase and various phospholipases (A_2, C and D)) (Darszon *et al.*, 1996; Florman *et al.*, 1998).

Early studies suggested that ZP3 was the main natural agonist for acrosome-intact sperm, whose binding would trigger the initial stages of the AR (Bleil and Wassarman, 1983; Florman *et al.*, 1999; O'Toole, 2000). However, subsequent studies have questioned this model when it appeared that cumulus cells induced this initial phase in the mouse; where sperm, retained both penetrating and fertility abilities (Jin *et al.*, 2011). However, perhaps it is not surprising that the AR should be initiated at this point as there is intimate contact between the ZP and cumulus cells (Gadella, 2013).

The initial stages of the AR uncover a new layer of intra-acrosomal proteins, increasing the affinity of the acrosome reacting spermatozoa to the secondary receptor known as ZP2 (secondary zona pellucida binding) (Bleil *et al.*, 1988; Howes *et al.*, 2001; Gadella, 2010; Pérez-Aguirreburualde *et al.*, 2012; Gadella, 2013; Caballero *et al.*, 2014). At the same time, some evidence has suggested that ZP2 may be also involved in facilitating sperm penetration as a

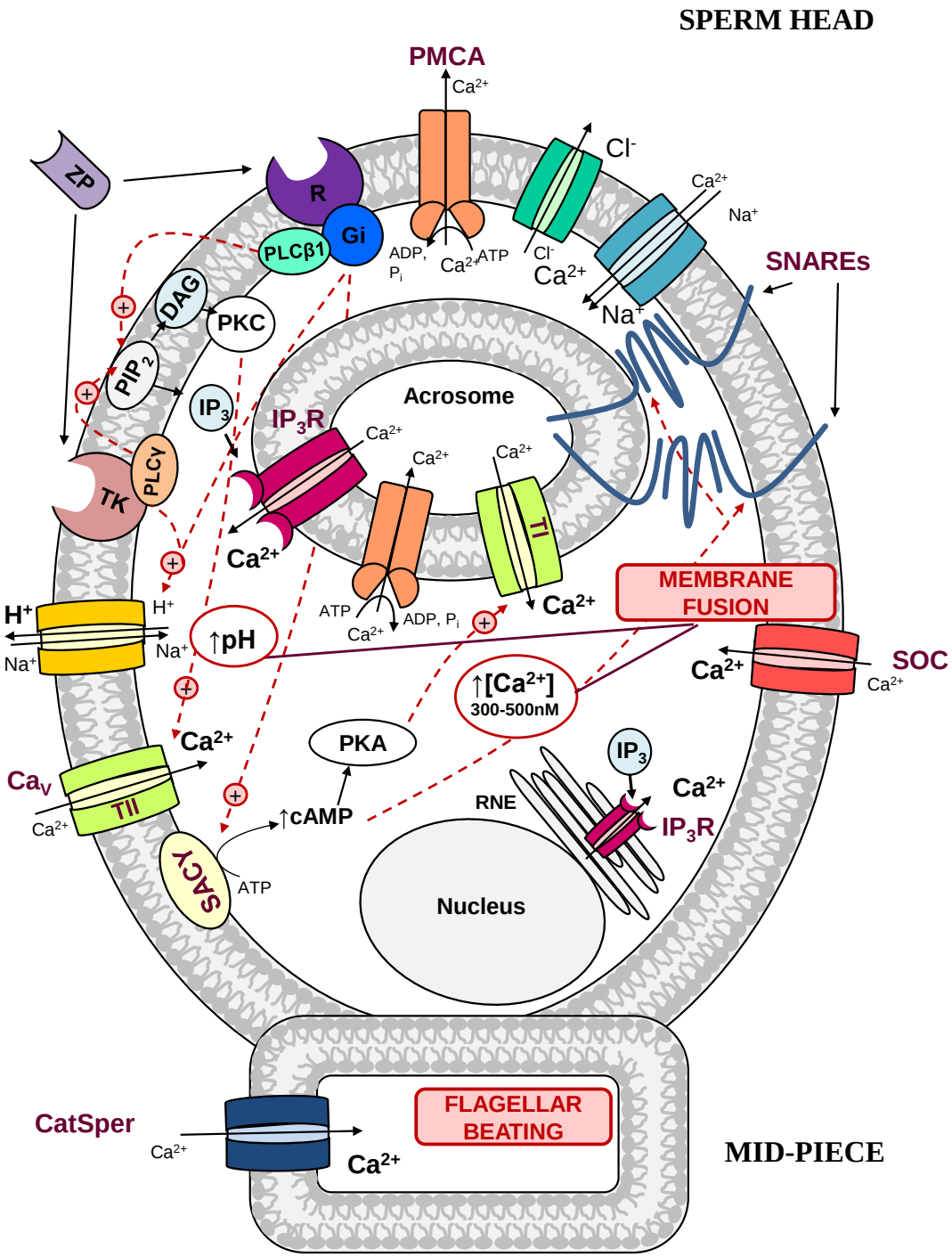


Figure 1.2 Hypothetical signaling model for mammalian sperm acrosome reaction (AR). Interaction of sperm receptors (R= receptor; TK= Tyrosine Kinase) with ZP provokes an increase of cytoplasmic Ca^{2+} levels and alkalization, leading to multipoint fusions between outer acrosomal membrane and plasma membrane by Soluble NSF Apptachment Protein Receptors (SNAREs) and subsequent release of acrosomal content. Ca^{2+} influx and efflux is mediated by production of Inositol 1,4,5-trisphosphate (IP_3) and Diacylglycerol (DAG) and activation of certain Protein Kinases (PKA, PKC).

consequence of the progressive release of hydrolytic acrosomal enzymes which would cleave ZP2 and would allow the sperm to burrow through the ZP in combination with hyperactive motility acquired at the end of capacitation (Moreno *et al.*, 1999; Wassarman *et al.*, 2001; Gupta *et al.*, 2012). The same ZP2 function, linked to secondary zona pellucida binding, appears to be conserved in pig, cow and human (Gadella, 2010).

1.1.2.2 Gamete fusion and polyspermy block

Once the acrosome reacted spermatozoon arrives to the perivitelline space after crossing the ZP, it situates parallel to the oolemma in order to facilitate binding between specific ligands exposed on the sperm equatorial surface and receptors located on the oolemma. This leads to the plasma membrane fusion of both gametes and fertilisation of the egg (Gadella and Evans, 2011; Gadella, 2013).

In the mouse, sperm-oolemma binding seems to be mediated by several proteins but only a few have been described as essential to enable fertilisation. For sperm, it is noteworthy to mention the proteins IZUMO1 and ADAMs, which are also present in human, however, they have not been identified in the cow and pig yet (Inoue *et al.*, 2011; Bromfield and Nixon, 2013). With regards to the oolemma, it is essential the expression of CD9 and CD81 tetraspanins (Rubinstein *et al.*, 2006).

Immediately after the incorporation of sperm into the oocyte (fertilisation fusion), some sperm factors activate a signaling cascade in the egg, leading to the initiation of Ca^{2+} oscillations and depolarization of oocyte membrane which culminates in the induction of the cortical reaction (Gadella, 2013), classified as an important mechanism to block polyspermy. Extrusion of cortical granules appears to modify structural properties of ZPs, named ZP hardening, which reduce their affinity for spermatozoa and prevent future sperm bindings (Gadella and Evans, 2011).

Studies in the mouse have shown that ZP hardening is initiated following the cortical reaction when ZP3 and ZP2 are modified into truncated forms designated as ZP3f and ZP2f, respectively (Gadella, 2010). Nevertheless, in the pig and cow only ZP2 is truncated (Hatanaka *et al.*, 1992; Noguchi *et al*, 1994) and, the remodelling of the ZP following the cortical reaction in these species seems to require the participation of proteases (Hoodbhoy and Talbot, 1994). Other particularities of bovine and porcine ZP hardening are that the loss of affinity in sperm-zona binding appears to be caused by the removal of carbohydrate residues (Velásquez *et al*., 2007; Gadella, 2013). Its efficiency in blocking polyspermy may be affected by the degree of oocyte maturation (Santos *et al.*, 2008; Coy *et al.*, 2008; Canovas *et al.*, 2009). In humans, however, it has been demonstrated that ZP hardening does not alter the biological activities of ZP, such as sperm-binding affinity and induction of voltage-dependent calcium influx but, as a result of this process, the ZP becomes impenetrable in a similar way to other mammalian species (Patrat *et al.*, 2006).

Apart from exocytosis of cortical granules implicated in reduction of polyspermy, the oocyte itself could act as a block, preventing sperm entrance. Apparently, the depolarization suffered by the oocyte membrane after sperm fusion, together with the secretion of cortical granules to the perivitelline space, modify oocyte receptivity, reducing its affinity for spermatozoa (Gadella and Evans, 2011). Numerous spermatozoa found in the pervitelline space in many mammalian species have been considered as proof of the efficiency of this mechanism prevention of polyspermy. Therefore, mammals could regulate sperm-egg interactions mainly through these two prevention mechanisms, i.e. membrane and ZP block. However, in some species such as the rabbit, pika and pocket gopher, where more than one hundred sperm have been observed in the perivitelline space, the membrane block seems to be more developed than the ZP block. In the human, mouse, cow and certain other species both appear to be used equally; with only a few sperm observed in the perivitelline space (Hunter, 1990; Sengoku *et al.*, 1995; Hunter *et al.*, 1998). Finally, the oviduct also plays a role in regulating polyspermy through specific glycoproteins; however, further research is needed in this field (Coy *et al.*, 2012).

1.1.3 Egg activation

After sperm fusion, mammalian oocytes which are arrested at Metaphase II (MII) undergo a sequence of cellular changes which enable them to exit from MII, complete meiosis and undergo embryo development (Alberio *et al.*, 2001; Fissore *et al.*, 2002; Urnov and Wolffe, 2002). These cellular processes are known as "oocyte activation" and Schultz and Kopf (1995) classified them into two categories: (i) early events, which are maturational stage-dependent and are promoted by a Ca^{2+}_i increase, including cortical granule exocytosis, recruitment of maternal mRNAs and resumption of meiosis with extrusion of the second polar body, and (ii) late events, which are affected by the sperm's ability to induce long-term Ca^{2+} oscillations in order to assure the formation of male and female pronuclei, DNA synthesis and the first meiotic cleavage (Fig. 1.3) (Schultz and Kopf, 1995; Fissore *et al.*, 2002).

1.1.3.1 Induction of calcium oscillations

It is widely accepted that the immediate series of transitory Ca^{2+}_i increases registered in the mammalian oocyte shortly after sperm entry, and required for egg activation, appears to be induced by the release of a soluble sperm factor which diffuses into the egg cytoplasm (Stricker, 1999; Nomikos *et al.*, 2013). These Ca^{2+} fluctuations define a characteristic oscillatory pattern which seems to be essential to ensure genetically competent embryo development up to and beyond implantation (Ducibella *et al.*, 2002; Ozil *et al.*, 2005; Rogers *et al.*, 2006; Ozil *et al.*, 2006; Ross *et al.*, 2009). In mammals, cytosolic Ca^{2+} oscillations are generated by a similar pathway to that observed in somatic cells. It consists of an increase in inositol-1,4,5-trisphosphate ($InsP_3$) production through the hydrolysis of phospholipid phosphatidylinositol bisphosphate (PIP_2) (Fig. 1.3) (Fissore *et al.*, 1995; Malcuit *et al.*, 2005; Malcuit *et al.*, 2006a). The pattern (oscillation frequency) of this series of Ca^{2+} transients is determined by the sperm and its duration can vary considerably among species (Nomikos *et al.*, 2013; Sanders and Swann, 2016). However, it should be noted that recent findings indicate that cytosolic Ca^{2+} levels may play a more relevant role in setting the pace of these oscillations than the

release of internal Ca^{2+} stores. They would be involved in the simultaneous regulation of $InsP_3$ receptors and sperm factor activities (Sanders *et al.*, 2018).

Phospholipase C zeta (PLCζ) has been proposed to be the most likely candidate to be the physiological sperm factor, based on the observation that its presence in eggs induces Ca^{2+} release from internal stores via increased production of $InsP_3$ (Jones *et al.*, 1998; Rice *et al.*, 2000). PLCζ is expressed as RNA in spermatids and is present as a protein in mature sperm (Saunders *et al.*, 2002). Microinjection of either recombinant PLCζ protein or complementary RNA (cRNA) causes prolonged Ca^{2+} oscillations with a similar pattern to that observed at fertilisation and this effectively activates oocytes from different mammalian species (i.e. mouse, human, pig, cow and horse), leading to subsequent embryo development (Rogers *et al.*, 2004; Swann *et al.*, 2006; Bedford-Guaus *et al.*, 2008; Ross *et al.*, 2008, 2009; Bedford-Guaus *et al.*, 2011; Sato *et al.*, 2013; Yoneda and Watanabe, 2015). Homologous PLCζ protein sequences in non-mammalian species, such as chickens and medaka fish also activate oocytes (Coward *et al.*, 2005; Ito *et al.*, 2008). Finally, immunoprecipitation studies have demonstrated that removal of PLCζ from sperm extracts is associated with a depletion of sperm-extract ability to initiate Ca^{2+} oscillations or hydrolyse PIP_2 (Escoffier *et al.*, 2015).

Although PLCζ is a derived phospholipase C isozyme, several peculiarities distinguish it such as its high sensitivity to Ca^{2+}_i, being stimulated its activity at very low Ca^{2+} concentrations (Kouchi *et al.*, 2004; Nomikos *et al.*, 2005), is only active in eggs (Phillips *et al.*, 2011) and its unusual localization on small vesicles spread across all cytoplasmic egg (Yu *et al.*, 2012). All these features of PLCζ present strong arguments in favour of this PLC isozyme as the possible sperm factor, and lead to a hypothetical model as to how it would activate the egg.

Apparently, PLCζ can diffuse into the ooplasm after sperm-egg fusion, causing an increase in the amount of $InsP_3$ after hydrolysis of PIP_2 from these small cytoplasmic vesicles, rather than from the plasma membrane (Yu *et al.*, 2008a; Yu *et al.*, 2012). Once $InsP_3$ binds to its receptor in the endoplasmic reticulum

(ER), it promotes a Ca^{2+} influx into the cytoplasm, which generates Ca^{2+} oscillations. It has been shown that the time which it takes to generate initial Ca^{2+} transients after fertilisation, and the frequency of those oscillations, may be related to the degree of solubility and amount of PLCζ, respectively, which also varies among species (Kashir *et al*., 2014; Swann and Lai, 2015; Sanders and Swann, 2016). It is noteworthy to mention that PLCζ quantity apparently is proportional to oocyte volume (Swann and Lai, 2016), and the microinjection of excessive PLCζ amounts have been demonstrated to be detrimental for embryo development (Ross *et al.*, 2008; Yu *et al.*, 2008b).

PLCζ localization also appears to vary among species. For example, in sperm from mouse, hamster and boar, it seems to be localized in acrosomal and post-acrosomal regions whereas, in humans it has been also found on the equatorial region (Nomikos *et al.*, 2013; Sanders and Swann, 2016). In cattle, however, PLCζ has been identified only on equatorial sperm portion (Yoon and Fissore, 2007; Ross *et al.*, 2008). The available evidence suggests that these variations could be related to differential functional roles for each species (Cooney *et al.*, 2010).

Nevertheless, the generation of Ca^{2+} oscillations is a multivariable process where not only PLCζ plays a key role. In fact, last findings point the existence of an alternative and independence mechanism to PLCζ or a secondary sperm factor involvement to activate the mammalian oocytes (Hachem *et al.*, 2017; Nozawa *et al.*, 2018), however, further research is required. Additionally, oocyte also needs to acquire the ability to respond properly to this sperm factor. This appears to be highly influenced by the extent of cytoplasmic and nuclear maturation of the oocyte (Swain and Pool, 2008). ER reorganization, number and sensitivity of $InsP_3$ receptors, $Ca^{2+}{}_i$ concentration stored in ER and redistribution of calcium-binding ER proteins, are all relevant variables whose involvement seems to be necessary for successful fertilisation (Ajduk *et al.*, 2008; Goud *et al.*, 1999, 2002; Vanderheyden *et al.*, 2009).

1.1.3.2 Early and late events associated with calcium oscillations

At fertilisation, generation of Ca^{2+} oscillations requires a series of biochemical changes, inducing the activation of key cytosolic and mitochondrial proteins involved in cell cycle regulation, allowing meiosis to resume (Whitaker and Patel, 1990). Meiotic arrest is maintained by high levels of both M-phase promoting factor (MPF) and the proteins responsible of its stabilization such as mitogen-activated protein kinase (MAPK) or mos proto-oncoprotein (Mos) which are found as a complex named cytostatic factor (CSF) (Sagata *et al.*, 1989; Haccard *et al.*, 1993; Kubiak *et al.*, 1993). Therefore, meiosis resumption requires a reduction of MPF activity which seems to be caused by calmodulin-dependent protein kinase II (CAMKII) whose stimulation has been associated with $Ca^{2+}{}_i$ oscillations (Markoulaki *et al.*, 2004). Single Ca^{2+} rise temporarily reduces MPF levels but prolonged $Ca^{2+}{}_i$ fluctuations are needed to maintain this low activity (Tatone *et al.*, 2002), enabling the completion of meiosis.

Simultaneously, Ca^{2+}oscillations also result in the induction of the cortical reaction, described in previous section (1.1.2.2). This apparently occurs through a different signaling pathway than that involved in meiosis resumption (Sanders and Swann, 2016). Moreover, other relevant aspect concerning Ca^{2+} oscillations is that the number of transients would be decisive for the initiation of molecular, structural and genetic events which lead to embryo transition (Wakai *et al.*, 2011; Armant, 2015). For instance, it has been reported in the mouse that while cortical reaction is triggered immediately after the first $Ca^{2+}{}_i$ rise, exit from meiotic arrest, polar body extrusion and -de novo maternal protein biosynthesis and pronuclear formation requires 4, 8 and 24 oscillations, respectively (Fig. 1.3) (Ducibella *et al.*, 2002; Gonzalez-García *et al.*, 2014).

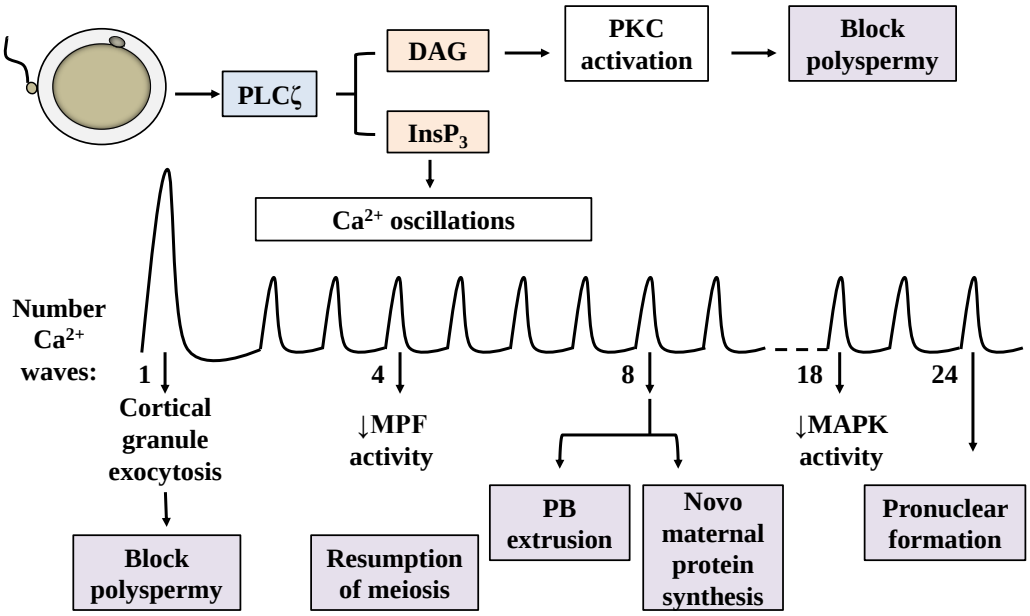


Figure 1.3 Diagram of mouse egg activation following gamete fusion. Once sperm factor protein (PLCζ) is released into ooplasm hydrolyses PIP_2 from small cytoplasmic vesicles leading to DAG and $InsP_3$. An increase in $InsP_3$ levels evokes influx of Ca^{2+} from internal stores, drawing a specific Ca^{2+} pattern. The number of Ca^{2+} oscillations is crucial to activate sequentially all events of egg activation. While the other product of PIP_2 hydrolysis, DAG, is involved in blocking polyspermy by stimulation of Protein Kinase C (PKC).

Nevertheless, implications of $Ca^{2+}{}_i$ go even further. $Ca^{2+}{}_i$ signaling seems to be involved in key pathways during the different embryo stages during preimplantation development (Armant, 2015). This would include the initiation of compaction (Pey *et al.*, 1998), stimulation of cavitation, influencing cell number at blastocyst stage (Stachecki and Armant, 1996), modulation of embryo-endometrium invasion during implantation (Carson *et al.*, 2000; Hynes, 2002; Wang *et al.*, 2002), active participation in *de novo* gene expression (Ashby and Tepikin, 2002), and formation of many growth factors and cytokines secreted by blastocysts to ensure a successful embryo implantation (O'Neill, 1997, 1998).

1.2 ART IN FARM ANIMAL BREEDING

Nowadays, livestock is in high demand and one of the fastest growing sectors of agricultural economy, representing 40% of the global value of agricultural output, supplying the food needs of almost a 1.3 billion people according with the Food and Agriculture Organization (FAO) of the United Nations. It is therefore essential to maximise the productivity of this industry. Advances in agricultural research together with the development of innovative biotechnologies will play a crucial role going forward (Verma *et al.*, 2012). These biotechnologies, known as Advanced Reproductive Technologies (ART), are focused mainly on improving reproductive efficiency, which is the biggest cause of economic loss in this sector. However, ART provide important tools to improve genetic selection and alter the sex for dairy production systems. They take the form of artificial insemination (AI), *in vitro* embryo production (IVP), embryo cryopreservation, sexing of semen and embryos, cloning by somatic cell nuclear transfer (SCNT) and embryo transfer (ET) (Moore and Thatcher, 2006; Urrego *et al.*, 2014).

1.2.1 AI and IVP

In recent decades, AI has made a significant contribution to animal breeding, being extensively used as the preferred ART with over 100 million of cattle AIs per year (approximately 80% in Europe and North America), followed by 40 million in pigs (more than 90% in Europe and North America), 3.3 million in sheep and 0.5 million in goats. Major benefits associated with the use of AI are increased rates of genetic improvement, which facilitates the exploitation highly valuable males, enable breeding between animals with spatial-temporal with reduction in sexually transmitted diseases (Morrell, 2011; Verma *et al.*, 2012).

There have also been progressive improvements in *in vitro* culture conditions that enabled *in vitro* maturation (IVM) of oocytes, together with the development of non-invasive transvaginal techniques for ovarian follicular aspiration (ovum pick-up; OPU) which allows direct retrieval of oocytes from

growing follicles from living donor female. Following *in vitro* fertilisation (IVF), these techniques lead to large production of transferable embryos (Fig. 1.4) of high genetic merits. These contribute to accelerating to dissemination of superior genetic traits (Moore and Thatcher, 2006). Moreover, possible combination of IVP with other emerging technologies (sex-sorted semen, discussed next), enables sex selection and further enhances the value of these technologies (Boa-Amponsem and Minozzi, 2006; Verma *et al.*, 2012).

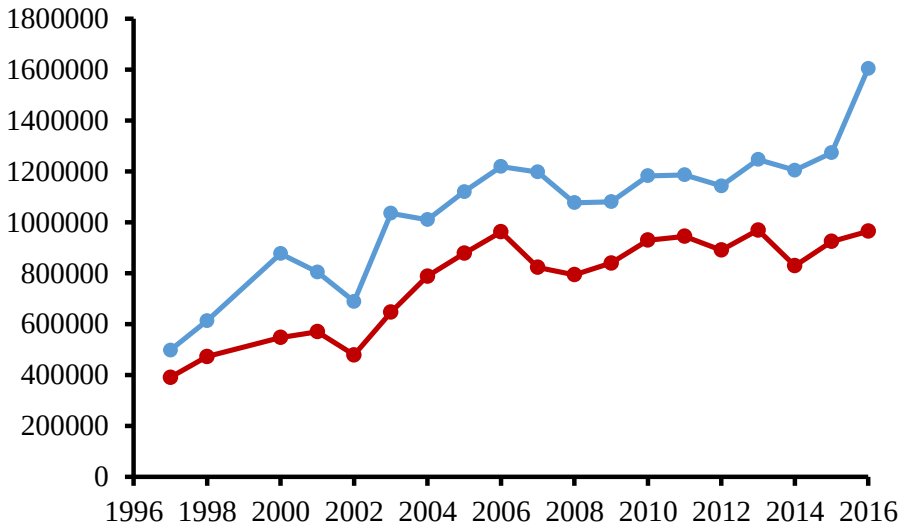


Figure 1.4 Collected (—●—) and transferred (—●—) embryos produced by AI and IVF in the global cattle industry over the past two decades. Data collected from IETS Statistics 2016 (Perry, 2018).

1.2.1.1 Use of sex-sorted semen

Fertilisation of oocytes with sex-sorted spermatozoa constitutes one of reproductive strategies performed in animal production systems to produce sexed embryos, leading to the control of optimal proportions of males and females. Its use is particularly advantageous for the cattle industry due to its economic benefits (Rath *et al.*, 2013; Butler *et al.*, 2014). Replacement females are the limiting factor in the dairy cattle breeding, so are in greater demand. In contrast, males are usually more desirable for beef production (Espinosa-Cervantes and Córdova-Izquierdo, 2013).

Currently, the only commercially viable method for producing sex-sorted spermatozoa in mammals is by flow cytometry based on the detection of small differences in DNA content between X- and Y-sperm chromosomes (3.8% in cattle); which can be undertaken with high accuracy (~90%) (Xu *et al.*, 2009; Kaimio *et al.*, 2013; Butler *et al.*, 2014; Xu, 2014). This powerful tool has been successfully demonstrated in many species, including rabbits (Johnson *et al.*,1989), sheep (Evans *et al.*, 2004), pigs (Rath *et al.*, 1997), horses (Lindsey *et al.*, 2002) and cattle (Seidel *et al.*, 1999).

Nevertheless, the sorting process has been associated with several negative implications such as slow speed of sorting (Raph *et al.*, 2013), low number of sexed spermatozoa per straw (Garner and Seidel, 2008) and sperm damage including altered motility patterns, a reduced life span, acceleration of the acrosome reaction, and increases in the proportion of capacitated sperm as a result of sperm exposure to chemical and mechanical stresses during the sorting process (Bermejo-Álvarez *et al.*, 2008; Carvalho *et al.*, 2010). Consequently, reduced fertilisation success and poor embryo development have been reported following AI or IVF with sexed semen compared to non-sorted sperm (Sartori *et al.*, 2004; Mocé *et al.*, 2006; Butler *et al.*, 2014; Mikkola *et al.*, 2015). Therefore, the high cost and inefficiency of sorting process seems to compromise the wide application of sexed semen in livestock breeding, especially when it is combined with AI or IVF.

1.2.1.2 Intra-Cytoplasmic Sperm Injection (ICSI)

ICSI classified as a part of ART has been a revolutionary advance in the reproduction field, in particular for human fertility treatment where it is employed as the first choice in cases of male infertility (Kohda *et al.*, 2011). In the livestock industry, the main interest is the production of genetically superior animals of the desired sex, biodiversity conservation or as an alternative to overcome problems associated with IVF following the use of sex-sorted semen (Morozumi and Yanagimachi, 2005; Jo *et al.*, 2014; Arias *et al.*, 2015).

Since the first performance of ICSI in mammals by Uehara and Yanagimachi in 1976, its application has been reported in several species such as mice (Kimura and Yanagimachi, 1995), rabbits (Hosoi *et al.*, 1988), cattle (Goto, 1990), sheep (Catt *et al.*, 1996), non-human primates (Chan *et al.*, 2000), humans (Palermo *et al.*, 1992), horses (Cochran *et al.*, 1998), pigs (Martin, 2000) and cats (Pope *et al.*, 1998). In contrast to the wide and successful use of ICSI in mice and humans, this technique has enjoyed limited success in the majority of domestic animals as a consequence of low success of fertilisation and poor embryo development, except horses (Suttner *et al.*, 2000; Oikawa *et al.*, 2005; Galli *et al.*, 2014; Arias *et al.*, 2014; Jo *et al.*, 2014; Salamone *et al.*, 2017).

For these reasons, initial interest in bovine and ovine ICSI was mainly limited to research. The potential benefits of using sex-sorted semen and further improvements in instrumentation, however, have rekindled interest in this technique in domestic species, particularly cattle (Garner and Seidel, 2008; García-Roselló *et al.*, 2009). In contrast, porcine ICSI has been restricted basically to research purposes whose principal aims related to the study of genetic modifications in farm animals (Garcia-Vazquez *et al.*, 2010), xenotransplants for future human biomedical application (Niemann *et al.*, 2005) and, basic science into the role of human spermatozoa within the oocyte (Canovas *et al.*, 2007). In the case of the horse, however, all efforts have focused on the development of optimal and efficient protocols for ICSI as it is the only viable option of ART for the horse-breeding industry as IVF remains challenging (Palmer *et al.*, 1991; Galli *et al.*, 2014; Salamone *et al.*, 2017). Currently, ICSI in the horse is considered a key ART for commercial breeding programmes (Hinrichs, 2005).

1.3 LIMITATIONS OF ICSI IN DIFFERENT SPECIES

Low efficiency of bovine ICSI is linked to a failure in pronuclei formation as a result of bull sperm inability to trigger repeated Ca^{2+} oscillations responsible

for ensuring proper oocyte activation; but also difficulty sperm chromatin decondensation (Rho *et al.*, 1998a; Chung *et al.*, 2000; Malcuit *et al.*, 2006b; Arias *et al.*, 2015). In sheep, the main problem related to ICSI relates to problems in the early embryo development which is compromised by Day 3-4 (between 8 to 16 blastomeres) when the majority of ICSI embryos arrest (Gómez *et al.*, 1998a). However, principal reasons involved in the limited ICSI success in porcine seems to relate to oocyte maturation. There are still no optimal IVM protocols for pig oocytes that would enable them to acquire full competency to be fertilised and respond to the sperm factor. Moreover, porcine embryo culture systems remain suboptimal, leading to low blastocyst development (García-Roselló *et al.*, 2009; Li *et al.*, 2013; Nakai *et al.*, 2016b; Salamone *et al.*, 2017).

These variations in ICSI efficiency among species (Table 1.1) require ICSI protocols to be tailored to each. However, technical challenges with ICSI itself, the development of adequate IVP systems that enable optimal IVM and embryo culture, together with the source of oocytes and spermatozoa which are affected by animal breed, climate and nutrition should also been taken into account (Leroy *et al.*, 2008a; Leroy *et al.*, 2008b; García-Roselló *et al.*, 2009; Leroy *et al.*, 2011; Salamone *et al.*, 2017).

1.3.1 Sperm factors

The major factors affect negatively fertilisation and embryo development following ICSI include suboptimal egg activation, delayed sperm nuclear decondensation and introduction of hazardous acrosomal content (Katayama *et al.*, 2005; Morozumi and Yanagimachi, 2005; García-Roselló *et al.*, 2009; Vanden-Meerschaut *et al.*, 2014). However, inherent sperm features that characterise each species such as the gamete size, resistance capacity of sperm membranes and protamine type may be play a critical role in ICSI success (Table 1.1).

Tabla 1.1 Features of gametes from several mammalian species [a].

	OOCYTE FEATURES					SPERM FEATURES			
	IVM time (h)	Diameter (µm)	Lipid content (ng)	Egg activation	Sperm:oocyte ratio (volume)	Length and width sperm head (µm)	PLCζ amount (fg)[b]	PLCζ localisation[b]	Protamine type (P2/P1 ratio)[b]
Mouse	14	~ 72	2	--	$2.57 \cdot 10^{-4}$	L≈ 7.4 W≈ 3.6	20-50		P1-P2 (2:1)
Pig	40-44	~105	160	X	$1.94 \cdot 10^{-4}$	L≈ 9 W≈ 5	350		P1
Horse	28-30	110-120	NA	--	$0.49 \cdot 10^{-4}$	L≈ 6.5 W≈ 3.4	Orthologue		P1-P2 (NA)
Cattle	20-24	110-130	60	X	$1.16 \cdot 10^{-4}$	L≈ 9.1 W≈ 4.7	105-165		P1
Human	24-40	~120	20-30	--	$0.27 \cdot 10^{-4}$	L≈ 4.6 W≈ 3.2	Orthologue		P1-P2 (1:1)
Sheep	26-27	150-180	90	X	$0.29 \cdot 10^{-4}$	L≈ 8.1 W≈ 4	NA	NA	P1

[a] Information collected by Hirao *et al.*, 1994; Goudet *et al.*, 1997; Otoi et al., 1997; Corzett *et al.*, 2002; Dell'Aquila *et al.*, 2003; Garner, 2006; Griffin *et al.*, 2006; Balhorn, 2007; Yoon and Fissore, 2007; Sturmey *et al.*, 2009; Cooney *et al.*, 2010; Nakai *et al.*, 2011; Varea-Sánchez *et al.*, 2013; Santos *et al.*, 2014

[b] NA (Not Available)

1.3.1.1 Introduction of sperm membranes into oosplasm

In contrast to what happens *in vivo*, during ICSI, the sperm plasma membrane as well as the acrosome are introduced into the oocyte. These sperm components may interfere with the adequate release and rapid diffusion of PLCζ across the oosplasm which could lead to incomplete oocyte activation, with subsequent failure in the generation of transient and persistent Ca^{2+}_{i} rises (García-Roselló *et al.*, 2009; Vanden-Meerschaut *et al.*, 2014). Moreover, several investigations have revealed that acrosomal content could be potentially hazardous to embryo development (Morozumi and Yanagimachi, 2005; García-Roselló *et al.*, 2009; Kashir *et al.*, 2014); and removal of both plasma and acrosomal sperm membranes before ICSI improves fertilisation rates and embryo development in the pig (Katayama *et al.*, 2005), mouse (Morozumi *et al.*, 2006) and sheep (Anzalone *et al.*, 2016). These factors could account for low success, especially in cattle because bull spermatozoa have the largest head size of any domestic species (Table 1.2).

Tabla 1.2 Spermatozoa head dimensions in several mammalian species.

	Bull	Boar	Ram	Rabbit	Cat	Mouse	Dog	Horse	Man
									
Length (µm)	9.1	9.0	8.1	7.7	7.7	7.4	7.0	6.5	4.6
Width (µm)	4.7	5.0	4.0	4.5	3.2	3.6	3.5	3.4	3.2
Area (μm^2)	34.5	37.5	26.6	28.0	19.0	21.6	20.9	15.2	10.8

Adaptation from Garner, 2006.

1.3.1.2 Sperm DNA decondensation

The initial phase of sperm chromatin remodelling consists of protamine replacement by maternal histones in order to reduce the level of DNA

compaction (Swain and Pool, 2008). Upon gamete fusion, sperm nuclear content is released into the egg cytoplasm where is easily accessible to cytoplasmic oocyte factors responsible for the reduction of protamine disulphide bonds, an indispensable prerequisite for the initiation of sperm DNA decondensation (Perreault *et al.*, 1984; Perreault *et al.*, 1987; Luberda, 2005; Julianelli *et al.*, 2012). There is strong evidence that indicates that part of the oocyte's ability to process sperm components and remodel its genetic material leading to pronuclei stage relates to its maturity. The other factor relates to egg activation and with the peculiarity that this capacity appears to last for only a limited period of time after gamete fusion (Usui and Yanagimachi, 1976; Perreault *et al.*, 1988; McLay *et al.*, 2002; Swain and Pool, 2008).

ICSI bypasses gamete fusion and therefore avoids the removal of sperm membranes. This could delay sperm decondensation as a consequence of an additional step of permeability necessary to enable the interaction between the sperm nucleus and oocyte-derived factors involved in this process and, ultimately, lead to fertilisation failure (Goud *et al.*, 1998). Actually, it has been shown that microinjection of spermatozoa in the absence of its nuclear membranes increases sperm decondensation rate in the hamster (Perreault *et al.*, 1987; Yanagida *et al.*, 1991).

1.3.1.3 Other factors related to ICSI efficiency

Sperm head dimension has a particular relevance in ICSI, especially for species with the largest head size as is the case in domestic farm animals (Table. 1.2). Because of the need to use microinjection needles with a higher inner diameter (8-10 μm) than commercial pipettes employed routinely in human fertility clinics (4-6 μm), increasing the probability of oocyte damage during penetration and breakage of oolemma. Therefore, it was introduced an alternative microinjecting system known as piezo-drill which is based on the generation of ultrasonic mechanical pulses for puncture a hole in the ZP and break the oolemma, being less traumatic than conventional ICSI procedure. The improvement of ICSI success after the application of this microinjecting adaptation has been reported in several species such as mouse, horse and cow

(Choi *et al.*, 2002; Wei and Fukui, 2002; Galli *et al.*, 2003; Yoshida and Perry, 2007).

Resistance of sperm membranes which differs between species (most resistant: bull>sheep>pig>horse; Salamone *et al.*, 2017) is another important factor to consider. The necessity to break the sperm tail prior to ICSI is apparently important to provide optimal conditions, enabling and facilitating the procedure. One essential function associated with this breakage is sperm immobilization to avoid possible detrimental effects that the microinjected spermatozoon could have in the oocyte cytoplasm as a result of its motility. Also, it is believed that breakage could facilitate the release of sperm content into the egg, which may enhance sperm chromatin decondensation (Dozortsev *et al.*, 1995).

Finally, it is noteworthy that the other variable that might interfere with fertilisation success following ICSI is the type and proportion of protamines (Table 1.1) which seem to be primarily responsible for determining decondensation speed of the sperm nucleus (Perreault *et al.*, 1987, 1988). Protamines compact and stabilize sperm chromatin through formation of intra- and intermolecular disulfide bridges as a result of cysteine residue oxidation during final stages of sperm maturation in the epididymis (Perreault *et al.*, 1984; Balhorn, 2007). Disruption of these bridges, therefore, is necessary during fertilisation to decondense sperm genetic material and reactivate the sperm genome, leading to pronuclear formation and syngamy (Perreault *et al.*, 1988; Julianelli *et al.*, 2012). There are two types of protamines in mammalian sperm, classified as protamine 1 (P1) and 2 (P2). They differ mainly in their cysteine content and consequent number of disulfide bridges, with P1 having higher residues of cysteine which leads to a greater degree of compaction and stability than P2 (Balhorn, 1982). Furthermore, P1 appears to be present in all mammalian spermatids investigated so far, whereas P2 has been identified only in few species, including primates, many rodents, rabbits, hares, horses, zebras, tapirs and elephants (Balhorn, 1989; Lee and Cho, 1999). The proportion of P2 varies among species and, apparently, P1/P2 ratio is the main regulating factor of sperm DNA decondensation, increasing significantly the decondensation

rate in species with both types of protamine compared to those with only P1 such as the bull, boar or ram (Perrault *et al.*, 1988).

1.3.2 Oocyte factors

Failure or low efficiency on IVP post-ICSI could also be attributed to oocyte-derived factors involved in the direct or indirect acquisition of oocyte competency, and ability to respond to sperm activating stimulus following gamete fusion. Prominent among these factors are source of oocytes (OPU or slaughterhouse), oocyte maturation (IVM) and intrinsic factors such as the genetic background (Swain and Pool, 2008; Galli *et al.*, 2014).

1.3.2.1 Abattoir vs OPU

Originally, the only available source of oocytes for IVP was abattoir material. During the late 1980s an alternative approach known as OPU (ultrasound guided, transvaginal follicular aspiration) was introduced and used in farm animals, especially in the cattle industry (Merton *et al.*, 2003). Slaughterhouse ovaries have the advantage of collecting substantial quantity of oocytes. However, these are collected at different stages of ovarian follicular development and from different genetic backgrounds, resulting in variable post-fertilisation developmental efficiencies and low reproducibility (Karadjole *et al.*, 2010). OPU also offers the prospect of preserving genetic traits from valuable live donors and increasing the rate of genetic improvement as a consequence of reducing the generational interval (Galli *et al.*, 2001; Merton *et al.*, 2003). However, its effectiveness is determined by hormonal-stimulation treatment, aspiration efficiency and frequency, diameter of the aspirant needle and level of operator training (Pieterse *et al.*, 1992; Gibbons *et al.*, 1994; Scott *et al.*, 1994; Bols *et al.*, 1995).

Oocyte developmental competency is determined during folliculogenesis (Merton *et al.*, 2003). A positive correlation between ovarian follicle size and developmental competency of oocytes to produce embryos capable of reaching the blastocyst stage *in vitro* exists in the cow; it is termed “oocyte quality”.

Lager follicles (> 13mm diameter) give rise to more developmentally competent oocytes, producing greater numbers of blastocyst than oocytes from smaller follicles (3-8 mm diameter) (Hagemann *et al.*, 1999). Granulosa cell secretions are essential to acquire complete competence at the end of oocyte growth (Kastrop *et al.*, 1991; Bruynzeel *et al.*, 1997; Scaramuzzi *et al.*, 2011). For all that, these two parameters could be of particular importance in the choice of oocyte retrieval method.

Several studies have investigated variations in IVP efficiency with respect to oocyte source, revealing that it is possible to retrieve more blastocyst from slaughterhouse-derived oocytes (Lopes *et al.*, 2006). This may be related to the recovery of a larger number of competent oocytes as a result of full accessibility to all follicles and preservation of almost entire cumulus cell masses associated with post-mortem effects that appear to reduce the union affinity between the follicle and oocyte (Mullaart *et al.*, 1999). In contrast, the number of oocytes collected by OPU is lower due to equipment resolution, and cumulus-oocyte-complexes (COCs) could be damaged during aspiration in the absence of optimal conditions (Hashimoto *et al.*, 1999; Merton *et al.*, 2003). Nevertheless, when Karadjole *et al.* (2010) undertook a comparative study with similar numbers and quality of oocytes, they observed more developmentally competent oocytes following OPU than slaughterhouse retrieval.

1.3.2.2 Maturation

Oocyte maturation is probably one of the most important factors influencing fertilisation success and embryo development, due to the fact that it provides the oocyte with the ability to respond to PLCζ and, initiate events related to egg activation (Vanden-Meerschaut *et al.*, 2014). Oocyte maturation encompasses events that occur in both the nucleus and cytoplasm. Nuclear maturation is characterized by dissolution of intact nuclear envelope known as germinal vesicle (GV), formation of the meiotic spindle with the subsequent, meiosis progression from prophase I stage where oocyte is arrested at the time of birth to metaphase II stage (MII) and extrusion of the first polar body (PB) (Swain and Pool, 2008). Cytoplasmic maturation includes a series of structural

changes, such as alterations to the Golgi complexes, cortical granules, mitochondrial re-distribution and lipid storage, together with accumulation and post-translational modifications of certain mRNAs and proteins (Brevini-Gandolfi and Gandolfi, 2001; Smith, 2001).

Completion of both, nuclear and cytoplasmic maturation is equally necessary for successful oocyte maturation. It has been demonstrated that incomplete oocyte cytoplasmic maturation, as a result of reduced protein production or abnormal signaling pathways, appears to lead to unsuccessful fertilisation with a failure in mechanisms to block the polyspermy (Swain and Pool, 2008). In this respect the follicular environment has a crucial role for acquisition of full oocyte competency. This specific microenvironment promotes an increase in oocyte size and contributes to its development through the production of hormones and metabolites implied in modulating nuclear maturation. Interactions with cumulus cells are essential for continuous influx of nutrients and maturation-regulating factors via gap junctions (Fair *et al.*, 1995; Arlotto *et al.*, 1996).

Oocyte maturation has particular relevance in the success of ICSI as this technique bypasses normal gamete interactions. The process of sperm chromatin decondensation, sperm nuclear swelling, maternal and paternal genome remodelling, pronuclear formation and syngamy (Perreault *et al.*, 1988; Julianelli *et al.*, 2012) all are highly depend on the maturational stage of oocytes (Gioia *et al.*, 2005; Swain and Pool, 2008). The antioxidant glutathione (GSH), present in the ooplasm, is the main factor responsible for the reduction process that takes place during sperm DNA decondensation and formation of the male pronucleus (Perreault *et al.*, 1987; Perreault *et al.*, 1988; Luberda, 2005; Julianelli *et al.*, 2012). Its level increases during the course of oocyte maturation, reaching a maximum value at MII, before decreasing during subsequent embryo development (Usui and Yanagimachi, 1976; Komar, 1982; Perreault *et al.*, 1988; Furnus *et al.*, 1998). Several studies which quantified intracellular GSH concentrations during different stages of oocyte maturation under *in vivo* and *in vitro* conditions have suggested that GSH could be used as a valuable indicator of cytoplasmic maturation, and that GSH levels are

significantly lower when maturation occurs *in vitro* compared to *in vivo* (Abeydeera *et al.*, 1998; Furnus *et al.*, 1998; Yamauchi and Nagai, 1999; Brad *et al.*, 2003; Zuelke *et al.*, 2003; Luberda, 2005). These studies indicate that further research is required to identify key regulating factors involved in the control of oocyte maturation, in order to refine IVM protocols, in particular for livestock species where it is still a challenge.

1.3.2.3 Intrinsic factors

Other causes which could also have an impact in oocyte quality, interfering in fertilisation success and developmental capacity of embryos, relate to intrinsic characteristics of females which included factors such genetic background, nutrition and, body temperatures (Galli *et al.*, 2014).

Genetic background seems to be the main factor responsible for determining reproductive success. For example, findings reported by comparative studies have shown greater IVP success (higher oocyte quality and number as well as blastocyst yields) in *Bos indicus* compared to *Bos taurus*, the two of the major sub species of cattle (Pontes *et al.*, 2010; Sartori *et al.*, 2010; Sudano *et al.*, 2014; Gimenes *et al.*, 2015; Sales *et al.*, 2015; Sartori *et al.*, 2016). *Bos indicus* is widely located in tropical regions due to a series of adaptations that allow these breeds to tolerate the effects of heat stress and certain tropical diseases on reproductive function. In contrast, *Bos taurus* is more sensitive and is restricted mainly to template regions (Adeyemo *et al.*, 1979; Bennett *et al.*, 1985; Rocha *et al.*, 1998; Barros *et al.*, 2006).

These differences are based on genotypic variations which have led to important physiological alterations such as average of antral follicle count (Bastos *et al.*, 2010), size of dominant follicle diameter at deviation together with its growth rate (Gimenes *et al.*, 2008; Sartori *et al.*, 2010), volume of corpus luteum (Sartori *et al.*, 2016), and circulating concentration of certain hormones (Anti-Müllerian Hormone (AMH), progesterone, estradiol, Follicle Stimulating Hormone (FSH), insulin, Insulin-like Growth Factor 1 (IGF-1)) and cholesterol (Álvarez *et al.*, 2000; Sartori *et al.*, 2010; Sartori and Barros,

2011; Sartori *et al.*, 2013; Baldrighi *et al.*, 2014; Batista *et al.*, 2014; Sartori *et al.*, 2016). With subspecies, genetic selection for production traits have been found to have had both a positive (Snijders *et al.*, 2000; Leroy *et al.*, 2008b) and negative effect on reproductive performance (Kadzere *et al.*, 2002; Veerkamp *et al.*, 2003; Horan *et al.*, 2005); the latter due in part to inbreeding depression.

At the same time, nutrition appears to have a significant influence on oocyte maturation and subsequent embryo development (Leroy *et al.*, 2011). For example, high-energy diets known as glucogenic diets promote ovarian follicular development (Gong, 2002; Van Knegsel *et al.*, 2005), but have a detrimental effect on oocyte quality (Armstrong *et al.*, 2001; Adamiak *et al.*, 2005). Diets enriched in certain saturated long chain fatty acids, such as palmitic or stearic acids, have also been associated with inhibition of oocyte maturation (Lorey *et al.*, 2008a). Some evidence suggests that nutritional influence in oocyte development appears to be influenced by the body condition of the animal (Adamiak *et al.*, 2006).

Similarly, specific hormones such as insulin like growth factor I (IGF1) and progesterone can modulate fertility by influencing oocyte maturation, embryo development, implantation rate and pregnancy (McEvoy *et al.*, 1995; Moreira *et al.*, 2000; Mann and Lamming, 2001). Finally, body temperature as a result of heat or metabolic stresses can have a detrimental effect on oocyte quality and pre-implantation embryo development (Edwards and Hansen, 1997; Rocha *et al.*, 1998; Chebel *et al.*, 2004).

1.3.3 Artificial oocyte activation (AOA) by chemical stimuli

In several of the afore mentioned species, including human (Catt and Rhodes, 1995), rabbit (Keefer, 1989), horse (Squires *et al.*, 1996), hamster (Uehara and Yanagimachi, 1976) and mouse (Kimura and Yanagimachi, 1995), the injection procedure itself is apparently sufficient to activate the oocyte, initiating the calcium response required to enable further embryonic development. However, a failure or incomplete activation appear to be among

main causes of low success of fertilisation and/or suboptimal embryo development following ICSI in many domestic species. Therefore, a great variety of chemical treatments have been developed to induce egg activation considered as an essential step of the ICSI procedure in cattle (García-Roselló *et al.*, 2009; Galli *et al.*, 2014; Salamone *et al.*, 2017). In sheep, the necessity AOA protocols lies in the improvement of embryo development to blastocyst stage rather than fertilisation rates (Gómez *et al.*, 1998b; Pereyra-Bonnet *et al.*, 2008), whereas in the pig the benefits of AOA following ICSI are still not clear. However, blastocyst numbers obtained by several studies using AOA following porcine ICSI indicate that it has value (Kikuchi *et al.*, 2002; Yong *et al.*, 2006; Li *et al.*, 2013).

1.3.3.1 Different approaches

Increased cytosolic Ca^{2+} concentrations and low MPF activity are the two main strategies by which different AOA treatments are based in order to mimic PLCζ activity. With regards to Ca^{2+}_i, this can be induced by chemical agents that provoke a single and sustained calcium spike such as ethanol (Eth; Horiuchi *et al.*, 2002). This causes an extracellular calcium influx together with an increase in $InsP_3$. Ionomycin (Io; Rho *et al.*, 1998a; Suttner *et al.*, 2000) is involved in the release of intracellular calcium stores; and the calcium ionophore A23187 (CaIo; Kline and Kline, 1992) generates both an intracellular and extracellular calcium flux. Other chemical agents like strontium chloride ($SrCl_2$) and thimerosal, a sulfhydryl-oxidising agent, are responsible for triggering several persistent calcium spikes and can activate mouse and bovine eggs (Fissore *et al.*, 1995; Wakayama *et al.*, 1998). However, thimerosal has not been widely used due to its deleterious effects on the meiotic spindle (Cheek *et al.*, 1993).

A single calcium increase appears not to be sufficient to assure the first embryonic cell cycle after the resumption of meiosis as this transition requires several hours in mammalian species (Wakai and Fissore, 2013). During this period, low levels of MPF are necessary. This can be achieved by either continuous degradation of one of its subunits, known as cyclin B, or by

regulating its phosphorylation status through the activity of proteins which constitute CSF (Alberio *et al.*, 2001). For this reason, it has become common to combine single-calcium-spike inducers with protein synthesis inhibitors like cycloheximide (CHX) that inhibits cyclin B synthesis, or phosphatase-inhibitors such as 6-dimethylaminopurine (6-DMAP) responsible for inhibiting phosphorylation of important kinases such as cdc2 (the other MPF subunit), Mos and MAPK, implicate in the control MPF activity (Fernandes *et al.*, 2014; Lee *et al.*, 2015). For example, 7% Eth or Io (5µM) combined to 6-DMAP (1.9mM) or CHX (10µg/ml) has been employed as a first choice for activating eggs following bovine ICSI due to their successful results on embryo development and the report of healthy born calves (Rho *et al.*, 1998b; Hamano *et al.*, 1999; Horiuchi *et al.*, 2002; Galli *et al.*, 2003; Oikawa *et al.*, 2005). Whereas in species where AOA treatments are not essential, such as sheep and pigs, standard protocols are less common (García-Roselló *et al.*, 2009; Salamone *et al.*, 2017); except in humans where it is CaIo or Io widely used in cases of complete failure or low fertilisation success following ICSI (Vanden-Meerschaut *et al.*, 2014; Ebner and Montag, 2016).

1.3.3.2 Strontium as an effective exogenous activator

As mentioned in section 1.1.3.1, Ca^{2+} oscillations induced following mammalian sperm entrance traces a unique pattern which is indispensable not only to promote oocyte-to-embryo transition but, also to ensure genetically normal development of embryos (Ducibella *et al.*, 2002; Ozil *et al.*, 2006). Consequently, it is crucial to reproduce as reliably as possible this characteristic Ca^{2+} pattern when oocytes are stimulated *in vitro*.

Strontium (Sr^{2+}) has been described to cause a multi-oscillation Ca^{2+} pattern similar to that induced by the spermatozoon during normal fertilisation in mouse oocytes (Bos-Mikich *et al.*, 1997; Yamazaki *et al.*, 2005). It persists for long period, therefore promoting embryo development (Vitullo and Ozil, 1992). In the mouse, $SrCl_2$ has been used successfully as a conventional activator following the application of certain ART, leading to the birth of healthy offspring (Yamazaki *et al.*, 2005; Choi *et al.*, 2013). According to

mouse experiments performed by Zhang *et al.* (2005), Ca^{2+} signaling generated by Sr^{2+} seems to be regulated by $InsP_3$ receptors but through a different mechanism than that associated with normal fertilisation. However, in this case, previous activation of PLC and joint action by $InsP_3$ is required to enable Sr^{2+} evokes Ca^{2+} oscillations.

Nevertheless, $SrCl_2$ has not been established as routine AOA in certain domestic animals or in human which need assisted oocyte activation. There is limited knowledge about how Sr^{2+} is transported into the cell and its mechanism of action in these species (Cheek *et al.*, 1993; Zhang *et al.*, 2005). Calcium transients following $SrCl_2$ activation have only been reported in the mouse and rat (Day *et al.*, 2000; Tomashov-Matar *et al.*, 2005), and absence of optimal conditions such as concentration, incubation time, media requirements, and the use of other activating agents perhaps have contributed to its limited use. However, several studies have improved $SrCl_2$ protocol in different species such as bovine, porcine and ovine, showing comparable activating rates than conventional treatments (Méo *et al.*, 2005; Yamazaki *et al.*, 2005; Che *et al.*, 2007; Méo *et al.*, 2007; Choi *et al.*, 2013). In humans there have been reports of healthy births after its application in cases where conventional AOA (CaIo) did not improve fertilisation rates (Kyono *et al.*, 2008; Kim *et al.*, 2012).

1.4 DEVELOPMENTAL OUTCOMES FOLLOWING ICSI

Since the development of ICSI in humans (Palermo *et al.*, 1992), this technique has revolutionised assisted reproduction, leading to the birth of over two million babies worldwide (Palermo *et al.*, 2017). Its rapid diffusion and clinical application has relied on its great potential to overcome various types of human sterility, together with its repeatable and successful results on fertilisation (~72 to -74%), embryo development up to blastocyst stage (~63 to -65%) and pregnancy rate (~28 to -33%) which are very close to IVF values (~65 to -70%, 70 to -73 and 33 to -35%, respectively) (Langley *et al.*, 2014; Palermo *et al.*, 2015; Ferraretti *et al.*, 2017; Kim *et al.*, 2017).

A few years after the benefits of human ICSI emerged, the incorporation of piezo- drill as an adaptation to mouse ICSI by Kimura and Yanagimachi (1995) led to a surprising increase in mouse oocyte survival rates accompanied by high percentages of fertilisation (~92%), cleavage (~90 to -92%) and blastocysts (~75 to -78%) (Mizuno *et al.*, 2002; Piotrowska-Nitsche and Chang, 2013). Consequently, mouse ICSI has been used extensively as a model in reproductive research to study and modify gametes and embryos between species (Lyu *et al.*, 2010). Furthermore, piezo-drill introduction in conjunction with the improvement of culture media have enabled an increase in ICSI efficiency in horses; with reports of variable blastocyst percentages (~0 to -42%) but with high potential pregnancy rates (~50 to -80%) (Hinrichs, 2013; Galli *et al.*, 2014; Choi *et al.*, 2016; Rader *et al.*, 2016; Salamone *et al.*, 2017). Therefore, equine ICSI plays a crucial role in the breeding industry (Hinrichs, 2005).

Nevertheless, the low repeatability and unsatisfactory results obtained in the other domestic species following ICSI have limited its application. For example, although pigs and sheep show acceptable fertilisation and cleavage rates, blastocyst yields rarely exceed 20-25% in any species, even working under optimal conditions (García-Roselló *et al.*, 2009; Shirazi *et al.*, 2011; Anzalone *et al.*, 2016).

1.4.1 ICSI in cattle

Bovine ICSI is one of the most challenging ART due to extremely low fertilisation rates, suboptimal embryo development (Table 1.3) and reduced number of healthy live births (Table 1.4) since first performed by Goto (1990).

Table 1.3 Bovine embryo development following ICSI under different working conditions.

ICSI type	Oocyte source/ Maturation[a]	Sp[a]	Sperm treatment[ab]	Activation[ab]	2PN (%)	Cleav. (%)	Blast. (%)	References
Normal	S-In vitro	F	Hep	A23187 (1µM)	28	28	-	Keefer et al., 1990
				-	11	4		
Normal	S-In vitro	F	Bovine Epithelial Cells	A23187 (10µM)	44	-	-	Heuwieser et al., 1992
				-	9	-	-	
	In vivo			-	74	-	-	
Normal	S-In vitro	F	-	-	25	-	-	Catt and Rhodes, 1995
Piezo	S-In vitro	F	-	A23187 (50µM-10')	72	-	-	Katayose et al., 1999
				-	72			
Normal				A23187 (50µM-10')	19			
				-	5			
Normal	S-In vitro	F	Hep (50µg/ml)	A23187 (50µM-10')	33	-	-	Wei and Fukui, 1999
			Hep+Caffeine		46			
			A23187 (10µM)		60			
			DTT		61			
Normal	S-In vitro	F	DTT	Io+6-DMAP	38	8	3	Chung et al., 2000
Piezo	S-In vitro	F	DTT	A23187 (5µM-10')	19	28	0	Suttner et al., 2000
				Io+6-DMAP	31	44	17	
Normal				A23187 (5µM-10')	13	32	5	
				Io+6-DMAP	19	59	19	
Piezo	S-In vitro	F	Pentoxifylline	Eth (7%)	91	51	14	Fujinami et al., 2004
				-	71	13	4	
Normal	S-In vitro	F	Hep	Io (8')+6-DMAP	21	76	19	Li et al., 2004
Normal	S-In vitro	F	DTT	Io+CHx	51	52	10	Rho et al., 2004
		Fr			55	70	16	
Normal	S-In vitro	F	-	Io+6-DMAP	-	51	6	Vichera et al., 2010
Normal	S-In vitro	F	GSH+Hep (80µM)	A23187 (5µM)+6-DMAP (4h)	34	84	20	Sekhavati et al., 2012
			DTT		25	72	13	
Normal	S-In vitro	F	DTT	Io+CHX	-	71	22	Arias et al., 2014
			NaOH (1mM)		-	69	20	
Normal	S-In vitro	F	Hep (20µg/ml)	Io (2mM)+6-DMAP (5mM-4h)	73	93	29	Jo et al., 2014
		F-S			75	79	25	
Piezo	S-In vitro	F	DTT	3 times Io+ Eth (7%)	73	83	18	Lee et al., 2015
			GSH(8mM)+Hep		84	80	28	
			GSH+Hep+LPC (300µg/ml)		69	74	17	
Piezo	OPU-In vitro*	F	DTT	-	70	65	29	Oikawa et al., 2016
Normal	S-In vitro	F	LPC (500µg/ml)	Io+CHX	77	65	29	Zambrano et al., 2016
			Triton (0.05%)		76	66	27	
Normal	S-In vitro	F	MβCD (1mM)	Io+CHX	54	84	24	Águila et al., 2017a
Normal	S-In vitro	F	GSH+Hep (80µM)	Io+6-DMAP	77	61	19	Canel et al., 2017
Normal	S-In vitro	F	GSH	Io+CHX	64	74	26	Zambrano et al., 2017
			GSH+LPC(500µg/ml)		64	80	27	
			GSH+Triton (0.05%)		54	78	28	

[a] S (Slaughterhouse); OPU (Ovum Pick Up); Sp (Sperm); F (Frozen); Fr (Fresh); F-S (Frozen Sorted); Cleav. (Cleavage); Blast. (Blastocyst); PN (Pronuclei); Hep (Heparin); DTT (Dithiothreitol); GSH (Glutathione); LPC (Lysophosphatidylcholine); MβCD (Metyl-β-cyclodextrin); Io (Ionomycin); 6-DMAP (6-Dimethylaminopurine); CHX (Cycloheximide); NA (Not Available)

[b] Standard conditions: Heparin= 10 µg/ml; Caffeine= 5 mM; Pentoxifylline= 10 mM; DTT= 5 mM; GSH= 15 mM; Io= 5 µM-4 or 5 min; 6-DMAP= 1.9 mM-3h; CHX= 10 µg/ml-5h

*OPU oocytes from donor with low embryo production

Table 1.4 Embryo development, pregnancies and deliveries following bovine ICSI using different sperm pre-treatments and egg activation protocols.

ICSI type	Oocyte source/ Maturation[a]	Sperm[a]	Sperm treatment[ab]	Activation[ab]	2PN (%)	Cleavage (%)	Blastocyst (%)	Live born (sum total)[ac]	References
Normal	S-In vitro	Frozen	Killed	NA	-	12	8	Yes (NA)	Goto, 1990
Normal	S-In vitro	Frozen	DTT	Io+6-DMAP	-	61	24	Pregnancy[1]	Rho *et al.*, 1998b
Normal	S-In vitro	F-Sorted	Sonification	Eth (7%)	-	47	7	Yes (10)	Hamano *et al*., 1999
Normal	S-In vitro	Frozen	Hep+Caffeine	ES	57	48	13	Pregnancy[2]	Hwang *et al.*, 2000
Piezo	S-In vitro	Frozen	Pentoxifylline	Eth (7%)	70	42	20	Yes (5)	Horiuchi *et al.*, 2002
Piezo	S-In vitro	Frozen	-	Lipid polarization	78	72	23	Yes (4)	Wei and Fukui, 2002
Piezo	S-In vitro	Frozen	-	Io+CHX	-	67	22	Yes (1)	Galli *et al*., 2003
Piezo	S-In vitro	Frozen	Caffeine (10mM)	Io+6-DMAP	-	84	40	Yes (1/10)	Oikawa *et al*., 2005
				Eth (7%)	-	76	20	Yes (9/10)	

[a] S (Slaughterhouse); OPU (Ovum Pick Up); PN (Pronuclei); F-Sorted (Frozen Sorted); ES (Electrical stimulation); Hep (Heparin); DTT (Dithiothreitol); Io (Ionomycin); 6-DMAP (6-Dimethylaminopurine); CHX (Cycloheximide); NA (Not Available)

[b] Standard conditions: Heparin= 10 µg/ml; Caffeine= 5 mM; Pentoxifylline= 10 mM; DTT= 5 mM; Io= 5 µM-4 or 5 min; 6-DMAP= 1.9 mM-3h; CHX= 10 µg/ml-5h

[c] Pregnancy [1]= it was reported pregnancy but, there was not delivery of live calves; Pregnancy [2]= collected data until sixth month of pregnancy

1.5 DEVELOPMENT OF WORKING HYPOTHESIS AND SPECIFIC OBJECTIVES

1.5.1 Working hypothesis

Major efforts by cattle industry to increase the rate of genetic improvement for a range of commercially important traits require overcoming low reproductive rate in this species. AI and IVP-ET offer many advantages in this respect, particularly with the use of sex-sorted semen. However, the main disadvantage associated with the sex-sorted semen that the sorting procedure reduces sperm concentration and quality, limiting its success in AI and IVF. Therefore, there is a renewed interest to combine ICSI with this type of sperm in order to improve post-fertilisation development and pregnancy outcomes.

In contrast to the success of ICSI in certain mammalian species such as the human, mouse and horse, the employment of this technique in cow is limited as a consequence of low fertilization and poor embryo developmental rates. The principal problem responsible for these disappointing results is a failure in pronuclei formation, which seems to be directly related to the inability of bull sperm to activate the egg and decondense its sperm chromatin. For this reason, the main line of investigation in this thesis is focused on developing sperm pre-treatments which facilitate the release of sperm components and make paternal chromatin more accessible to oocyte. Also, this thesis seeks to improve existing AOA protocols to promote the oocyte-to-embryo transition through the generation of appropriate calcium responses and persistence of low MPF low activity for the required period of time.

Taking all of this into account, together with the fact that the bull spermatozoon possesses one of the largest heads with sperm chromatin highly compacted due to the presence of only type I protamines and, accordingly with findings from ICSI studies where the removal of sperm membranes have improved its success, this thesis presents the following hypothesis: That the absence of bull sperm membranes and/or a previous sperm decondensation at the time of microinjection followed by the application of a suitable AOA

protocol would enhance pronuclear formation and ensure a high proportion of competent blastocysts following ICSI.

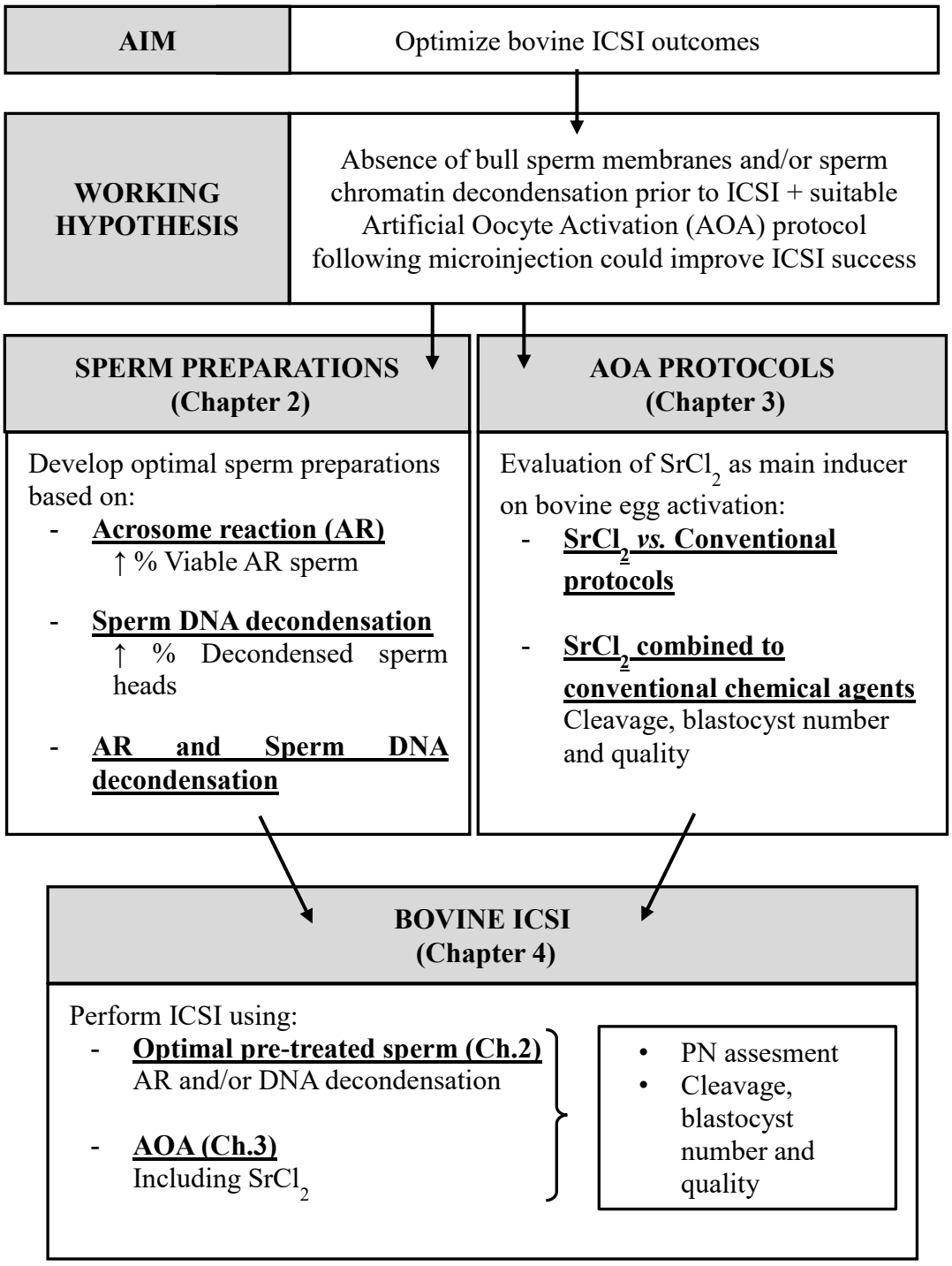
1.5.2 Broad objectives and specific aims

The initial objective of this thesis was to develop an optimal sperm preparation protocol for bovine ICSI based on the disruption of bull sperm membranes and DNA decondensation. The proportion of viable fully AR spermatozoa following the addition of AR inducers under optimal working conditions were recorded. Similarly, the percentage of decondensed sperm heads after incubation of sperm with a specific reagent involved in the reduction of protamine bridges was assessed. Finally these two sperm parameters were assessed following the combination of both treatments.

The second objective of this thesis concerned the improvement of existing bovine AOA protocols by investigating the benefits of incorporating novel combinations of reagents. Consequently, the initial study evaluated the efficiency of conventional AOA treatments whilst at the same time assessing the effectiveness of $SrCl_2$ as an AOA agent. Next, it investigated the synergistic effect of $SrCl_2$ combined to single-calcium-spike inducers and protein synthesis inhibitors on subsequent embryo development in absence of sperm (i.e. parthenogenesis).

Finally, this thesis evaluated bovine ICSI success following the application of optimal sperm pre-treatments and AOA protocols. It assessed pronuclear formation, cleavage and blastocyst development and quality after microinjection of pre-treated spermatozoon with AR and/or DNA decondensation agents and oocyte activation using the optimal AOA containing $SrCl_2$.

1.5.3 Thesis structure diagram



CHAPTER 2:

Sperm preparation for bovine ICSI: Acrosome reaction and DNA decondensation

2.1 INTRODUCTION

ICSI overcomes several critical steps of fertilisation process such as sperm-ZP binding, removal of acrosome membrane and gamete fusion. However, strong evidence indicates that the limited success of bovine ICSI may be largely attributed to the introduction of sperm membranes into the oocyte which impedes appropriate egg activation and delay sperm nucleus decondensation (García-Roselló *et al.*, 2009; Vanden-Meerschaut *et al.*, 2014). Furthermore, oocyte exposure to hydrolytic enzymes from the acrosome of microinjected spermatozoon has a detrimental effect on its cytoskeleton and compromises embryo developmental potential (Morozumi and Yanagimachi, 2005; García-Roselló *et al.*, 2009; Kashir *et al.*, 2014; Zambrano *et al.*, 2016), particularly in those species with large sperm head, as in the case of bull spermatozoa (Garner, 2006).

Several artificial agents have been developed to remove both sperm plasma and acrosomal membranes based on recreating the scenario that capacitated spermatozoa undergo when reach the oocyte during *in vivo* fertilisation. These induce key physiological processes which lead to AR (Section 1.1.1.2 and 1.1.2.1). One of them is Lyshophosphatidylcholine (LPC), also known as lysolecithin, which activates a series of physiological pathways to trigger AR when bovine spermatozoa are previously exposed to heparin (Hep) (Parrish *et al.*, 1988). Hep is a glycosaminoglycan (GAG) that acts as a ligand for a receptor located in the sperm plasma membrane, modulating capacitation in bull spermatozoa. Once Hep binds to its sperm receptor, it stimulates an increase in Ca^{2+}_{i} concentrations, pH and cAMP (Srivastava *et al.*, 2013; Yadav *et al.*, 2013). LPC induces an increase of acrosin activity, an important acrosomal protease involved in hydrolysing ZP in bovine oocytes. It also induces sperm membrane fusion as a result of the formation of micelles into

the aggregated membranes, destabilising then their structural consistency; both these processes are regulated by tyrosine kinase activity (Lucy, 1978; Parrish *et al.*, 1988; Purdy and Graham, 2004; Costa *et al.*, 2010; Pérez-Aguirreburualde *et al.*, 2012).

Another group of relevant AR inducers are the calcium ionophores, due to their capacity to increase membrane permeability to Ca^{2+} ions. The presence of these essential ions is required for the release of actin and the activation of several proteins (PKA, PKC, PLC and PLA_2) involved in promoting membrane fusion during AR (Breitbart and Spungin, 1997), as well as acquisition of hyperactive motility pattern (Tateno *et al.*, 2013). Therefore, calcium ionophores such as A23187 have been used in several mammalian species to induce a significant rise of $Ca^{2+}{}_i$ levels in spermatozoa (Talbot *et al.*, 1976; Jamil and White, 1981; Purdy and Graham, 2004; Tateno *et al.*, 2013). An excessive $Ca^{2+}{}_i$ increase, however, seems to be associated with an adverse collateral effect on sperm motility, immobilising temporary the majority of spermatozoa for unknown reasons but, once Ca^{2+} excess is removed from the media, spermatozoa restore their motility with a hyperactive pattern without any apparent damage (Hong *et al.*, 1985; Suarez *et al.*, 1987; Tateno and Mikamo, 1987). Moreover, Tateno *et al.* (2013) demonstrated that, following exposure to high doses of A23187, motile spermatozoa also retained their ability to fertilise intact cumulus cell oocytes in the mouse through an alternative pathway. This alternative pathway does not require $HCO^-{}_3$ nor activation via the cAMP-dependent pathway.

On the other hand reducing agents, which focus on breaking disulfide bonds of protamines, have been used to decondense sperm DNA such as dithiothreitol (DTT) and glutathione (GSH) (Rho *et al.*, 1998b; Suttner *et al.*, 2000; Sekhavati *et al.*, 2012; Lee *et al.*, 2015; Canel *et al.*, 2017; Zambrano *et al.*,2017). The improvement of bovine ICSI success following the microinjection of DTT-pre-treated spermatozoa has been controversial. Some studies have reported an increase in fertilisation, cleavage and blastocyst proportion (Rho *et al.*, 1998b; Wei and Fukui, 1999; Rho *et al.*, 2004; Oikawa

et al., 2016), while others have not found significant effects (Chung *et al.*, 2000; Suttner *et al.*, 2000; Galli *et al.*, 2003; Sekhavati *et al.*, 2012; Arias *et al.*, 2014; Lee *et al.*, 2015). In general, blastocyst development when DTT is employed as a sperm reducing agent prior to ICSI is low and there have been no reports of live births following its use. This may be related to the harmful effects of DTT on sperm DNA which compromise the structural integrity of paternal chromatin and, ultimately, interferes with embryo development (Cheng *et al.*, 2009; Sekhavati *et al.*, 2012). In contrast, GSH is part of the physiological machinery that the oocyte has available to unpack sperm chromatin by the reduction of protamine disulfide bridges (Calvin *et al.*, 1986; Perreault *et al.*, 1987; Luberda, 2005; Julianelli *et al.*, 2012). However, the second step of chromatin decondensation involves protamine-histone exchange and this requires the presence of a protamine acceptor. This is a high molecular weight molecule with negative charge, and heparin sulfate (HS) seems to be a strong candidate to be the biological acceptor (Romanato *et al.*, 2005 and 2008; Julianelli *et al.*, 2012).

Hep also participates in the solubilisation of sperm nuclei through the release of histones which has been described as a pre-requisite to finish the decondensation process (Courvalin *et al.*, 1982). Consequently, Hep has been used to induce sperm DNA decondensation due to its structural and functional similarities to HS and its affinity for histones (Sánchez-Vázquez *et al.*, 2008; Julianelli *et al.*, 2012). In combination with GSH it has also been used to treat bull spermatozoa prior to ICSI, leading to encouraging results (Sekhavati *et al.*, 2012; Canel *et al.*, 2017).

Nevertheless, the large variation in fertilisation and blastocyst rates following bovine ICSI, and the absence of a standard successful sperm pre-treatment protocol giving reproducible results, means that further research is required. Therefore, this experimental chapter is focused on investigating optimal combinations of AR inducers and reducing agents in order to develop a suitable sperm pre-treatment that could be used routinely for bovine ICSI.

2.1.1 Objectives

The main aim of this study was to optimize sperm pre-treatment procedures, involved in the removal of acrosomal membranes and sperm chromatin decondensation for bovine ICSI. The first study focused on inducing the proportion of AR bull spermatozoa whilst retaining their viability. Specifically, it sought to optimise working conditions (incubation times and concentrations) of LPC combined with Hep and Calcium Ionophore A23187 (CaIo). The second study sought to establish the optimal conditions for L-glutathione (GSH) mediated sperm decondensation when combined with the aforementioned AR inducers (LPC+Hep and CaIo).

2.2 MATERIALS AND METHODS

Chemicals and reagents were purchased from Sigma-Aldrich Company Ltd. (Dorset, England), unless otherwise indicated.

2.2.1 Study design, experiments and replication

2.2.1.1 Study 2A: Removal of acrosomal membrane

2.2.1.1.1 Expt. 2Ai. Effect of Hep on the proportion viable bull sperm over time

In order to establish an optimal time slot for Hep exposure without compromising sperm viability, washed bull spermatozoa were incubated in 10 µg/ml Hep for 0, 2, 4 and 6 hours (h), immediately after bovine gradient-density separation (no Hep incubation). Then, sperm viability was recorded after each of these four incubation periods by eosin-nigrosin staining.

2.2.1.1.2 Expt. 2Aii. Effect of LPC on AR and sperm viability

Next, the effect of LPC on the proportion of AR sperm after a previous incubation with Hep (optimised in Expt. 2Ai) was evaluated. Following 2 h in 10 µg/ml Hep, sperm were incubated in LPC (100 µg/ml) for 15 min and then acrosomal status was assessed and compared to the control group (absence of exposure to LPC after 2 h of heparin incubation). Viability was examined using two different staining approaches: fluorescence staining (PI/Hoechst 33258) and eosin-nigrosin; while AR was evaluated by triple fluorescence staining (PI/Hoechst 33258/PNA-FITC).

2.2.1.1.3 Expt. 2Aiii. Effect of LPC on the proportion of acrosome status and viability after different incubation times in Hep (10 µg/ml)

To study if the efficacy of LPC is affected by pre-incubation time with Hep, bull spermatozoa were exposed to the same Hep concentration (10 µg/ml) as used in previous experiments but for 15, 60 and 120 min followed by LPC (100 µg/ml, 15 min). Acrosomal status and sperm viability were examined

after each of these periods and immediately after bovine gradient density separation, with the absence of Hep and LPC (control group).

2.2.1.1.4 Expt. 2Aiv. Effect of Hep dose (for 15 min) on the proportion of AR and viable sperm after LPC treatment

Different Hep concentrations were tested (40, 80 and 100 µg/ml) for 15 min incubation before the addition of LPC (100 µg/ml) for a further 15 min to investigate its impact on the ability of LPC to trigger the AR. Sperm viability and proportion of AR sperm were estimated for each treatment and compared to the control group; which it used the standard Hep dose (10 µg/ml).

2.2.1.1.5 Expt. 2Av. Effect of Calcium Ionophore A23187 (CaIo) dose on percentage AR and sperm viability

To evaluate the optimal CaIo dose and post-incubation time (motility recovery period) after CaIo exposure to achieve the greatest proportion of viable acrosome reacted spermatozoa, bull spermatozoa were exposed to 10, 15 and 20 µM CaIo for 10 min. After that time, CaIo was removed and immotile treated sperm were resuspended and incubated for 30 or 60 min in fresh modified BWW media containing higher BSA concentration (3%) to recover sperm motility. The proportion of AR sperm and sperm viability were then assessed using the techniques described in the preceding sections of this chapter.

2.2.1.2 Study 2B: Sperm DNA decondensation

2.2.1.2.1 Expt. 2Bi. Combined effect of GSH and Hep on the proportion of sperm head decondensation after different incubation periods

To test the effect of Hep dose and co-incubation time with GSH on the efficiency of GSH to decondense the nuclear sperm DNA, bull sperm were incubated simultaneously with different Hep concentrations (40 and 80 µM) and GSH (15 mM) for 3, 7 and 20 h. A Hep dose of 4.4 µM (~80 µg/ml) was

also included and acted as a control. Decondensed sperm heads were counted with a phase contrast microscope.

2.2.1.2.2 Expt. 2Bii. Synergistic effect of GSH with either LPC or CaIo on the percentage of sperm head decondensation and AR

This study sought to evaluate how the prior removal of sperm plasma- and acrosome- membranes affects GSH activity. After the application of optimal sperm pre-treatments to induce AR (LPC+Hep or CaIo), spermatozoa were co-incubated with GSH (15 mM) and Hep (80 µM) for 20 h. Finally, viability, proportion of AR and proportion decondensed sperm heads were recorded.

2.2.2 Sperm preparation

Commercially available South Devon semen straws from a single sire were thawed at 37ºC for 90 s in water bath. Depending on the experiment, either semen from a single straw was used or semen was pooled from several straws from the same bull. Sperm samples were evaluated, before and after the treatments, for concentration and motility. The percentage total motile sperm was determined subjectively. A drop of semen was placed into a glass-slide and examined under a bright-field microscope at 200X magnification. Sperm concentration was determined using a haemocytometer at 1:20 dilution.

BoviPure density gradients (80% and 40%) were prepared by mixing BoviPure™ and BoviDilute™ stock solutions (Nidacon International AB, Sweden). Exactly 0.5 ml of 40% Bovipure was overlaid onto 0.5 ml of 80% Bovipure in a 1.5 ml Eppendorf tube. Then, 250 µl of sperm sample was placed delicately on top of the layers. After 15 min of centrifugation at 300 x g and 25ºC, the pellet was washed with BoviWash Sperm Dilution for 5 min at 300 x g at the same temperature. Finally, the pellet was suspended in 200 µl of Fert-TALP media (see Appendix II.a) containing the appropriate dose of Hep (see Appendix IV.e) and sperm concentration was adjusted to $7.5 \cdot 10^5$ sperm/ml.

In first experiments (Expt. 2Ai-iv), after the Hep exposure to different times and concentrations, 200 µl sperm sample was washed in 1 ml fresh Fert-TALP media for 5 min at 300 x g and 25ºC. Next, 75 µl pellet was resuspended in 125 µl Fert-TALP media containing LPC (100 µg/ml, see Appendix IV.a) and was incubated for 15 min at 38.5ºC in a humidified atmosphere of 5% CO_2 in air.

In next series of experiments (Expt. 2Av), 50 µl washed sperm sample by bovine gradients was transferred to 950 µl modified BWW media (see Appendix II.c) supplemented with different doses of CaIo (10, 15 and 20 µM, see Appendix IV.k) and incubated for 10 min at 38.5ºC with 5% CO_2 in air, according to the protocol cited by Tateno and Mikamo (1987). Then, in order to remove the chemical agent, sperm were washed for 5 min at 550 x g and 25º C. Finally, 30 µl pellet was transferred into 600 µl modified BWW media supplemented with 3% BSA. This is a higher concentration than standard (0.3%), necessary to eliminate the excess CaIo by the high affinity properties of BSA for CaIo in order to restore sperm motility (Suarez *et al.*, 1987). Treated sperm were incubated for 30 or 60 min under the same conditions as described previously.

In study 2B, 50 µl of sperm pellet was added to 50 µl Fert-TALP media containing GSH (15 mM, see Appendix IV.f) and co-incubated with different Hep concentrations (4.4, 40 and 80 µM) for 3, 7 and 20 h at 38.5ºC in a humidified atmosphere of 5% CO_2 in air, following a modified protocol from Romanato *et al.* (2003) and Canel *et al.* (2017) (Expt. 2Bi). In last set of experiments (Expt. 2Bii), 50 µl of sperm treated previously by Hep/LPC (Hep: 80 µg/ml, 5 min; LPC: 100 µg/ml, 15 min) or CaIo (10 µM) were exposed to 50 µl Fert-TALP media containing GSH/Hep (15 mM and 80 µM, respectively) for 20 h at 38.5ºC in a humidified atmosphere of 5% CO_2 in air. Before fixing the sperm sample in 2% paraformaldehyde (PFA) for 5 min, it was washed in 1 ml fresh Fert-TALP media and centrifuged at 210 x g and 25ºC for 7 min. Then, 2 µl of sperm sample was smeared onto a glass slide and air dried. At least 200 sperm randomly distributed across the slide were counted at a magnification of 400X under a phase contrast microscope to

calculate the proportion of DNA decondensed sperm. When spermatozoa showed a normal size head with defined membranes (Fig. 2.1-Blue arrow) then spermatozoa were classified as a "non-decondensed". They were classified as "decondensed" when they had enlarged heads (several sizes) with non-defined membranes (Fig. 2.1-Red arrow).

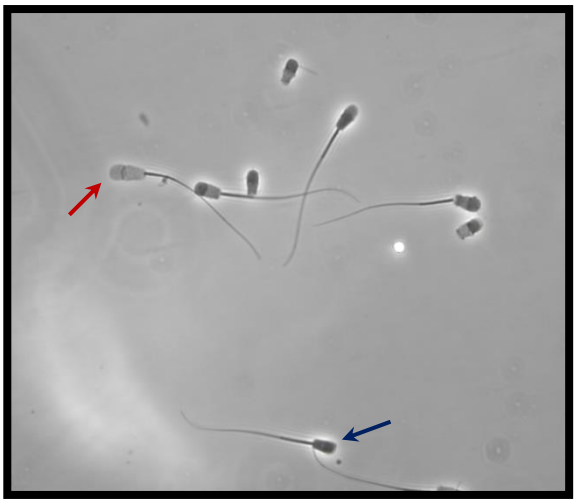


Figure 2.1 Different grades of sperm DNA decondensation after 20 h incubation in GSH+Hep. Red arrow: spermatozoon with the head completely decondensed; Blue arrow: normal spermatozoon.

2.2.3 Sperm viability assessment: Eosin-nigrosin vital staining

Eosin-nigrosin is a vital stain based on the integrity of sperm membranes. Eosin binds to basic or eosinophilic cellular components containing positive charges (proteins) from cytosol when the cell membranes are damaged. Therefore, eosin marks dead cells. Nigrosin is used as a background stain, to increase the contrast between faintly stained cells and otherwise bright background (Björnadahl *et al.*, 2003).

The eosin-nigrosin solution was prepared as described by Mortimer (Mortimer, 1985 and 1994). Briefly, 0.67 g of eosin Y yellow and 0.90 g of sodium chloride were dissolved in 90 ml of distilled water with gentle heating. When dissolved, 10 g of nigrosine (Thermo Fisher Scientific, Belgium) was added, heating until reach boiling point. The solution was allowed to cool to room temperature prior to make up the total volume to 100 ml. Finally, the

solution was filtered and stored in a sealed bottle in the dark. Staining was carried out by mixing approximately equal volumes of semen sample (30 µl) and stain solution (30 µl). The suspension was incubated for 30 s at room temperature. Then, a 20 µl droplet was transferred to a glass slide where it was smeared by sliding a cover slip in front of it. The smear was air dried and examined directly. At least 200 sperm were assessed at a magnification of 400X with a bright field objective. Sperm that were white (unstained) were classified as live and those that showed any pink or red colouration were classified as dead (Mortimer, 1994).

2.2.4 Sperm viability and acrosome status evaluation: Triple fluorescence staining (PNA-FITC/PI/Hoechst 33258)

Viability and acrosomal status were assessed simultaneously using florescent probes and fluorescence microscopy according to an adaptation of methods described previously (Martínez-Pastor *et al*., 2010; Álvarez *et al*., 2012).

One of the most common dyes used for viability stains is the membrane impermeable probe propidium iodide (PI) because it is easy and rapid to use. This dye enters cells which have a broken plasmalemma, emitting red fluorescence (λ= 636 nm) when binding to nucleic acids (Gillan *et al.*, 2005). On the other hand, Hoechst 33258 nucleic acid stain is a popular cell-permeant nuclear counterstain that emits blue fluorescence (λ= 461 nm) when bound to DNA double helix (Drobyshev, 1999). When PI penetrates a membrane damaged spermatozoa, it displaces or quenches Hoechst 33258 fluorescence (Martínez-Pastor *et al*., 2010). For acrosomal status assessment, lectins, which bind to glucosidic residues in different parts of the acrosomal membrane, have been widely used in different species. Arachis hypogaea (peanut) agglutinin (PNA) (Nagy *et al*., 2003) has the advantage that it is a specific binder of β-galactose residues located on the outer acrosomal membrane, resulting in less nonspecific fluorescent signal (Lybaert *et al.*, 2009). PNA is conjugated with the green fluorochrome fluorescein isothiocyanate (FITC) and, when an

acrosome is green stained, indicates that it is damaged or reacted (Martínez-Pastor *et al*., 2010).

Briefly, after the application of the different treatments, sperm samples were washed with 2 ml of Phosphate Buffered Saline (PBS, see Appendix I.a) for 5 min at 300 x g. This, pellet was suspended in 193 µl of PBS; and incubated with 4.5 µl of PI (final concentration: 33 µM), 1.7 µl of Hoechst 33258 (final concentration: 83 µl/ml, see Appendix IV.c) and 0.7 µl of PNA-FITC (final concentration: 3.3 µl/ml, see Appendix IV.b) for 15 min at 38.5ºC under dark conditions. To remove unbound probes, sperm samples were washed with 7.5 ml of warmed PBS for 10 min at 300 x g, and then fixed in 2% PFA for 5 min. Sperm samples were smeared onto glass slides by cytospin. Finally, spread samples were washed again to reduce the background, covered with a coverslip and sealed with nail varnish. When samples could not be analysed immediately, they were stored frozen (-20ºC) in the dark.

To estimate the proportion of AR sperm, at least 200 spermatozoa were evaluated in randomly selected fields at 400X magnification under a fluorescent microscope (Leica DMR). The spermatozoa were classified into six different subpopulations based on PI/Hoechst 33258/PNA-FITC staining: blue stain (viable sperm, intact acrosome; Fig. 2.2-A), blue and intense green stain above sperm head (viable, capacitated sperm; Fig. 2.2-B), blue and weak spotted green stain above sperm head (viable, complete acrosome reacted sperm; Fig. 2.2-C), red stain (nonviable sperm, intact acrosome; Fig. 2.2-D), red and intense green stain above sperm head (nonviable, capacitated sperm; Fig. 2.2-E), and red and weak spotted green above sperm head (nonviable, completely acrosome reacted sperm; Fig. 2.2-F). As a note of caution, the PNA-FITC stain can have a low percentage of false negative, because spermatozoa that have completely lost their acrosome cannot be stained by PNA-FITC (Martínez-Pastor *et al*., 2010; Álvarez *et al*., 2012).

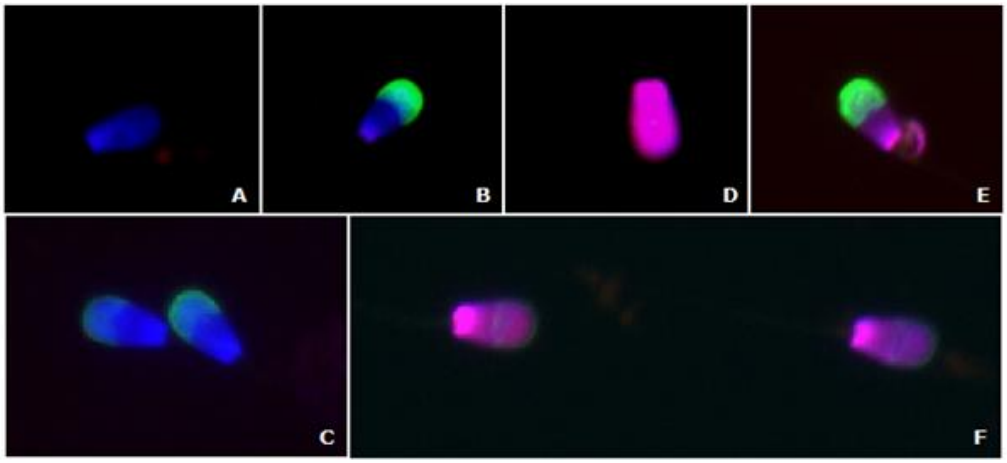


Figure 2.2 Different subpopulations obtained after triple fluorescence staining (PI/Hoechst 33258/PNA-FITC) of bull spermatozoa. A) Viable spermatozoa with intact acrosome; B) Capacitated viable spermatozoa; C) Completely acrosome reacted and reacting viable spermatozoa from left to right; D) Dead spermatozoa with intact acrosome; E) Capacitated dead spermatozoa and F) Fully acrosome reacted dead spermatozoa.

2.2.5 Statistical analysis

All proportion data were analysed using generalized linear regression models that assumed binomial errors and used logit link functions. Statistical analyses were performed using the GenStat statistical package (17th Edition, VSN International, 2011). Differences were considered significant at $P < 0.05$. Differences between individual means were established using Least Significant Differences (LSD) derived from residual errors of the statistical models. Proportions were converted to percentages for the purposes of data presentation and discussion.

2.3 RESULTS

2.3.1 Study 2A: Removal of acrosome membrane

2.3.1.1 Expt. 2Ai. Effect of Hep on the proportion viable bull sperm over time

Bull sperm viability decreased (P<0.01) following 2 h Hep incubation (from 65% to 26%; Table 2.1). The decline thereafter was less pronounced. For this reason, 2 h of incubation with Hep was selected as the initial time point to perform the next series of experiments with LPC.

Table 2.1 Percentage of viable bull sperm (mean ± s.e.) during different incubation times with Hep (10 µg/ml).

Hep incubation period (h)	Viable sperm (%)
0	65.3±1.33[a]
2	25.5±1.22[b]
4	20.2±1.23[c]
6	18.6±1.08[c]

The experiment was replicated three times. Viability was assessed by eosin-nigrosin stain**.** [a-c]**Values within the same columns with different superscripts differed at P< 0.05**.

2.3.1.2 Expt. 2Aii. Effect of LPC on AR and sperm viability

LPC treatment (100 µg/ml, 15 min) after 2 h of pre-incubation in Hep almost doubled (P=0.007) the percentage of AR and fully AR over total spermatozoa (viable and non-viable) compared to the control group (Table 2.2). When the viable sperm population (36.8%) was analysed a similar effect of LPC was observed. Therefore, LPC worked as an effective AR inducer following a previous exposure to Hep.

Table 2.2 Percentage Acrosome Reacted (AR) and sperm viability (mean ± s.e.) in the presence or absence of LPC (100 µg/ml) after incubation for 2h with Hep (10 µg/ml).

Treatment	% AR of Total	% Fully AR of Total	% Viable AR of viable	% Viable Fully AR of viable
	Total		Viable	
Hep	42.1±1.55[a]	37.1±2.01[a]	38 ±3.20[a]	33.6±2.68[a]
Hep+LPC	85.9±1.15[b]	73.6±1.97[b]	82.3±2.54[b]	69.8±2.57[b]

Each treatment was replicated three times. AR (Acrosome Reacted) = Fully AR + Capacitated (viable and non-viable sperm). Fully AR= AR only (viable and non-viable). Viable AR = Fully AR (viable) + capacitated (viable). Viable Fully AR = viable AR only. **[a-b]Values differed at P< 0.05.**

2.3.1.3 Expt. 2Aiii. Effect of LPC on the proportion of acrosome status and viability after different incubation times in Hep (10 µg/ml)

As a consequence of the low percentage of viable sperm achieved in previous experiment, it was decided to reduce the incubation time in Hep (10 µg/ml) from 120 to either 60 or 15 min prior to treatment with LPC (100 µg/ml, 15 min). Reducing the incubation time with Hep from 120 to 15 min increased ($P<0.001$) the percentage of viable sperm, irrespective of the staining method employed to assess sperm viability (Table 2.3).

Table 2.3 Effect of different incubation times with Hep (10 µg/ml) on percentage viable bull sperm for LPC treatment (100 µg/ml for 15 min).

Incubation times (min)	Fluorescence Stain (%)	Eosin-Nigrosin Stain (%)
0	66.5±3.94[a]	70.3±2.62[a]
15	67.3±3.93[a]	69 ±2.64[a]
60	42.3±3.99[b]	49.4±3.36[b]
120	34.1±4.04[b]	30±2.63[c]

Each treatment was replicated five times. [a-c]Values differed at P< 0.05.

The importance of Hep exposure in terms of subsequently inducing AR is clearly illustrated in Table 2.4. A previous Hep exposure increased (P<0.001) the percentage of AR sperm following LPC treatment. However, duration of exposure to Hep (15 to 120 min) did not affect the percentage AR sperm.

Table 2.4 Effect of LPC on acrosome reacted and viable sperm (mean ± s.e.) after different incubation times with Hep (10 µg/ml).

Time (min)	% AR of Total	% Fully AR of Total	% Viable AR of viable	% Viable Fully AR of viable
	Total		Viable	
0	33.4±3.72[a]	30.8±3.43[a]	26.1±3.43[a]	24.8±3.27[a]
15	71.5±3.81[b]	61.6±3.71[b]	67.5±3.88[b]	59.4±3.83[b]
60	71.2±3.59[b]	61.7±3.54[b]	64.6±4.58[b]	58.4±4.55[b]
120	83.2±3.27[c]	68.7±3.62[b]	74.1±5.11[b]	63.6±5.3[b]

Each treatment was replicated five times. AR (Acrosome Reacted) = Fully AR + Capacitated (viable and non-viable sperm). Fully AR= AR only (viable and non-viable). Viable AR = Fully AR (viable) + capacitated (viable). Viable Fully AR = viable AR only. **[a-c]Values within the same columns with different superscripts differed at P< 0.05.**

2.3.1.4 Expt. 2Aiv. Effect of Hep dose (for 15 min) on the proportion of AR and viable sperm after LPC treatment

Having identified 15 min as the optimal duration for Hep exposure, it was necessary to next establish the optimal Hep dose prior to LPC treatment. Data presented in Table 2.5 indicated that increasing the dose of Hep from 10 to 40 µg/ml increased (P<0.01) the efficiency of LPC to induce AR. Although the percentage of AR of total sperm increased further at 80 µg/ml Hep, this effect was not consistent across the other parameters assessed. However, the highest values but not significant, were consistently for 80 µg/ml Hep, hence we elected to use this concentration of Hep for future experiments. This experiment showed, therefore, that LPC activity was also affected by Hep dose.

Table 2.5 LPC effects on acrosome status and viability (mean ± s.e.) after 15 min pre-incubation in different Hep concentrations.

Hep dose (µg/ml)	% AR of Total	% Fully AR of Total	% Viable AR of viable	% Viable Fully AR of viable
	Total		Viable	
10	69.7±2.09[a]	61.3±2.60[a]	64.1±2.67[a]	57.3±2.60[a]
40	83.8±1.68[b]	75.8±2.30[b]	82.7±2.04[b]	77.5±2.13[b]
80	90.3±1.35[c]	81.1±2.10[b]	89.2±1.68[b]	80±2.02[b]
100	86.4±1.52[bc]	80.9±2.02[b]	83.3±1.93[b]	79±1.98[b]

Each treatment was replicated three times. AR (Acrosome Reacted) = Fully AR + Capacitated (viable and non-viable sperm). Fully AR= AR only (viable and non-viable). Viable AR = Fully AR (viable) + capacitated (viable). Viable Fully AR = viable AR only. **[a-c]Values within the same columns with different superscripts differed at P<0.05.**

2.3.1.5 Expt. 2Av. Effect of Calcium Ionophore A23187 (CaIo) dose on percentage AR and sperm viability

Alternative protocols exist that make use of CaIo instead of Hep and LPC for terminal sperm capacitation and AR induction. The first series of experiments established the optimal period of time (i.e. 30 vs 60 min) required for optimal viability and to recover motility after treatment with different doses of CaIo (10, 15 or 20 µM). The period of recovery had no effect on either sperm motility or viability (Fig. 2.3), although numerically viability was greater for 30 than 60 min. Similarly, there was no effect of CaIo dose on motility or percentage viable and fully AR sperm (Fig. 2.3 and Table 2.6). However, CaIo was also an effective agent inducing AR and, in subsequent experiments, therefore, 10 µM was selected and the recovery period chosen was 30 min.

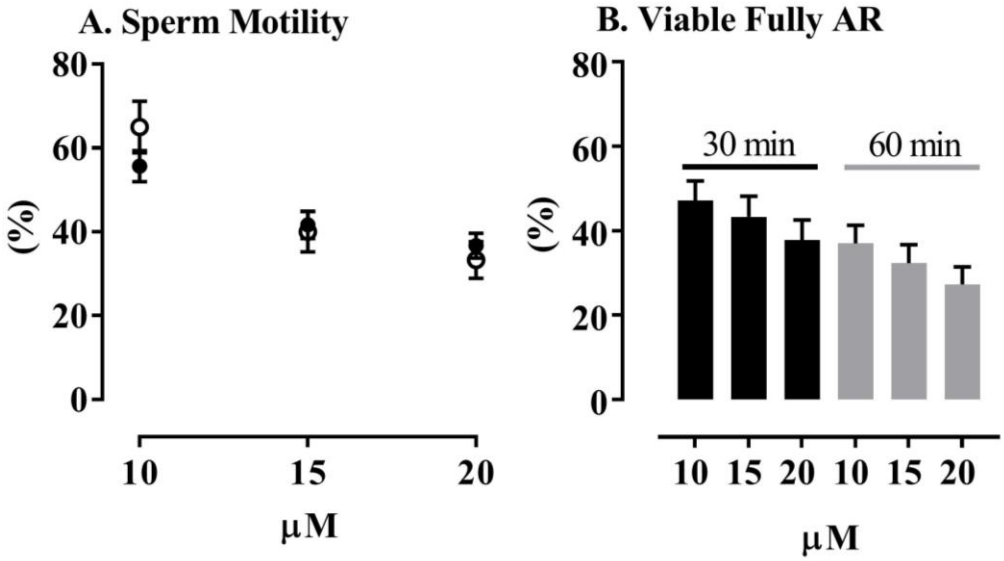


Figure 2.3 Percentage motile sperm (A) and percentage viable and fully acrosome reacted (FAR) sperm (B) after exposure to different concentrations of CaIo for 10 min. Sperm were incubated for either 30 or 60 min in modified BWW media containing 3% BSA at 38.5ºC with 5% CO_2 in air. The experiment was replicated three times. For (A) closed circles = 30 min post-incubation time, opened circles = 60 min.

Table 2.6 Dose effect of CaIo on acrosome status and viability (mean ± s.e.) after 10 min exposure and 30 min post-incubation in modified BWW media.

CaIo dose (μM)	% AR of Total	% Fully AR of Total	% Viable AR of viable	% Viable Fully AR of viable
	Total		Viable	
10	92.4±2.69	81.2±2.86	89.1±3.72	78.1±5.13
15	95.9±2.11	79.3±3.10	93.8±3.15	75.5±5.76
20	91.4±2.95	79.2±3.10	85.9±4.55	72.5±6.09

Each treatment was replicated three times. AR (Acrosome Reacted) = Fully AR + Capacitated (viable and non-viable sperm). Fully AR= AR only (viable and non-viable). Viable AR = Fully AR (viable) + capacitated (viable). Viable Fully AR = viable AR only. **There were no treatment effects.**

2.3.2 Study 2B: Sperm DNA decondensation

2.3.2.1 Expt. 2Bi. Combined effect of GSH and Hep on the proportion of sperm head decondensation after different incubation periods

GSH (15 mM) has been shown to induce sperm DNA decondensation when used in conjunction with the appropriate dose of Hep (80 µM, Delgado *et al.*, 2001). The next series of experiments sought to confirm this effect in our hands, and to identify the optimal dose of Hep and duration of co-incubation with our sire.

In the first of these experiments the percentage decondensed sperm heads increased with both dose of Hep ($P<0.001$) and duration of co-incubation with GSH ($P=0.001$) (Fig. 2.4). There was no interaction between dose of Hep and duration of culture. Therefore, the highest percentage decondensed heads occurred when sperm were incubated with 80 µM Hep/15 mM GSH for 20 h.

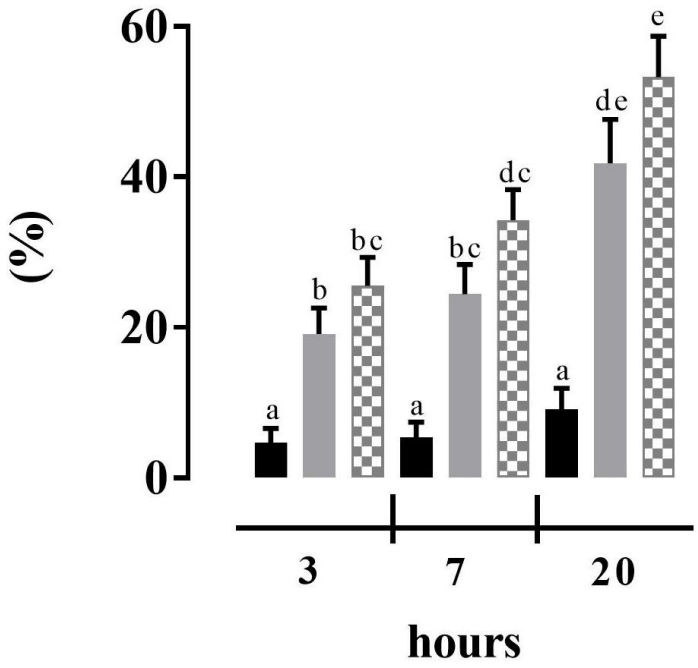


Figure 2.4 Percentage decondensed sperm heads following exposure to 15 mM GSH and 4 (black bar), 40 (grey bar) and 80 (chequered bar) µM Hep for 3, 7 and 20 h co-incubation at 38.5ºC with 5% CO_2 in air. The experiment was replicated three times. Values with different superscripts differed at $P<0.05$.

2.3.2.2. Expt. 2Bii. Synergistic effect of GSH with either LPC or CaIo on the percentage of sperm head decondensation and AR

In contrast to GSH alone and LPC+GSH, CaIo+GSH increased (P=0.003) decondensed sperm as a percentage of total sperm (Table 2.7). Under our experimental conditions most sperm (~92%) AR, but the effects of LPC or CaIo is to increase (P=0.009) this figure further (~99%). LPC+GSH increased (P<0.05) fully AR sperm (that had undergone sperm-head decondensation) as a percentage of the total population of sperm that had undergone DNA decondensation. However, there was no differential effect of treatment on fully AR sperm (with non-decondensed (or normal) heads) as a percentage of non-decondensed (or normal) sperm.

Table 2.7 Combined effect of GSH (15mM) + Hep (80µM) and LPC (100µg/ml) or CaIo (10 µM) on the percentage of Acrosome Reacted (AR) and viable sperm (mean values ± s.e.) following 20 h of incubation.

Sperm treatment	% DH of total	% AR of Total	% Fully AR of DH	% Fully AR-N
	Total		Total DH	Total N
GSH	43.2±2.31[a]	92.2±1.94[a]	80.1±5.66 [a]	69.1±8.08
LPC+GSH	43.5±4.11[a]	98.8±1.28[b]	97.5±3.49 [b]	95.3±6.59
CaIo+GSH	67.8±2.15[b]	99.2±0.61[b]	65.1±4.74 [a]	77.1±10.1

Each treatment was replicated three times. AR (Acrosome Reacted) = Fully AR + Capacitated (viable and non-viable sperm). Fully AR= AR only (viable and non-viable). Viable AR = Fully AR (viable) + capacitated (viable). Viable Fully AR = viable AR only, DH (viable and non-viable sperm with decondensed head, including fully AR and capacitated), N (viable and non-viable sperm with normal head, including fully AR and capacitated). **[a-b]Values within the same columns with different superscripts differed at P< 0.05.**

2.4 DISCUSSION

This study confirmed that Hep+LPC and CaIo are AR inducers with similar efficiency under optimal working conditions, leading to a high percentage of viable spermatozoa (~55-65%; Table 2.3 and Fig. 2.3) which has undergone fully AR (~80%; Table 2.5 and 2.6). Furthermore, GSH acts as an effective reducing agent, inducing nuclear decondensation in more than 50% of total population when it is co-incubated with high Hep dose (Fig 2.4). Finally, LPC followed by GSH/Hep exposure seems to be more efficient in inducing full AR compared to CaIo under the same conditions. However, CaIo appears to have a synergistic effect on sperm DNA decondensation in the presence of GSH/Hep (Table 2.7).

2.4.1 Study 2A: Removal of acrosome membrane

2.4.1.1 Hep followed by LPC sperm treatment

This series of experiments sought to optimise working conditions for Hep in order to attain the maximum proportion of viable AR sperm following LPC treatment. The dose of LPC selected (100 µg/ml) was based on studies which reported that higher concentrations than this reduced sperm motility and increased damage of sperm DNA (Parrish *et al.*, 1988; Zambrano *et al.*, 2016), whereas the chosen period for LPC incubation (15 min) was the optimal time established by Parrish *et al.* (1988) when they exposed pre-treated ejaculated bull spermatozoa (Hep, 10 µg/ml for 4 h) to 100 µg/ml LPC for several periods of time. They also found that the ability of LPC to induce AR was dependent on previous incubation with Hep, which is required for spermatozoa to undergo capacitation. Similar effects were observed in the current study (Table 2.2), which achieved a similar percentage of AR to Parrish *et al.* (1988) but with a shorter Hep incubation time (2 h). These differences might be attributed to sperm source as frozen-thawed spermatozoa are more sensitive to be capacitated than ejaculated sperm as a result of the cryopreservation process. Purdy and Graham (2004) also noticed the same positive correlation between Hep pre-incubation time and LPC efficiency, and obtained similar results to Parrish *et al.* (1988). In addition, they also observed

that LPC activity was dependent on Hep concentration; a fact that was confirmed in the present study (Table 2.5).

The current study established optimal conditions for inducing the AR (i.e. ≥ 40 µg/ml Hep for 15 min; 100 µg/ml LPC for 15 min) for frozen-thawed sperm for the single sire under investigation. It is recognised, however, that these conditions would need to be optimised for other bulls (Parrish *et al.*, 1988; Molnarova *et al.*, 2006; Gervasi *et al.*, 2011; Pérez-Aguirreburualde *et al.*, 2012).

2.4.1.2 CaIo sperm treatment

This study optimised CaIo dose as well as post-incubation time required to recover sperm motility following removal of CaIo in order to obtain the maximum percentage of viable, fully AR spermatozoa. In this case, it was not necessary to include an additional pre-incubation with capacitating agents because of the high Ca^{2+}_{i} induced by CaIo; this induced AR directly thereby bypassing capacitation (First and Parrish, 1987).

Although the three assessed CaIo doses were equally efficient at inducing AR, the 10 µM dose was selected because, numerically, it provided the highest percentage of viable fully AR (Table 2.6) following incubation for a minimum period (30 min) in modified BWW media to recover their motility (Fig. 2.3). Interestingly, bull spermatozoa were immobilised rapidly in presence of high doses of CaIo in agreement to previous studies which reported a similar effect in mouse, sheep, human and also, in cattle (Shams-Borhan and Harrison, 1981; Hong *et al.*, 1985; Suarez *et al.*, 1987; Tateno and Mikamo, 1987).That could be related to the fact that iophonores transport Ca^{2+} ions continuously into sperm cytoplasm in the presence of external millimolar Ca^{2+} levels. This would increase Ca^{2+}_{i} excessively until it became toxic to the gamete, interfering with proper cellular function such as mitochondrial production of ATP. As a consequence, the loss of flagellar movement might be associated with a reduction of available ATP (Campbell, 1983; Suarez *et al.*, 1987).

Nevertheless, empirical studies with erythrocytes demonstrated that the addition of albumin removed A23187 from their plasma membranes, reversing the ionophore effect (Sarkadi *et al.*, 1976; Anderson and Lovrien, 1981). Subsequently, Suarez *et al.* (1987) restored mouse sperm motility that had been treated by A23187 by adding high concentrations of BSA. Accordingly, in the current study immotile bull spermatozoa, as a result of exposure to high CaIo doses (10, 15 and 20 µM), were able to regain sperm motility following an incubation period in modified BWW media where BSA concentration was higher (3%= 30 µg/ml) than standard (0.3%= 3 µg/ml).

2.4.2 Study 2B: Sperm DNA decondensation

2.4.2.1 GSH/Hep sperm treatment

This experiment was focused on verifying if optimal conditions (GSH/Hep timing and dosage) described previously by several studies in bull spermatozoa (Delgado *et al.*, 2001; Sánchez-Vázquez *et al.*, 2008; Sekhavati *et al.*, 2012; Canel *et al.*, 2017) were also the same for our sire. It was corroborated that GSH (15 mM) requires the proper Hep dose (80 µM) to decondense sperm DNA efficiently (Fig. 2.4), and their combined action seems to be time-dependent, reaching the maximum values of sperm decondensed heads (53%) following longer co-incubation periods (20 h). Similarly, GSH-Hep has been reported to decondense sperm nuclei in other mammalian species such as human, mouse, hamster, rat and guinea pig (Reyes *et al.*, 1989 and 1996; Sánchez-Vázquez *et al.*, 1996, 1998) under different optimal conditions which are highly dependent on protamine differences (type and proportion) in mammalian sperm chromatin (Section 1.3.1.2).

Therefore, the reproducibility of the GSH/Hep effect on *in vitro* sperm chromatin decondensation in several mammalian species, together with the fact that GSH and GAGs like Hep are present in both mammalian gametes, and in the case of GAGs are also in reproductive tracts and secretions (Luberda, 2005; Sánchez-Vázquez *et al.*, 2008), leads to the suggestion that the physiological mechanism of sperm DNA decondensation is mediated by the simultaneous contribution of GSH and Hep during *in vivo* fertilisation.

It should be noted, however, that there is strong evidence, based on comparative studies which evaluated the decondensation speed between full spermatozoa or isolated sperm nuclei, to postulate that intact sperm plasma membranes act as a physical barrier, hampering the accessibility of GSH and Hep to sperm chromatin and thus delaying its decondensation (Delgado *et al.*, 2001; Romanato *et al.*, 2005; Sánchez-Vázquez *et al.*, 2008). That could be one of the main reasons why it is necessary to employ high concentrations of these components when the sperm nucleus is decondensed *in vitro* in contrast to physiological levels during the *in vivo* process (Reyes *et al.*, 1989; Delgado *et al.*, 2001; Julianelli *et al.*, 2012). The requirement for high concentrations of GSH and Hep, however, could be also related to suboptimal oocyte IVM which could lead to insufficient intracellular accumulation (Section 1.3.2.2) (Abeydeera *et al.*, 1998; Furnus *et al.*, 1998; Yamauchi and Nagai, 1999; Brad *et al.*, 2003; Zuelke *et al.*, 2003; Luberda, 2005).

2.4.2.2 GSH/Hep combined to Hep+LPC or CaIo

In light of the foregoing findings, the last set of experiments evaluated the impact of disrupting sperm membranes prior to GSH/Hep treatment on the percentage of sperm decondensed heads. Interestingly, CaIo increased the percentage of total spermatozoa with decondensed heads (68%, Table 2.7), enhancing the baseline achieved by GSH/Hep (43%) in absence of any additional sperm pre-treatment, and in contrast to Hep+LPC which did not have any effect on sperm DNA decondensation (44%). It is thought that these findings could be related to the ability of CaIo to permeabilise cellular membranes to certain ions. In this case, besides increased Ca^{2+} permeability across sperm plasma membrane which improved AR efficiently (to 99%), perhaps it also permeabilised the nuclear envelope, facilitating the entrance of GSH and Hep into the sperm nucleus. In contrast, Hep+LPC would be only focused on disrupting sperm plasma and acrosomal membranes to trigger AR with the same efficiency as that of CaIo (99%). As few studies have investigated such combinations, however, it is difficult at this stage to speculate further on the current set of findings.

2.4.3 Conclusions

Heparin (Hep) in conjunction with LPC or high doses of calcium ionophore A23187 (CaIo) can be used as effective AR agents, whereas simultaneous exposure to GSH and heparin leads to effective sperm DNA decondensation in bull spermatozoa prior to bovine ICSI. In this chapter, optimal working conditions for all these reagents were optimised for frozen-thawed semen from a single sire. It was corroborated that LPC action is both time- and dose-dependent to previous Hep exposure. However, the effect of CaIo on AR induction is independent of its dose, at least with regard to the following three concentrations (10, 15 and 20 μM) which were evaluated in this study. Finally, it was confirmed that GSH requires the simultaneous action of a specific dose of Hep to be an effective reducing agent, and its action unpacking sperm chromatin is enhanced by longer incubation times. Moreover, GSH action is potentiated by previous exposure to high dose CaIo.

Subsequently, next chapter is focused on the study of artificial egg activation following ICSI which is a required step of bovine microinjection process.

CHAPTER 3:

Artificial oocyte activation (AOA): Strontium versus conventional treatments

3.1 INTRODUCTION

Fertilisation success and subsequent embryo development following ICSI is highly dependent on the ability of oocytes to trigger appropriate Ca^{2+} transients responsible for activating all cellular processes which mediate resumption of meiosis and enable oocyte-to-embryo transition (Section 1.3.2). Limited success of bovine ICSI seems to be associated principally to the absence of an adequate oocyte activation stimulus due to a failure to evoke sustainable Ca^{2+} oscillations, and the consequential inability to induce low MPF activity following sperm introduction (Fujinami *et al.*, 2004; Malcuit *et al.*, 2006b). Therefore, microinjected bovine oocytes are regularly exposed to AOA treatments as a part of the ICSI procedure in order to support embryo development (Section 1.3.3) (García-Roselló *et al.*, 2009; Galli *et al.*, 2014; Salamone *et al.*, 2017).

Among the great variety of treatments developed to induce AOA, ethanol (Eth, 7%) (Nagai, 1987) and calcium ionophores (CaIo) such as A18237 (Liu *et al.*, 1998) or ionomycin (Susko-Parrish *et al.*, 1994) combined to 6-dimethylaminopurine (6-DMAP) or cycloheximide (CHX) (Presicce and Yang, 1994; Rho *et al.*, 1998a; Saeki *et al.*, 1997; Hou *et al.*, 2009) stand out as being the most widely used for bovine ICSI (Section 1.3.3.1). Parthenogenetic studies reveal that a previous short period of exposure to Eth (7%, 5 min) before a longer period of incubation (5h) with CHX together with cytochalasin B (CB) a cell-permeable mycotoxin required to inhibit reversible actin filament polymerization, thus preventing the extrusion of second polar body (Carter, 1967); or similarly, CaIo exposure (5 min) prior to prolonged incubation (3-4h) in 6-DMAP, led to comparable high levels of cleavage and blastocyst development (Presicce and Yang, 1994; Wang *et al.*, 2008). Nevertheless, when these treatments are applied following ICSI, Eth alone (7%) seems to be

the most successful in terms of pregnancy and birth of healthy calves (Hamano *et al.*, 1999; Horiuchi *et al.*, 2002; Oikawa *et al.*, 2005). This is in contrast to the apparent reduced capacity of CaIo+6-DMAP to produce competent blastocysts capable of development to term (Oikawa *et al.*, 2005) (Table 1.4).

This may be related to the effects of protein synthesis/phosphorylation inhibitors used for AOA, whose low specificity for particular proteins involved in controlling cell-cycle exit from MII meiotic arrest could also inhibit important proteins or pathways implicated in cellular events, thereby negatively impacting on embryo development (Kharche and Birade, 2013). In particular, genetic analysis of bovine embryos derived from ICSI, SCNT or parthenogenesis have demonstrated that an increase in chromosomal abnormalities in embryos exposed to 6-DMAP during AOA (Szöllösi *et al.*, 1993; Avery *et al.*, 1998; Rho *et al.*, 1998; Bhak *et al.*, 2006; Felmer and Arias, 2015; Arias *et al.*, 2016), which ultimately led to elevated rates of embryo mortality and pregnancy loss (Hill *et al.*, 2000; Slimane-Bureau and King, 2002).

However, even if Eth and CaIo provoke membrane hyperpolarization accompanied by a sufficient Ca^{2+} rise to efficiently induce egg activation (Tosti *et al.*, 2002), multiple Ca^{2+} transients following this initial rise lasting and for at least 23 h is the required to assure the formation of male and female pronuclei, nuclear envelope breakdown and syngamy (Nakada *et al.*, 1995). Perturbation of normal Ca^{2+} signaling during egg activation has irreversible effects on developmental capacity of embryos that could undermine chances of pregnancy (Ducibella *et al.*, 2002; Ozil *et al.*, 2005; Rogers *et al.*, 2006; Ozil *et al.*, 2006; Ross *et al.*, 2009).

In contrast, strontium (Sr^{2+}), a multi-spike-Ca^{2+} inducer (Section 1.3.3.2), is associated with beneficial effects on later embryo development in the mouse, leading to the birth of healthy offspring (Yamazaki *et al.*, 2005; Choi *et al.*, 2013). Apparently, this is due to its ability to generate long-lasting Ca^{2+} oscillations with a similar pattern to that triggered by the spermatozoon following entrance to the oocyte during fertilisation (Bos-Mikich *et al.*, 1997;

Yamazaki *et al.*, 2005). Nevertheless, $SrCl_2$ is not established as main AOA treatment in other mammalian species which require exogenous egg activation stimulus. This is because its activity generating Ca^{2+} oscillations is restricted only to mouse and rat (Day *et al.*, 2000; Tomashov-Matar *et al.*, 2005). The reduced number of studies that have monitored Ca^{2+} oscillations induced by $SrCl_2$, have also contributed to our general limited understanding of its mechanism of action. And finally, the necessity of adjustments in dose, culture media requirements and incubation times among domestic species have led to restrict its use (Méo *et al.*, 2005; Yamazaki *et al.*, 2005; Che *et al.*, 2007; Méo *et al.*, 2007; Choi *et al.*, 2013).

In the specific case of cattle, the actions of Sr^{2+} seem to be improved in combination with a calcium ionophore, reflecting its beneficial effects on the blastocyst quality (Yamazaki *et al.*, 2005). However, bovine blastocyst rates from parthenogenetic or fertilized embryos by ICSI still remains low when $SrCl_2$ is used as the AOA (Méo *et al.*, 2004; Méo *et al.*, 2005; Méo *et al.*, 2007; Bevacqua *et al.*, 2010). For this reason, the primary objective of the current chapter was to devise a novel AOA protocol that includes $SrCl_2$, and to assess its effect on bovine embryo development following parthenogenetic activation.

3.1.1 Objectives

Principal purpose of this study is to develop an efficient AOA protocol to that leads to high rates of embryo development. Therefore, initial experiments evaluated oocyte responses to $SrCl_2$ alone as an activating agent for parthenogenesis and also to conventional treatments used commonly in bovine ART. Subsequent studies investigated combinations of $SrCl_2$ with other routinely used agents (i.e. Eth and CaIo (A23187)), and finally its co-incubation with CHX to induce parthenogenetic activation.

3.2 MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich Company Ltd. (Dorset, England) unless otherwise indicated.

3.2.1 Study design, experiments and replication

3.2.1.1 Study 3A: Strontium *vs* conventional treatments

3.2.1.1.1 Expt. 3Ai. Effect of strontium ($SrCl_2$) activation on parthenogenetic development of bovine oocytes

As an initial pilot study to test its efficacy in our hands, bovine MII oocytes were cultured with $SrCl_2$ (20mM) for 6 h either in the presence (diploid embryos) or absence (haploid embryos) of CB (7.5 µg/ml). Putative zygotes were cultured to the blastocyst stage. Development was assessed on both Day 2 and Day 8.

3.2.1.1.2 Expt. 3Aii. Embryo development following activation with either Ethanol (Eth) or Calcium Ionophore A23187 (CaIo) in presence of Cycloheximide (CHX) and Cytochalasin B (CB)

Again, we wished to check in our laboratory the efficacy of conventional AOA protocols employed in bovine, which consist of short exposure in Eth or CaIo followed by longer incubation periods in CHX. For this purpose, two treatment groups (Eth+CHX/CB and CaIo+CHX/CB) were compared together with two negative controls (i.e. DMSO+CHX/CB and DMSO alone), as CaIo dissolves more readily in the presence of DMSO (De Jonge and Barratt, 2013). Reagents were added at the following rates (Eth, 7%v/v; CaIo 5µM; CHX, 10µg/ml and CB, 7.5 µg/ml). Embryo development was assessed to the blastocyst stage, Day 8.

3.2.1.2 Study 3B: Strontium plus different inducers for AOA

3.2.1.2.1 Expt. 3Bi. Evaluation of bovine oocyte activation using combinations of $SrCl_2$ with either Eth and CaIo

This experiment sought to determine if combinations of the activating reagents used in Experiments 3Ai and 3Aii would lead to greater post-activation development to the blastocyst stage. Therefore combinations of $SrCl_2$ (20 mM) with either Eth (7%v/v) or CaIo (5µM) were compared to either Eth + CHX (10µg/ml) (considered a positive control) or $SrCl_2$ alone (considered a negative control). CB (7.5 µg/ml) was included in all cases. Embryo development was assessed to the blastocyst stage, Day 8.

3.2.1.2.2 Expt. 3Bii. Assessment of a novel combination of $SrCl_2$ with CaIo and CHX on activation of bovine oocytes

To determine if post-activation development could be improved further, the combined effect of $SrCl_2$ (20 mM) + CaIo (5µM) + CHX (10µg/ml) was compared to treatment combinations used in Experiments 3Aii (Eth+CHX and CaIo+CHX) and 3Bi ($SrCl_2$+CaIo), all in the presence of CB (7.5 µg/ml). Embryo development was assessed to the blastocyst stage, Day 8. Embryo quality was further assessed by immunostaining to determine total cell number and allocation to the inner cell mass (ICM) and trophectoderm (TE).

3.2.2 Oocyte collection and *in vitro* maturation (IVM)

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in warmed PBS. Before follicle aspiration, ovaries were rinsed first with Eth (70% v/v) and then several times with warmed PBS. Cumulus-oocyte-complexes (COCs) were aspirated from follicles with a diameter of 5 to 9 mm using an 18-gauge needle attached to a 10 ml syringe. Follicular fluid was deposited into 50 ml sterile centrifuge tubes at 39ºC for 15 min approximately. The pellet from aspirated follicular fluid was spread into a 100 mm Petri dish and COCs with more than four layers of cumulus cells and dark homogenous cytoplasm for IVM (Fig. 3.1-A) were selected. These COCs were washed twice in HEPES-buffered cell culture medium 199 (Hepes-TCM199,

M7528) supplemented with 5% heat-inactivated Fetal Calf Serum (FCS, F9665), 0.1 mg/ml glutamine and 50 units and 0.05 mg/ml of penicillin-streptomycin and then, in bicarbonate-buffered maturation (TCM199, M2154) supplemented with 10% FCS, 0.1 mg/ml glutamine, 0.01 mg/ml follicle stimulating hormone (FSH), 0.01 mg/ml luteinizing hormone (LH) and 50 units and 0.05 mg/ml of penicillin and streptomycin (Pen/Strep, P4333) (see Appendices I.b, IV.q,r). Groups of 20-25 COCs were cultured in 500 µl maturation media in four well-dishes (Nunc, Denmark) for 24 h at 38.5ºC in a humidified atmosphere of 5% CO_2 in air.

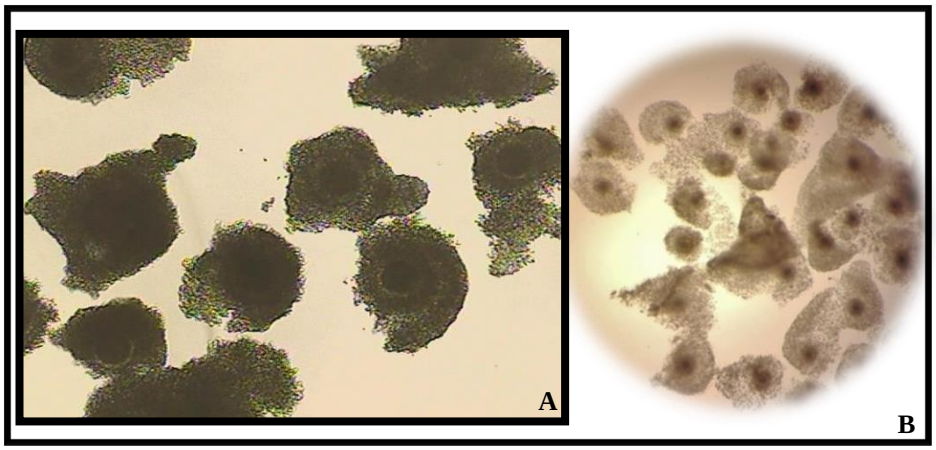


Figure 3.1 Bovine cumulus-oocyte-complexes (COCs): A) Grade 1 and 2, with more than four layers of cumulus cells and dark homogenous cytoplasm; B) expanded COCs after 24 h of IVM.

3.2.3 Parthenogenetic activation

After 24 h IVM (Fig. 3.1-B), cumulus cells were removed. First, COCs were incubated in 500 µL Hepes-TALP media supplemented with hyaluronidase (300 IU/ml, see Appendix IV.g) for 2 min at 39ºC and then, gently pipetted for a further 2 min. Denuded oocytes were washed twice in Hepes-TALP media (see Appendix II.d) and those oocytes with a visible first polar body were transferred to calcium and magnesium free Fert-TALP media (Ca^{2+}-Mg^{2+}-Free Fert-TALP, see Appendix II.b) under a humidified atmosphere of 5% CO_2 in air until oocyte activation. Oocytes were randomly distributed and activated by the following treatments: a) 7% v/v Eth dissolved in Hepes-TALP media for 5 min at room temperature and then incubated in 40 µl drops containing Fert-

TALP media supplemented with 10 µg/ml CHX and 7.5 µg/ml CB (see Appendices IV.h,i) under light mineral oil (Merk Millipore, United Kingdom) for 5 h at 38.5ºC in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 (Eth+CHX/CB); b) 5 µM CaIo reconstituted in 0.1% DMSO and dissolved in Hepes-TALP media for 5 min at room temperature followed by 5 h in Fert-TALP media with CHX and CB (CaI+CHX/CB); c) 20 mM $SrCl_2$ (see Appendix IV.j) in Ca^{2+}-Mg^{2+}-Free Fert-TALP media supplemented with CB for 6 h ($SrCl_2$/CB); d) 7% v/v Eth in Hepes-TALP media for 5 min, followed by 6 h in Ca^{2+}-Mg^{2+}-Free Fert-TALP media supplemented with $SrCl_2$ and CB (Eth+$SrCl_2$/CB); e) CaIo in Hepes-TALP media for 5 min following $SrCl_2$ and CB in Ca^{2+}-Mg^{2+}-Free Fert-TALP media for 6 h (CaI+$SrCl_2$/CB); f) $SrCl_2$, CB and CHX in Ca^{2+}-Mg^{2+}-Free Fert-TALP media for 6 h, immediately after CaIo activation for 5 min (CaIo+$SrCl_2$/CB/CHX). Oocytes from all treatments were washed three times in fresh warmed Hepes-TALP media and four times in Fert-TALP media before incubation for 5 h in Fert-TALP media supplemented with CHX and CB or for 6 h in Ca^{2+}-Mg^{2+}-Free Fert-TALP media with 20 mM $SrCl_2$; except $SrCl_2$/CB group where oocytes were incubate directly for 6 h without any extra wash step.

3.2.4 Embryo culture

Following oocyte activation, parthenotes were rinsed three times in Hepes-buffered synthetic oviduct fluid media (Hepes-SOF, see Appendix III.b) to remove completely the rest of CHX and CB. Afterwards, 15-20 embryos were cultured in 40 µl drops of modified SOF media (mSOFaaci, see Appendix III.a) covered with mineral oil at 38.5ºC in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. On Day 2 (Fig. 3.2), proportion cleaved together with the proportion of cleaved embryos with ≥ 4 cells was assessed. The proportion activated oocytes and cleaved zygotes that gave rise to blastocysts were assessed on Day 8. mSOFaaci media was replaced on Day 2 and Day 5.

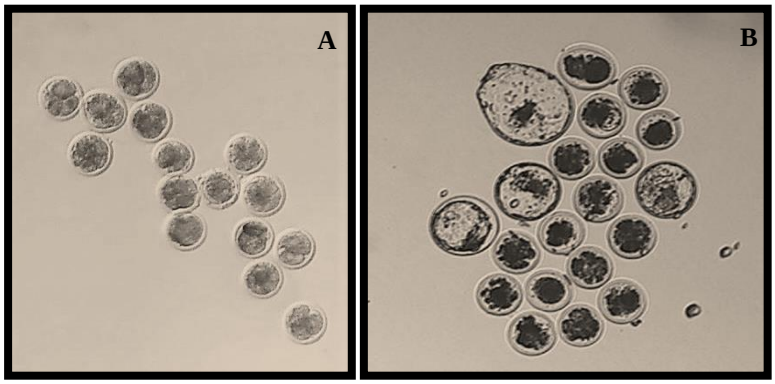


Figure 3.2 Bovine embryo culture: A) Parthenotes at 48 hpa; B) Blastocysts from oocyte activation protocols at 192 hpa.

3.2.5 Embryo immunocytochemistry staining

All parthenogenetic embryos which reached blastocyst stage by Day 8 underwent immunostaining to assess cell number (Total, ICM and TE). This study used the specific protein marker SOX2, which is restricted to the ICM at blastocyst stage in the bovine embryo (Fig. 3.3) (Goissis and Cibelli, 2014). For this purpose, blastocysts were fixed using 4% PFA in PBS for 15 min at room temperature following removal ZP with warmed Tyrode's acid (T1788) and rinsing in warmed PBS. Fixed embryos were washed twice in PBS supplemented with 1% BSA (each for 5 min) in order to eliminate PFA. They were permeabilized by incubation with 0.1% Triton X-100 in PBS at room temperature for 15 min. Embryos were then rinsed twice with 1% wt/v BSA-PBS for 5 min each before 1 h incubation in blocking solution (5% BSA-PBS wt/v, 10% Donkey serum and 0.3M Glycine) at room temperature to avoid non-specific immunoreactions. Then, blastocysts were incubated with a goat primary antibody against SOX2 (1:100, Santa Cruz Biotechnology, USA) overnight at 4ºC. The following day, embryos were washed four times in 1% BSA-PBS wt/v (each wash lasting 10 min), followed by 1 h incubation with anti-goat secondary antibody (Cy3, 1:500, Jackson Immuno Research Europe Ltd, UK) at room temperature in the dark. Embryos were then washed again four times in 1% BSA-PBS wt/v (each was lasting 10 min). Finally, stained embryos were mounted onto a slide with DAPI-fluoroshield mounting medium, covered with a small coverslip and sealed with nail varnish. Samples were

analysed under a fluorescent microscope (Leica DMR) after storage for 30 min at 4°C to enhance the fluorescence signal.

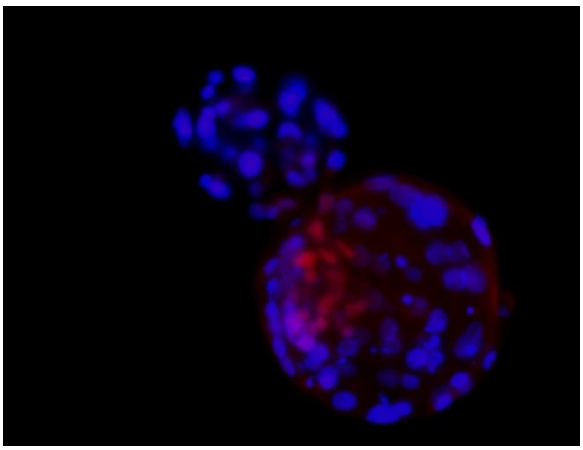


Figure 3.3 Hatched parthenogenetic blastocyst with the inner cell mass labelled in red and, trophectoderm in blue.

3.2.6 Statistical analysis

Cleaved zygote and blastocyst development from different parthenogenetic treatments were analysed by Generalized Linear Regression assuming either binomial errors, adjusted by logit link functions for proportions, or Poisson errors, adjusted by logarithm link functions for blastomere counts. All statistical analyses were performed within the GenStat statistical package (17th Edition, VSN International, 2011). Differences were considered significant at $P < 0.05$. Differences between individual means were established using Least Significant Differences (LSD) derived from residual errors of the statistical models. Proportions were converted to percentages for the purposes of data presentation and discussion.

3.3 RESULTS

3.3.1 Study 3A: Strontium vs conventional treatments

3.3.1.1 Expt. 3Ai. Effect of strontium ($SrCl_2$) activation on parthenogenetic development of bovine oocytes

The percentage of oocytes activated with $SrCl_2$ alone that cleaved and developed to the blastocyst stage was low in this study and was not dependent on the presence or absence of CB (Table 3.1).

Table 3.1 Effect of strontium on parthenogenetic development (Percentage, mean ± s.e.) of bovine oocytes.

Activation treatment	Treated oocytes (n)	Cleaved on Day 2 (% of activated)	≥4 Cells on Day 2 (% of cleaved)	Blastocysts on Day 8 (% of activated)
$SrCl_2$+CB	65	17 **(26.2±8.12)**	6 **(30.4±8.42)**	2 **(3.1±2.05)**
$SrCl_2$	66	17 **(25.7±8.01)**	7 **(45.2±8.28)**	2 **(3.1±2.05)**

Experiment was replicated four times. $SrCl_2$ (Strontium chloride, 20 mM in Ca^{2+}-Mg^{2+}-Free Fert-TALP media for 6 h), CB (cytochalasin B, 7.5 µg/ml).

3.3.1.2 Expt. 3Aii. Embryo development following activation with either Eth or CaIo in presence of CHX and CB

Both ethanol (Eth) and calcium ionophore (CaIo) in the presence of cycloheximide (CHX) and cytochalasin B (CB) were equally effective in activating oocytes and promoting development to Day 2, and both were greater ($P<0.001$) than DMSO alone or with CHX+CB (Table 3.2). However, activation with Eth+CHX+CB led to a significantly greater ($P=0.027$) percentage of blastocysts on Day 8. The solvent DMSO (0.1%) on its own or with CHX+CB activated a small number of oocytes, with one or two developing to the blastocyst stage.

Table 3.2 Cleavage and blastocyst development (Percentage, mean ± s.e.) following application of different activation protocols in bovine oocytes.

Activation treatment	Treated oocytes (n)	Cleaved on Day 2 (% of activated)	≥4 Cells on Day 2 (% of cleaved)	Blastocysts on Day 8 (% of activated)
Eth+ CHX+CB	62	49 **(78.7±7.03)[a]**	29 **(58.0±9.35)**	16 **(25.9±6.65)[a]**
CaIo+ CHX+CB	63	55 **(87.5±5.62)[a]**	32 **(58.6±8.78)**	6 **(9.5±4.63)[b]**
DMSO+ CHX+CB	62	12 **(19.5±6.88)[b]**	5 **(46.7±19.60)**	2 **(3.3±2.94)[b]**
DMSO	60	13 **(21.6±7.23)[b]**	6 **(44.3±18.29)**	1 **(1.7±2.14)[b]**

Experiment was replicated four times. Eth (Ethanol 7% in hepes-TALP media for 5 min), CHX (cycloheximide, 10 µg/ml)+CB (cytochalasin B, 7.5 µg/ml) in Ca^{2+}-Mg^{2+}-Free Fert-TALP media for 5 h, CaIo (Calcium ionophore A23187 dissolved in 0.1% DMSO, 5 µM in hepes-TALP media for 5 min), DMSO (Dimethyl sulfoxide, 0.1% in hepes-TALP media for 5 min). **[a-b]Values within columns with different superscripts differed at P< 0.05.**

3.3.2 Study 3B: Strontium plus different inducers for AOA

3.3.2.1 Expt. 3Bi. Evaluation of bovine oocyte activation using combinations of $SrCl_2$ with either Eth and CaIo

As $SrCl_2$ on its own led to poor rates of oocyte activation (Expt 3Ai), it was decided to assess if it could synergistically interact with other established activators to promote embryo development. The primary comparison of interest was $SrCl_2$+Eth+CB vs $SrCl_2$+CaIo+CB, with Eth+CHX+CB and $SrCl_2$+CB acting as positive and negative controls respectively. Although there were no significant differences between the different treatment combinations (Table 3.3), numerically, development to the blastocyst stage was superior for the $SrCl_2$+CaIo+CB combination than the $SrCl_2$+Eth+CB combination, and was similar to Eth+CHX+CB.

Table 3.3 Evaluation of different oocyte activation protocols involving combinations of strontium, ethanol and calcium ionophore on cleavage and blastocyst development (Percentage, mean ± s.e.).

Activation treatment	Treated oocytes (n)	Cleaved on Day 2 (% of activated)	≥4 Cells on Day 2 (% of cleaved)	Blastocysts on Day 8 (% of activated)
Eth+ CHX+CB	67	48 **(71.8±7.76)**	36 **(73.8±7.47)**	12 **(17.6±7.22)**
Eth+ $SrCl_2$+CB	68	31 **(45.9±8.53)**	16 **(53.1±10.4)**	5 **(7.3±4.94)**
CaIo+ $SrCl_2$+CB	72	39 **(54.2±8.23)**	27 **(68.5±8.64)**	13 **(17.7±6.95)**
$SrCl_2$+CB	42	16 **(37.3±10.77)**	8 **(53.4±14.5)**	3 **(7.7±6.81)**

Experiment was replicated four times. Eth (Ethanol 7% in hepes-TALP media for 5 min), CHX (cycloheximide, 10 µg/ml)+CB (cytochalasin B, 7.5 µg/ml) in Ca^{2+}-Mg^{2+}-Free Fert-TALP media for 5 h, CaIo (Calcium ionophore A23187 dissolved in 0.1% DMSO, 5 µM in hepes-TALP media for 5 min), $SrCl_2$ (Strontium chloride, 20 mM in Ca^{2+}-Mg^{2+}-Free Fert-TALP media for 6 h), DMSO (Dimethyl sulfoxide, 0.1% in hepes-TALP media for 5 min).

3.3.2.2 Expt. 3Bii. Assessment of a novel combination of $SrCl_2$ with CaIo and CHX on activation of bovine oocytes

As Méo *et al.* (2007) demonstrated that young oocytes (22-24h IVM) respond more efficiently to strong stimulus of AOA than matured (26-30h IVM) together with it has already established that addition of CHX or 6-DMAP following a previous induction of a great Ca^{2+}_i rise improve activation and blastocyst development (Bos-Mikich *et al.*, 1995; Alexander *et al.*, 2006); and taking into account that CHX leads to superior blastocysts in terms of normal diploidy when it is used in SCNT (Cha *et al.*, 1997; Winger *et al.*, 1997). The primary comparison in this study, therefore, was the new combination (i.e. $SrCl_2$+CaIo+CHX+CB) vs that used in Expt. 3Bi (i.e. $SrCl_2$+CaIo+CB). The inclusion of Eth+CHX+CB and CaIo+CHX+CB served as positive controls. Although percentage cleaved of activated and ≥4 cells of cleaved was similar between the four treatment combinations, the novel combination (i.e. $SrCl_2$+CaIo+CHX+CB) increased (P=0.026) percentage blastocysts of

activated (Table 3.4). Therefore, in all subsequent experiments this combination of agents was used for oocyte activation.

Table 3.4 Effect of a novel combination of $SrCl_2$ and other agents (CaIo, CHX and CB) on activation of bovine oocytes (Percentage, mean ± s.e.).

Activation treatment	Treated oocytes (n)	Cleaved on Day 2 (% of activated)	≥4 Cells on Day 2 (% of cleaved)	Blastocysts on Day 8 (% of activated)
Eth+ CHX+CB	107	86 **(80.4±3.36)[a]**	64 **(74.1±4.22)**	25 **(22.4±6.47)[a]**
CaIo+ CHX+CB	106	90 **(84.9±3.05)[a]**	56 **(62.4±4.54)**	16 **(16.6±5.82)[a]**
CaIo+ $SrCl_2$+CB	110	58 **(52.7±4.16)[b]**	39 **(68.1±5.43)**	13 **(17.1±5.72)[a]**
CaIo+$SrCl_2$ +CHX+CB	107	93 **(86.9±2.85)[a]**	59 **(63±4.45)**	36 **(35.8±7.43)[b]**

Experiment was replicated six times. Eth (Ethanol 7% in hepes-TALP media for 5 min), CHX (cycloheximide, 10 µg/ml)+CB (cytochalasin B, 7.5 µg/ml) in Ca^{2+}-Mg^{2+}-Free Fert-TALP media for 5 h, CaI (Calcium ionophore A23187 dissolved in 0.1% DMSO, 5 µM in hepes-TALP media for 5 min), $SrCl_2$ (Strontium chloride, 20 mM in Ca^{2+}-Mg^{2+}-Free Fert-TALP media for 6 h). **[a-b]Values within columns with different superscripts differed at P< 0.05.**

Total blastomere number, determined by immunostaining on Day 8, increased (P<0.001) with embryo stage but did not differ between the four activation protocols (Fig. 3.3-A and -B). Similarly, blastomere number within the ICM and TE did not differ between the four activation protocols.

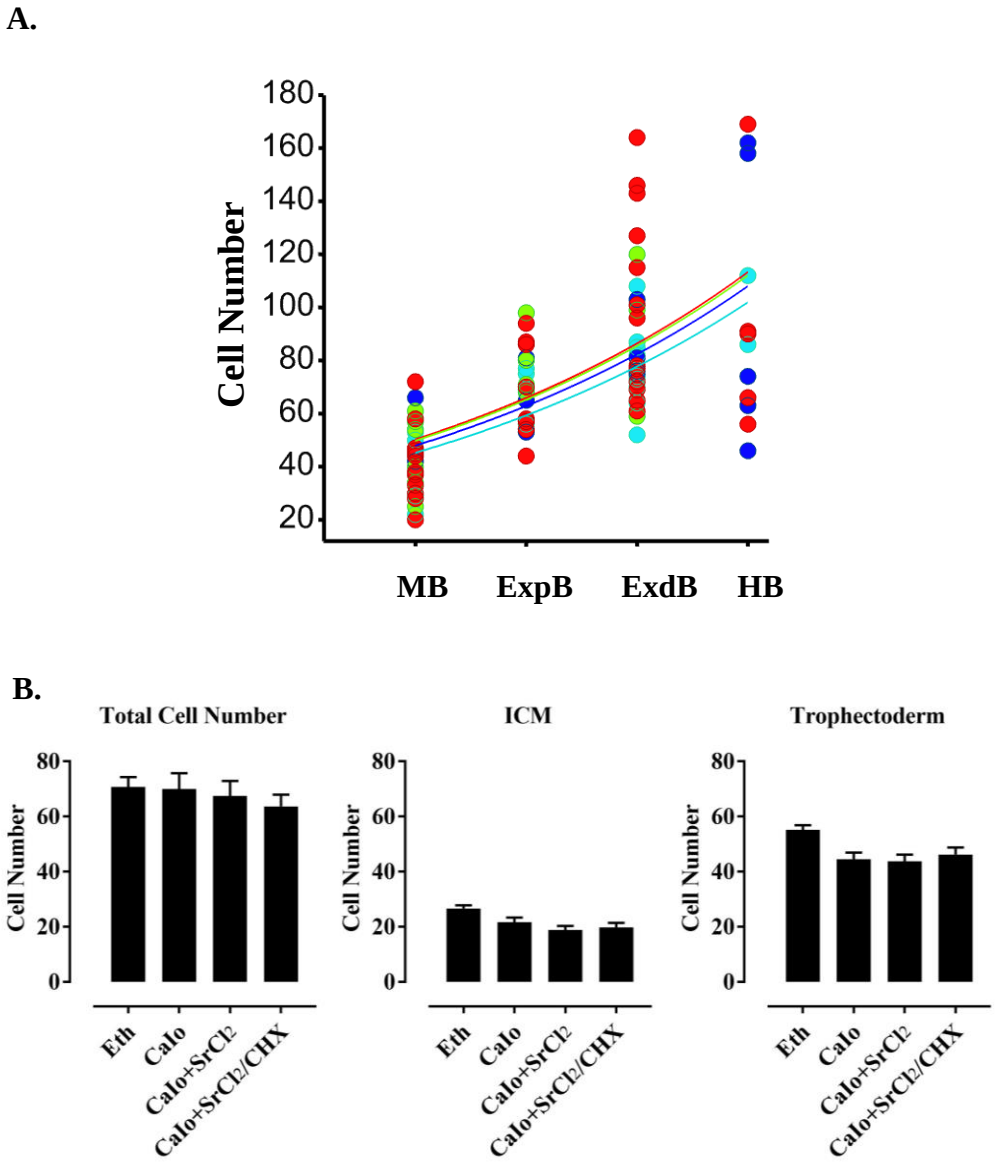


Figure 3.3 Embryo quality assessment after oocytes activated parthenogenetically. A) Total cell number increased (P<0.001) with embryo stage development on Day 8 (MB, mid-blastocyst; ExpB, expanding blastocyst; ExdB, expanded blastocyst; HB, hatched-blastocyst) from parthenogenetic embryos produced by: (●) Eth+CHX/CB, (●) CaIo+CHX/CB, (●) CaIo+$SrCl_2$/CB; (●) CaIo+$SrCl_2$/CHX/CB. B) Average number of total cells, cell in inner-cell mass (ICM) and trophectoderm (TE).

3.4 DISCUSSION

These series of experiments have developed a novel and efficient AOA protocol for bovine eggs. It includes $SrCl_2$, involved in inducing long term multi-Ca^{2+} oscillations from internal oocyte stores and combined with CHX, a protein synthesis inhibitor responsible for keeping low levels of MPF, following a previous short period of exposure to CaIo (A23187, a single-calcium-spike inducer through intracellular and extracellular calcium flux). This novel AOA protocol enhanced parthenogenetic blastocyst development (36%; Table 3.4) from activated bovine oocytes compared to more conventional treatments (Eth+CHX/CB= 22% and CaIo+CHX/CB= 16%) without detriment to blastocyst quality (Fig. 3.3).

3.4.1 Study 3A: Strontium *vs* conventional treatments

3.4.1.1 $SrCl_2$ as AOA inducer for bovine parthenogenesis

The activation potential of Sr^{+2} seems to be principally affected by oocyte age, Sr^{+2} concentration, time of exposure, media composition and its association with other chemical inducers; whose optimal values vary among species (Ma *et al.*, 2005; Méo *et al.*, 2005; Tomashov-Matar *et al.*, 2005; Che *et al.*, 2007; Choi *et al.*, 2013). Accordingly, as a starting point for initial experiments, the conditions described by Méo *et al.* (2005), who induced parthenogenetic activation in bovine oocytes with 20 mM $SrCl_2$ dissolved in Ca^{2+} and Mg^{2+} free culture medium for 6 h, were followed. This study, however, obtained very low levels of activation (cleavage on D2= 25%, Table 3.1) following $SrCl_2$ alone in presence of CB. This was well below that reported by Méo *et al.* (2005) (cleavage on D2 = 68%) under similar *in vitro* conditions, except they matured oocytes for 26h whereas IVM period was slightly shorter in this study (24h). The reason for this adjustment related to the design of prospective experiments described in the next chapter where it was planned to use this AOA treatment for bovine oocytes following ICSI. Oocytes from this group (ICSI) should be *in vitro* matured for 24h maximum to avoid detrimental effects associated with egg ageing and, therefore, this adaptation was required

to be able to use the same protocol in both treatment groups (ICSI *vs* Parthenogenesis).

Nevertheless, a preliminary experiment was undertaken where bovine oocytes were activated by $SrCl_2$ 24 and 26 hpm (data not shown) and a similar percentage of cleavage (25%) was observed. This discrepancy in results might be related to other factors such as age (Batista *et al.*, 2016), cattle genotype (Pontes *et al.*, 2010; Guerreiro *et al.*, 2014; Gimenes *et al.*, 2015) and nutritional status (Sartori *et al.*, 2016) which would determine initial oocyte quality and, indirectly, influence subsequent embryo development.

In addition, in first experiment (parthenogenetic activation of oocytes with $SrCl_2$ in the presence or absence of CB) also documented developmental competence differences between haploid and diploid embryos. Data revealed similar cleavage and blastocyst percentages between groups (25% and 3% respectively) (Table 3.1). Similarly, Ma *et al.* (2005) did not observe differences in activation percentage between haploid and diploid parthenotes following $SrCl_2$ parthenogenetic activation in mouse oocytes. However, in contrast to our findings, they noticed a marked increase in the percentage of diploid parthenogenetic blastocysts (56%) compared to haploid blastocysts (16%). This is in agreement with previous studies which demonstrated that diploid parthenotes have a greater ability to reach the blastocyst stage with a lower percentage of apoptotic cells compared to haploid parthenotes (Kaufman and Sachs, 1976; Cha *et al.*, 1997; Latham *et al.*, 2002; Liu *et al.*, 2002; Ma *et al.*, 2005). However, blastocyst rates in this experiment did not differ between oocytes activated with (diploid) or without (haploid) CB; possibly due to the low number of embryos that reached the blastocyst stage which was not enough to distinguish any statistical effect on embryo development.

3.4.1.2 Conventional AOA protocols for bovine parthenogenesis

The current study employed two conventional AOA protocols which included Eth or CaIo (A23187) combined with CHX-CB, whose high efficacy in

activating bovine eggs has already been established for different purposes such as parthenogenesis, SCNT and ICSI (Liu *et al.*, 1998; Rho *et al.*, 1998a; Bhak *et al.*, 2006; Shen *et al.*, 2008; Wang *et al.*, 2008; García-Roselló *et al.*, 2009; Korkmaz *et al.*, 2013; Felmer and Arias, 2015). In the current study, both treatments were used as control, as markers of initial oocyte quality. Tables 3.2 and 3.4 confirmed the equal potency of both protocols to activate successfully bovine eggs leading to high consistent and similar cleavage (~80%); however, blastocyst yields were always superior in Eth+CHX+CB treatment although no substantial differences were found in blastocyst quality (Fig. 3.3). This may be related to the use of different batches of oocytes and to variations in initial oocyte quality which would determine responses to AOA protocols.

3.4.2 Study 3B: Strontium plus different inducers for AOA

3.4.2.1 $SrCl_2$ with either Eth and CaIo for inducing AOA in bovine oocytes

In agreement with Yamazaki *et al.* (2005), this study showed that the action of $SrCl_2$ in activating bovine oocytes is enhanced following an exposure to CaIo (5 µM, 5 min). This combination seems also to be important in sheep and pigs, whereas it is not necessary in mice and rats (Ma *et al.*, 2005; Tomashov-Matar *et al.*, 2005; Che *et al.*, 2007; Choi *et al.*, 2013). Data from Expt. 3Bi showed that the combination of CaIo with $SrCl_2$ increased both cleavage (54%) and blastocyst (18%) percentages compared to $SrCl_2$ alone (37% and 7%, respectively) (Table 3.3).

Interestingly, Eth combined with $SrCl_2$/CB apparently had a negative effect on oocyte activation and further embryo development (Table 3.3) in contrast to its beneficial effect when it was combined with CHX/CB. Initially, it was thought that this combination (Eth+$SrCl_2$/CB) would be as efficient as CaIo+$SrCl_2$/CB because Eth causes a single great $Ca^{2+}{}_i$ rise as a result of extra- and intra-cellular Ca^{2+} flux, which would contribute to reduced MPF levels, allowing meiosis resumption (Vitullo and Ozil, 1992; Loi *et al.*, 1998). This would be similar to CaIo, apart from some differences found in peak levels and duration

of Ca^{2+} transient which defines the initial Ca^{2+} wave generated by each chemical inducer. CaIo generates a slightly high peak (426±51 nM) with a shorter period to return basal levels (~250 sec) than Eth (402±24 nM and 500 sec). However, in both cases, the Ca^{2+} rise differed from the original at fertilisation (521±39 nM and 150 sec, respectively) (Nakada and Mizuno, 1998). In addition, a characteristic feature associated with Eth activation is that it promotes a Ca^{2+} transient via $InsP_3$ (Alberio *et al.*, 2001). It is possible that this could explain why Eth has a detrimental effect before $SrCl_2$ incubation which was also reported by Méo *et al.* (2004).

Although there is limited knowledge regarding the molecular pathway by which Sr^{2+} induces the release of intracellular Ca^{2+} stores in order to evoke multiple Ca^{2+} oscillations, it is known that the mechanism of action of Sr^{2+} in mouse eggs appears to be mediated via $InsP_3$ receptors which requires simultaneous activation of PLC and $InsP_3$ (Zhang *et al.*, 2005). However, when Marshall and Taylor (1994) investigated the mechanisms implicated in the stimulation of $InsP_3$ receptors to mobilize intracellular Ca^{2+} stores in hepatocytes, they observed that these receptors were also permeable to several divalent ions including Ca^{2+}, Sr^{2+} and Ba^{2+}, although with a different degrees of selectivity (Ca^{2+}> Sr^{2+}> Ba^{2+}). Moderate elevations of Ca^{2+} and Sr^{2+} ions increased the affinity of $InsP_3$ receptors for their ligand ($InsP_3$), resulting in the stimulation of Ca^{2+} release. In contrast, higher concentrations of both ions (Ca^{2+}> 1μM; Sr^{2+}≥ 1mM) led to an inhibition of intracellular Ca^{2+} mobilization without affecting the ligand-affinity for the receptor. Therefore, in current study the high dose of Sr^{2+} used (20 mM) may have interfered with Eth activation, inhibiting the action of $InsP_3$ when bound to its receptor.

3.4.2.2 $SrCl_2$ with CaIo and CHX for inducing AOA in bovine oocytes

Nevertheless, activation rate and blastocyst development following the improved protocol (CaIo+$SrCl_2$/CB) remained low. As a consequence, the aim of the next step was to increase these percentages through the application of more potent AOA protocols based on the findings of Méo *et al.* (2007) who noticed that oocytes matured for 22 h required a stronger activation stimulus

(simultaneous action of three chemical agents) in order to obtain comparable results to oocytes matured for 26-30 h. This may be because "age" has a high impact on parthenogenetic activation (Vitullo and Ozil, 1992; Yang *et al.*, 1994; Liu *et al.*, 1998).

Méo *et al.* (2007) obtained the highest yield of blastocysts combining Io, $SrCl_2$ and 6-DMAP. The disadvantages of protein synthesis/phosphorylation inhibitors such as 6-DMAP or CHX is linked to detrimental effects on ploidy state and blastocyst development as a consequence of their low specificity for particular proteins during cell-cycle. It can alter the activity of a wide range of proteins including those responsible for preventing MPF activation; but also those with a key role during mitotic cell divisions and in the progression of chromosomal normal embryos with high capacity of development (Szöllösi *et al.*, 1993; Winger *et al.*, 1997; Loi *et al.*, 1998; Fernandes *et al.*, 2014; Lee *et al.*, 2015). However, bovine parthenogenetic and SCNT studies have suggested that these negatives effects are more remarkable when 6-DMAP is used in AOA protocol rather than CHX (Cha *et al.*, 1997; De La Fuente and King, 1998; Bhak *et al.*, 2006; Felmer and Arias, 2015).

Therefore, in this last experiment (Expt. 3Bii), the new and more comprehensive combination of agents for AOA bovine oocytes included CHX instead of 6-DMAP (i.e. CaIo+$SrCl_2$/CHX/CB). Interestingly, its addition during $SrCl_2$/CB co-incubation after a previous short exposure to CaIo enhanced oocyte activation and development of parthenogenetic blastocysts (Table 3.4). Cleavage (85%) was superior to CaIo+$SrCl_2$/CB in absence of CHX (54%) and comparable to that achieved with conventional treatments (Eth+CHX/CB= 80%; CaIo+CHX/CB= 85%). Also, blastocyst number from this new combination of AOA reached was greatest (36%) compared to the rest of treatments. Finally, when embryo stage (Fig. 3.3-A) and total cell number (Fig. 3.3-B) were analysed, no significant differences between AOA protocols were found.

However, it should be measured Ca^{2+} oscillations following the application of this new combination (i.e. CaIo+$SrCl_2$/CHX/CB) for bovine egg activation. It

would be necessary to assure that this improvement of parthenogenetic activation rates (cleavage and blastocyst development) is due to combined action of these reagents rather than other factors such as the ionic media composition. For example, several studies have reported that parthenogenetic activation in mice, rat and hamster can just occur by oocyte exposure to Ca^{2+}-free media (Miyamoto and Ishibashi, 1975; Kaufman *et al.*, 1977; Whittingham and Siracusa, 1978). Furthermore, the absence of extracellular calcium ions during egg activation also seems advantageous for the activity of calcium ionophore A23187. Bos-Mikich *et al.* (1995) showed that A23187 in Ca^{2+}-Mg^{2+}-free media induced a dual effect (increase cytoplasmic Ca^{2+} levels and inhibition of protein synthesis) in the mouse oocyte activation. On the other hand, it might be worthwhile to evaluate the ploidy state of these parthenogenetic embryos to get additional information about a possible negative effect of CHX on their normal competency to develop to the advanced embryo stages.

3.4.3 Conclusions

In conclusion, $SrCl_2$ combined with other egg-inducing agents (CaIo, CHX) could be employed efficiently as a suitable AOA protocol for bovine oocytes. In this chapter, the individual action of $SrCl_2$ as main inducer of AOA, together with its association with conventional agents such as Eth and CaIo, was evaluated. Once the beneficial action of exposing oocytes to CaIo prior to $SrCl_2$ incubation was demonstrated, a new combination was tested which also included the addition of CHX. This new AOA protocol (i.e. CaIo+$SrCl_2$/CHX/CB) showed encouraging results with regard to percentage of cleaved and blastocyst yields; which were higher than conventional AOA protocols. Therefore, a new AOA protocol has been developed to test on early and late embryo development as well as embryo quality following bovine ICSI in next chapter.

CHAPTER 4:

Developing bovine ICSI: Sperm preparations and artificial oocyte activation (AOA)

4.1 INTRODUCTION

The dairy cattle industry is keen to altering the sex ratio of calves born in favour of females with superior traits in order to maximise the productivity as cost-effectively as possible (Espinosa-Cervantes and Córdova-Izquierdo, 2013; Rath *et al.*, 2013; Butler *et al.*, 2014). Consequently, the use of sex-sorted semen represents a tremendous advantage if it could be employed for bovine embryo production by either AI or IVF in an efficient and cost-effective manner. Unfortunately, fertility rates with the use of sex-sorted semen are less than with unsorted semen as the sorting process itself has a detrimental impact on sperm physiology (Section 1.2.1.1).

Bovine ICSI offers the potential to overcome limitations associated with poor sperm viability and motility (e.g. with frozen-thawed sexed semen), but its success in cattle is limited. A problem commonly found in this species has been low fertilisation rates and limited embryo development following ICSI. This arises as a consequence of difficulties to decondense the sperm head and asynchronous pronuclei formation (Rho *et al.*, 1998a; Lagutina *et al.*, 2004; Xiao *et al.*, 2013). It is still not entirely clear if the main factor responsible for these constraints is the inability of the bull spermatozoon to induce consistent Ca^{2+} transients, thereby activating the oocyte, or if the problem lies with the oocyte itself which does not have full competency to respond adequately to the sperm stimulus (PLCζ) and to initiate events necessary to support the transition from oocyte to zygote (Rho *et al.*, 1998a; Chung *et al.*, 2000; Malcuit *et al.*, 2006a; Julianelli *et al.*, 2012; Vanden-Meerschaut *et al.*, 2014; Arias *et al.*, 2015; Salamone *et al.*, 2017).

The inability of the bull spermatozoon to fertilise the egg has been associated with the introduction of sperm membranes (sperm plasma membrane and

acrosome), as these act as physical barriers impeding the rapid release of PLCζ, and also hinder the exposure of sperm nuclear content to oocyte factors involved in chromatin unpacking. This leads to uncomplete egg activation and delays sperm DNA decondensation (Sections 1.3.1.1 and 1.3.1.2). This fact has been corroborated in several studies performed in different species including the pig, mouse and sheep where results showed higher fertilisation rates and embryo development following ICSI using spermatozoa in absence of sperm membranes (Katayama *et al.*, 2005; Morozumi *et al.*, 2006; Anzalone *et al.*, 2016). However, in addition, the incorporation of acrosomal content has a harmful effect on subsequent embryo development, especially in species with large heads such as bull spermatozoa (Morozumi and Yanagimachi, 2005; García-Roselló *et al.*, 2009; Kashir *et al.*, 2014).

On the other hand, because the majority of bovine oocytes fail to evoke Ca^{2+} oscillations following ICSI (Malcuit *et al.*, 2006a) there is already an established consensus on the use of artificial oocyte activation (AOA) protocols as a part of bovine ICSI procedure in order to improve blastocyst development (Rho *et al.*, 1998a; Chung *et al.*, 2000; Fujinami *et al.*, 2004; Oikawa *et al.*, 2005; García-Roselló *et al.*, 2009; Bevacqua *et al.*, 2010). It is quite likely that this failure is attributed, in part, to an inability on the part of the oocyte to initiate all events associated with egg activation following sperm entrance together with, the subsequent processing of sperm components which enables embryo development (Section 1.3.2). However, it is probably desirable that the selected AOA protocol should mimic closely the Ca^{2+} oscillations that occurs following gamete fusion. Although this pattern varies among species it appears to be crucial for subsequent development to term (Section 1.1.3) (Ducibella *et al.*, 2002; Ozil *et al.*, 2005; Rogers *et al.*, 2006; Ozil *et al.*, 2006; Ross *et al.*, 2009).

The success of ICSI in cows may be also influenced by certain attributes of bovine gametes such as the high resistance of both gamete membranes to be broken, large dimension of sperm head and high content of lipids contained in oocytes which hinders substantially the performance of ICSI and increases the probability of damage to the oocyte during sperm injection (Section 1.3.1.2).

Consequently, the piezo-drill was developed as a microinjecting system in order to simplify the oolemma breakage process and minimize the damage associated with it (Choi *et al.*, 2002; Wei and Fukui, 2002; Galli *et al.*, 2003; Yoshida and Perry, 2007). This alternative microinjecting system uses ultrasonic mechanical pulses to penetrate the ZP and break the oolemma, being less aggressive than conventional ICSI through ooplasm suction.

For these reasons, this chapter concerns the improvement of ICSI for bovine embryo production by employing different sperm pre-treatments which reproduce, as reliably as possible, events and processes that take place during *in vivo* fertilisation. Efforts were focused on triggering capacitation, AR and sperm DNA decondensation in order to remove both sperm plasma and acrosomal membranes and then, facilitate the unpacking of sperm chromatin before microinjection. On the other hand, the AOA treatment employed following ICSI was a new combination of chemical agents which included $SrCl_2$ as the principal activator. Interest in this particular chemical was based on findings in mice where $SrCl_2$ generated multiple long-term Ca^{2+} transients, mimicking the physiological pattern that spermatozoon evokes at fertilisation and thereby, enhancing mouse post-fertilisation development (Vitullo and Ozil, 1992; Bos-Mikich *et al.*, 1997; Yamazaki *et al.*, 2005).

4.1.1 Objectives

The principal objective of this chapter was to increase the pronuclear formation and blastocyst development following bovine ICSI through optimal sperm preparations and oocyte activation protocols developed in previous chapters. First, it was necessary to established optimal working conditions for ICSI protocol. Next, a series of experiments investigated the impact of removing the acrosome, decondense sperm DNA, or both combined on fertilisation success post-ICSI using a different sires. The final experiment in this series, evaluated the effect of optimal sperm treatments prior to ICSI on subsequent embryo development to the blastocyst stage.

4.2 MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich Company Ltd. (Dorset, England) unless otherwise indicated. All media and dishes were equilibrated for at least 2 h at 38.5ºC in a humidified atmosphere of 5% CO_2 at high or low O_2 tensions.

4.2.1 Study design, experiments and replication

4.2.1.1 **Study 4A:** Embryo development following piezo-assisted ICSI

After the required adjustments to use efficiently the piezo-drill with the laboratory micromanipulator system, initial assessments focused on evaluating embryo development after fertilisation of bovine oocytes by piezo-assisted ICSI combining the optimal sperm preparations (Hep/LPC) and novel egg activation protocol (CaIo+$SrCl_2$/CHX) developed in Chapters 2 and 3, respectively.

In the current series of experiments the ICSI pipette length which was longer (11.5 cm) than standard pipettes (5.5 cm) used regularly for normal or assisted microinjection by piezo-drill. Fluorinert replaced of mercury as a fill for the microinjecting pipettes. Such fluids provide consistency which is essential for the efficacy of the drilling process (Kimura and Yanagimachi, 1995; Stein and Schultz, 2010; Stein and Schneider-Eppendorf Userguide Nº 032). Finally, several piezo parameters such as speed, intensity and number of pulses were established following the recommendations of Yoshida and Perry (2007) and Stein and Schultz (2010).

4.2.1.2 **Study 4B:** Piezo-assisted vs conventional ICSI

Oocyte breakage potential was assessed visually following microinjection performed using a sharp pipette followed by the ooplasm suction (conventional ICSI) or with a blunt pipette by the generation of ultrasonic mechanical pulses (piezo-drill ICSI). To compare both techniques, a fluorescent dye (FITC-Dextran) was microinjected and, immediately afterwards, evaluated under ultraviolet fluorescence.

4.2.1.3 Study 4C: Alternative factors contributing to ICSI success

4.2.1.3.1 Expt. 4Ci. Glutathione (GSH) as an alternative agent for sperm preparation

To investigate the single and combined effect of removing acrosomal membranes and/or sperm DNA decondensation on fertilisation rate following ICSI, sperm were treated with LPC, GSH or LPC+GSH under the optimal conditions described previously (Study 2Aiii-iv and 2Bi-ii) before ICSI. Subsequently, microinjected oocytes were activated chemically following the protocol described in Study 3Bii (CaIo+$SrCl_2$/CHX). Pronuclear formation was evaluated 16 hpa. A non-treated sperm group served as a control.

4.2.1.3.2 Expt. 4Cii. Fertility of different bulls following ICSI with spermatozoa pre-treated with LPC+GSH

The fertility of semen from several bulls following the application of a common sperm treatment prior to ICSI was compared to that achieved with the baseline bull used in all previous experiments. Sperm from each sire were treated with LPC+GSH prior to microinjection. Pronuclear formation was recorded 16 h following chemical activation of microinjected oocytes. A control group (non-treated sperm) was added for every different bull.

4.2.1.3.3 Expt. 4Ciii. Effect of pre-treating sperm with CaIo, CaIo+GSH compared to LPC+GSH

In a final attempt to increase the proportion of two pronuclei (PN), another AR inducer, Calcium Ionophore A23187 (CaIo), was assessed. Prior to ICSI, sperm were treated with different concentrations of CaIo and, also combined with GSH to evaluate its synergistic action. The LPC+GSH group was used as a positive control and the haploid parthenogenetic activation treatment as a negative control. Pronuclear formation was assessed 16 hpa.

4.2.1.4 Study 4D: Embryo production after ICSI using pre-treated sperm

Once pronuclear formation rate following the application of different sperm pre-treatments was established, embryo development was assessed. Sperm

were treated with LPC+GSH or CaIo prior to ICSI. Following chemical activation of microinjected oocytes, presumptive zygotes were culture until the blastocyst stage, reporting cleaved and number of embryos ≥ 4 blastomeres on Day 2, and quantity of blastocysts on Day 8. An ICSI group with non-treated sperm and IVF were included. Diploid and Haploid parthenogenetic groups served as controls to verify initial oocyte quality and the embryo culture system.

Finally, embryo quality was assessed by the measurement of total cell number, inner cell mass (ICM) and trophectoderm (TE) from ICSI blastocysts on Day 8 by immunocytochemistry (SOX2 staining).

4.2.2 Oocyte collection and *in vitro* maturation (IVM)

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in warmed PBS solution. Before follicle aspiration, ovaries were rinsed first with Eth 70% v/v and then several times with warmed PBS. Cumulus-oocyte-complexes (COCs) were searched, classified and cultured for 22-24 h at 38.5ºC in a humidified atmosphere of 5% CO_2 in air under the same working conditions defined in Section 3.2.2 (Chapter 3).

4.2.3 Sperm preparation

Commercially available South Devon semen straws were thawed at 37ºC for 90 s in water bath. Semen from a single straw was used or semen was pooled from several straws from the same bull. Sperm samples were evaluated for concentration and motility, selected by BoviPure density gradients and treated before ICSI following the same criteria, steps and treatments described previously (Section 2.2.2, Chapter 2), except for sperm concentration which was adjusted to $5 \cdot 10^6$ sperm/ml.

In first experiment (Study 4A), sperm from a single sire were exposed to Hep (80 µg/ml for 15 min) followed by LPC (100 µg/ml for 15 min) to trigger AR. In subsequent experiments (Study C: Expt. 4Ci (1-3) and 4Ciii (3-5)) frozen-

thawed sperm from the same sire that used previously were exposed to the following treatments: (1) Hep (80 µg/ml for 15 min) followed by LPC (100 µg/ml for 15 min); (2) Co-incubation of Hep (80 µM) and GSH (15 mM) for 20 h to induce sperm DNA decondensation; (3) Combination of both treatments, LPC/Hep+GSH/Hep (LPC+GSH); (4) different concentrations of CaIo (10 and 20 µM) for 10 min to trigger AR by a different pathway to LPC/Hep and, followed by 30 min incubation to restore motility following the CaIo wash; (5) 10 µM CaIo (10 min) was also combined to GSH/Hep (15mM/80µM for 20 h). In an additional experiment in Study C (Expt. 4Cii) was studied the fertility ability of three different bulls when were pre-treated by the same protocol (LPC/Hep+GSH/Hep) that original sire. In last experiment (Study 4D), frozen-thawed sperm from sire used in first experiments were pre-treated exclusively by optimal pre-treatments (LPC/Hep+GSH/Hep *vs.* CaIo (10 µM)) to assess embryo development following bovine ICSI. All pre-treated sperm were kept at 38.5ºC in a humidified atmosphere of 5% CO_2 in air until microinjection.

4.2.4 Intra-Cytoplasmic Sperm Injection (ICSI)

Cumulus cells were removed following 22 h IVM. First, COCs were incubated in 500 µl Hepes-TALP media supplemented with hyaluronidase (300 IU/ml) for 2 min at 39ºC and then, gentle pipetting for other 2 min. Denuded oocytes were washed twice in Hepes-TALP media and those oocytes with a visible first polar body (PB), known as metaphase II oocytes (MII), were transferred to fresh TCM199 media until microinjection (22-24 h).

ICSI was performed at 400X magnification using an inverted microscope (Integra 3^{TM} Micromanipulator-RI) equipped with air syringes and a heating system. This keeps the plate temperature stable at 39ºC. Microinjection took place in an ICSI dish (Nunc, Thermo Scientific) with 10 µL droplets Hepes-TALP media supplemented with CB (7.5 µg/ml) to help prevent lysis of the egg. Groups of 6-8 MII oocytes were transferred just before ICSI, surrounded by 2.5 µL droplets 7% Polyvinylpyrrolidone (PVP, see Appendix IV.n) where pre-treated sperm were placed for selection and immobilisation prior microinjection. All media were covered with mineral oil.

Selected spermatozoa showed hyperactive motility with normal morphological features from head, intermediate piece and tail. However, when all spermatozoa were immotile as a result of using sperm treatments more aggressive (GSH/Hep alone or combined to LPC or CaIo), the sperm selection criteria was based mainly on normal morphological features. Then, appropriate spermatozoa were placed in a 7% PVP droplet and, immobilised by pressing the sperm tail to the bottom of the dish with the spiked injection pipette (8 µm inner diameter, RI), and then quickly withdrawing this pipette until a clear bend in the middle of the flagellum was observed. Finally, spermatozoa were aspirated into the pipette tail-first in, readiness for injection.

MII oocytes were held with the PB at the 12 or 6 o'clock position. The injection pipette with the spermatozoon was inserted into the oocyte at the 3 o'clock position after the spermatozoon was moved forward to the tip of the pipette (Fig. 4.1 A-B). When the injection pipette was in the middle of the oocyte, the ooplasm was aspirated gently until its rupture. This was recognized by a change observed in the aspiration speed (Fig. 4.1 C-E). Immediately after oolemma breakage, all cytoplasmic content was returned into the oocyte (Fig. 4.1 F), including the spermatozoon with the minimum volume of PVP (7%).

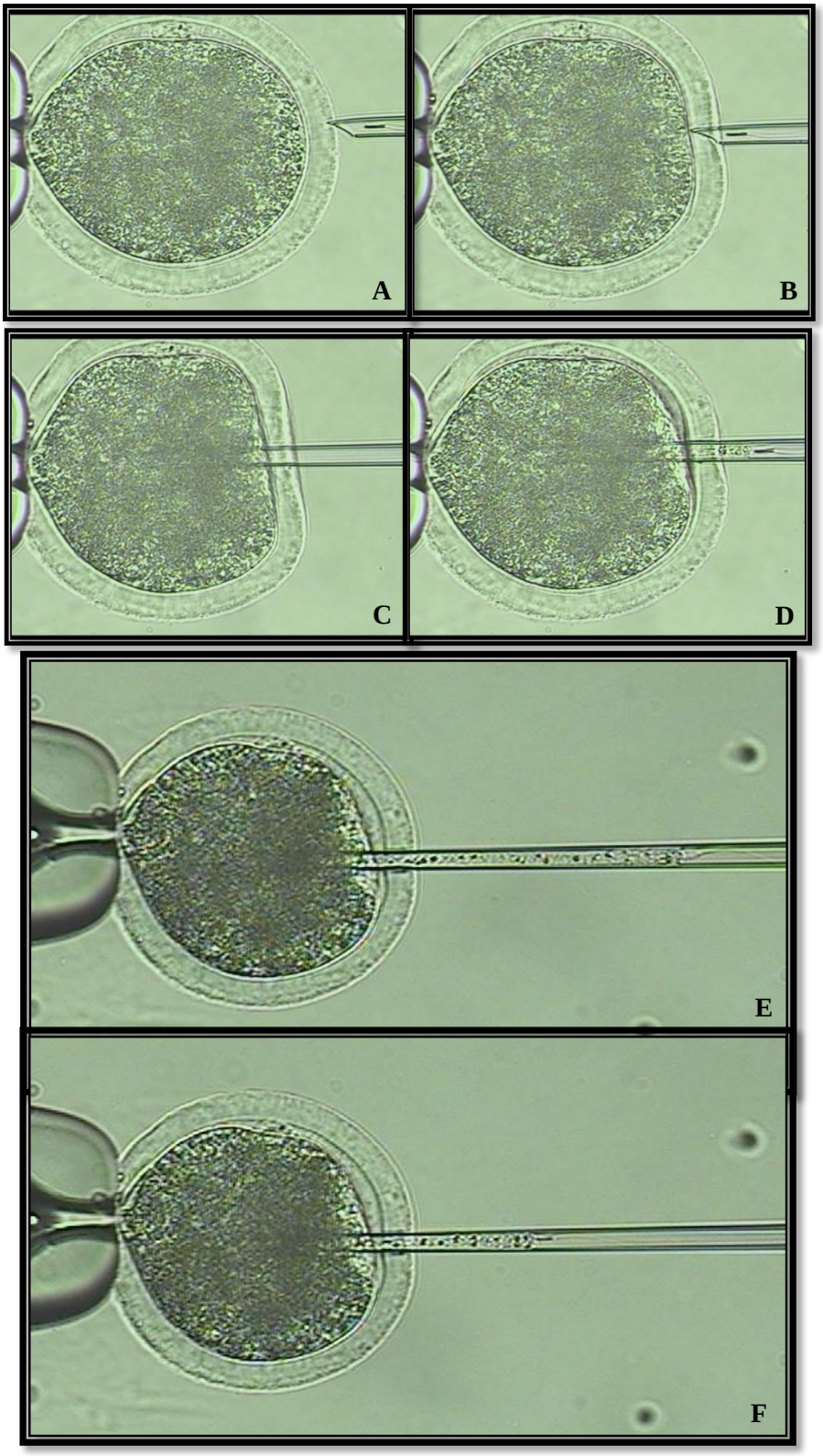


Figure 4.1 Bovine ICSI procedure. A-F) Sequential steps involved in the injection of a spermatozoon across the membrane of a MII oocyte with the PB at 12 o'clock position.

4.2.5 Egg activation

Immediately following ICSI, microinjected oocytes were activated chemically according the protocol described in Chapter 3 (Section 3.2.3). For this, oocytes were rinsed three times in 300 µl fresh warmed Hepes-TALP media before exposure to 5 µM CaIo for 5 min at 39ºC. Oocytes were then washed three times in 50 µl drops containing fresh warmed Hepes-TALP media and four times in 50 µl drops Ca^{2+}-Mg^{2+}-Free Fert-TALP media before 6 h incubation in Ca^{2+}-Mg^{2+}-Free Fert-TALP media supplemented with $SrCl_2$ (20 mM) and CHX (10 µg/ml) at 38.5ºC in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. The diploid parthenogenetic group (Parth-Diploid) was activated using the same protocol described above but in the presence of CB (7.5 µg/ml).

4.2.6 *In Vitro* Fertilisation (IVF)

After 22 h IVM, expanded COCs were transferred to 35 mm culture dish (Nunc, Thermo Scientific) containing 1 ml Hepes-TALP media to wash and partially denude COCs by pipetting several times. This process was repeated again with fresh Hepes-TALP media and later, with 1 ml fresh Fert-TALP before placing them in a four well dish (Nunc, Thermo Scientific) containing 400 µl Fert-TALP media. Groups of 20-25 COCs were inseminated with 100 µl capacitated sperm (final sperm concentration = $1 \cdot 10^6$ sperm/ml) for 18-24 h at 38.5ºC in a humidified atmosphere of 5% CO_2 in air.

4.2.7 Embryo culture

Following oocyte activation or 20 h post-insemination (hpi), presumptive zygotes were rinsed three times in Hepes-SOF media to eliminate chemical agents or cumulus cells, respectively. Groups of 15-20 embryos were then cultured in 40 µl drops mSOFaaci media in culture dishes, overlaid with mineral oil for 192 hpa/hpi at 38.5ºC in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. mSOFaaci media was replaced on Day 2 and Day 5, whereas cleaved and blastocyst numbers were determined on Day 2 and 8 respectively.

4.2.8 Fertilisation assessment: Pronuclear formation

The protocol used was an adaptation from several papers (Arias *et al.*, 2014; Jo *et al.*, 2014; Lee *et al.*, 2015). After 16 hpa, microinjected oocytes were fixed in 4% PFA for 10 min at room temperature. Subsequent to three washes in 1% PVP-PBS, oocytes were incubated with 10 µg/ml Hoechst 33342 (see Appendix IV.d), a permeable fluorescent dye with high affinity for DNA, for 10 min at room temperature under dark conditions to stain pronuclei (PN). Stained zygotes were then rinsed three times in 1% PVP-PBS to reduce background, and were mounted onto glass slides, sealing with nail varnish. Pronuclear formation was visualized at 400X magnification under a fluorescent microscope (Leica DMR) (Fig. 4.2).

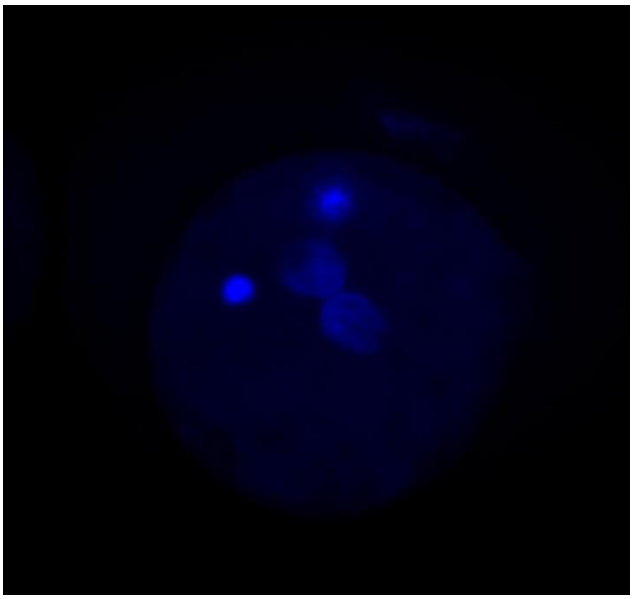


Figure 4.2 Evaluation of bovine pronuclear formation after 16 hpa by Hoechst 33342 staining. Zygote showed two opposite pronuclei in the middle of cytoplasm together with two PBs in a different focal plane.

4.2.9 Embryo immunocytochemistry staining

Embryo quality from normal and parthenogenetic blastocysts on Day 8 were evaluated by immunocytochemistry staining described in Section 3.2.5

(Chapter 3), assessing similar embryo parameters such as total cell number, number of cells in the ICM and TE.

4.2.10 Statistical analysis

Differences in cleavage and blastocyst development from different parthenogenetic treatments were compared using generalized linear regression model that assumed binomial distributions, adjusted by logit link functions. In the case of blastocyst cell numbers Poisson distributions were assumed which were adjusted using log link functions. All statistical analyses were performed within the GenStat statistical package (17th Edition, VSN International, 2011). Differences were considered significant at $P < 0.05$. Differences between individual means were compared using Least Significant Differences (LSD) derived from residual errors of the statistical models. Proportions were converted to percentages for the purposes of data presentation and discussion.

4.3 RESULTS and DISCUSSION

4.3.1 Study 4A: Embryo development following piezo-assisted ICSI

The initial study sought to assess developmental outcomes following piezo-assisted ICSI where sperm were prepared using protocols described in Sections 2.3.1 and 2.3.2 (Chapter 2), and egg activation used protocol described in Section 3.3.2 (Chapter 3). In six replicated experiments, the proportion cleaved and ≥4 cells on Day 2 of culture was greater ($P<0.001$) following parthenogenetic activation or standard IVF, than following either ICSI treatment (Table 4.1). Similarly, the proportion blastocysts of activated/inseminated was greater for both parthenogenetic activation and IVF than for either of the two ICSI groups. However, the proportion cleaved and blastocysts of activated, were each greater ($P<0.05$) for the LPC-ICSI than non-treat ICSI treatment groups.

The fact that ICSI-embryos were morphologically less advanced, and had reduced cell numbers (Fig. 4.3-A and -B), indicated that they may have been chromosomally compromised. For reasons to be discussed later, it was suspected that the piezo-drill may not have been effective in facilitating penetration of the oolemma, therefore leading to a relative high proportion of haploid embryos.

Table 4.1 Evaluation of LPC+Hep sperm pre-treatment on cleavage and embryo development (Percentage, mean ± s.e.) after piezo-ICSI in cattle.

Treatments	Treated oocytes (n)	Cleaved on Day 2 (% of activated)	≥4 Cells on Day 2 (% of cleaved)	Blastocysts on Day 8 (% of activated)
LPC-ICSI	112	77 **(68.5±2.72)^{a}**	35 **(45.2±7.18)^{a}**	16 **(14.4±3.34)^{a}**
Non-treat-ICSI	79	47 **(57.9±3.47)^{b}**	18 **(39.4±9.15)^{a}**	3 **(3.7±2.10)^{b}**
Parth (Diploid)	118	109 **(92.4±1.53)^{c}**	86 **(79.0±4.95)^{b}**	37 **(31.7±4.31)^{c}**
IVF	155	133 **(86.4±1.70)^{d}**	107 **(80.25±4.41)^{b}**	38 **(24.5±3.47)^{ac}**

Experiment was replicated six times. Sperm were pre-treated according to Section 2.3.1 (Chapter 2) prior to ICSI: (i) LPC (100 µg/ml) for 15 min following Hep exposure (80 µg/ml) for 15 min and (ii) untreated sperm (non-treat). Two controls were included: (iii) Diploid parthenogenotes (Parth-Diploid) and (iv) IVF. Microinjected oocytes were activated by exposure to 5 µM CaIo for 5 min followed by 20 mM $SrCl_2$ in Ca^{2+}-Mg^{2+}-Free Fert-TALP media supplemented with CHX (10 µg/ml) for 6 h. Diploid-Parth used the same activation protocol plus addition of CB (7.5 µg/ml). **$^{a-d}$Values within columns with different superscripts differed at P< 0.05.**

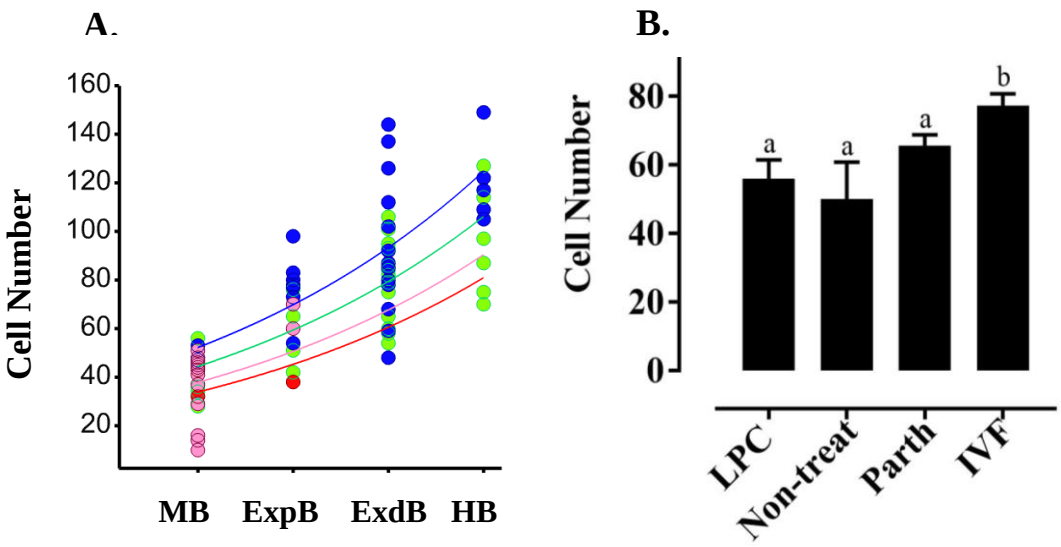


Figure 4.3 Embryo quality assessment after ICSI with pre-treated spermatozoa. (A) Total cell number related to embryo stage of development on Day 8 (MB, mid-blastocyst; ExpB, expanding blastocyst; ExdB, expanded blastocyst; HB, hatched-blastocyst) for embryos produced by: (●) LPC-ICSI, (●) Non-treated-ICSI; (●) Diploid parthenogenotes; (●) IVF. (B) Average total cell.

4.3.2 Study B: Piezo-assisted vs conventional ICSI

Given the results reported in Section 4.3.2, Study A, in order to assess the efficacy of piezo-assisted ICSI in our hands, 48 matured oocytes underwent either conventional or piezo-assisted ICSI using FITC-Dextran (green fluorescent) dye. Results indicated that, compared to conventional ICSI, only very low percentage of oocytes contained this green dye following piezo-assisted ICSI (Table 4.2). This clearly indicated that the piezo-drill microinjection method was ineffective in our hands (possible reasons are discussed later). For all subsequent experiments, therefore, it was decided to use conventional ICSI, even though this led to a slight increase in the proportion of degenerate oocytes (Table 4.2).

Table 4.2 Comparison of cytoplasmic penetration following either conventional or piezo-drill assisted ICSI.

Type of ICSI	Microinjected oocytes (n)	Green (%)	Colourless (%)	Deg (%)
Conventional	30	23 **(76.7)**	7 (23.3)	5 (16.7)
Piezo	18	2 **(11.1)**	16 (88.9)	1 (5.6)

Experiment was undertaken on a single occasion. All oocytes were microinjected with FITC-Dextran (green fluorescence) to evaluate the penetration potential of conventional ICSI vs piezo-ICSI. For reasons discussed later, the latter was performed with longer pipettes than standard; Green (penetrated oocytes); Deg (degenerate oocytes).

4.3.3 Study 4C: Alternative factors contributing to ICSI success

4.3.3.1 Expt. 4Ci. GSH as an alternative agent for sperm preparation

At this point in the proceedings, a group from Argentina published a study (Canel *et al.*, 2017) which presented promising results with bovine ICSI using GSH as a sperm preparation agent. This strategy was based on the premise of enhanced sperm chromatin decondensation prior to microinjection following long co-incubation (20 h) in the presence of high concentrations of GSH (15 mM) and Hep (80 µM). In that study, the egg activation protocol involved activating oocytes with Ionomycin (5 µM, 4 min; Appendix IV.l) combined

with 6-DMAP (1.9 mM, 3 h; Appendix IV.m) after 3 h culture in Fert-TALP media. Consequently, it was decided to test this combined sperm-preparation and egg activation protocol for ICSI.

In the experiment described in Table 4.3, the number 2 PN zygotes was assessed at 16 hpa by Hoechst 33342 staining for the ICSI treatment group. Following microinjection, oocytes were activated by the AOA protocol that Canel *et al.* (2017) employed (Io+6-DMAP). Development to the blastocyst stage was assessed in the parthenogenetic group. In this experiment percentage 2PN following ICSI was considerably less (Table 4.3) than that reported previously by Canel *et al.* (2017) (77%). Post-activation development to the blastocyst stage for diploid parthenotes (at around 21%) was modest by our laboratory standards (e.g. see Table 4.1), and half that (42%) reported by Canel *et al.* (2017). This indicates that a contributing factor to our results was the quality of oocytes and/or protocol used for *in vitro* maturation of oocytes (discussed later).

Table 4.3 Evaluation of pronuclear formation (Percentage, mean ± s.e.) after conventional ICSI using spermatozoa pre-treated by GSH/Hep and following egg activation (Io+6-DMAP) according to Canel *et al.* (2017), in comparison to parthenogenetic development.

Treatments	Treated oocytes (n)	2 PN (%)	Cleaved on Day 2 (% of activated)	≥4 Cells on Day 2 (% of activated)	Blastocysts on Day 8 (% of activated)
GSH Io+6-DMAP	49	**3 (6%)**	-	-	-
Parth (Diploid)	66	-	51 **(77%)**	35 **(69%)**	14 **(21%)**

Experiment was repeated three times. Sperm were treated for 20 h with GSH (15 mM) and Hep (80 µM) prior to ICSI and then microinjected (M) and/or activated (A). Oocytes were activated with 5 µM Io (Ionomycin) for 4 min followed by 3 h in Fert-TALP media and then 3h exposure to 1.9 mM 6-DMAP (6-Dimethylaminopurine). The Parthenogenetic Diploid group, however, was not cultured in Fert-TALP media for 3 h. Pronuclear formation was evaluated 16 hpa (hours post-activation); PN (pronuclei).

In light of these findings it was decided to investigate if a protocol that integrated LPC with GSH pre-treatment would lead to better results following ICSI. In the following experiment, one control group consisted LPC/Hep treatment described in Section 4.2.3 and reported in Section 4.3.1 (Table 4.1). Treatment groups consisted GSH/Hep pre-treatment of sperm (as described above and reported in Table 4.3), and a combination of LPC/Hep and GSH/Hep. The negative control group consisted sperm that received no pre-treatment. In all cases, oocytes were activated following ICSI using the protocol (i.e. CaIo+$SrCl_2$/CHX) first described and reported in Chapter 3, and reported again in this chapter (Section 4.3.1).

Data presented in Fig. 4.4 indicated a clear benefit of pre-treating sperm with a combination of both LPC and GSH. The levels of Hep used in this combined treatment group matched those used for LPC (4 µM) and GSH (80 µM) alone.

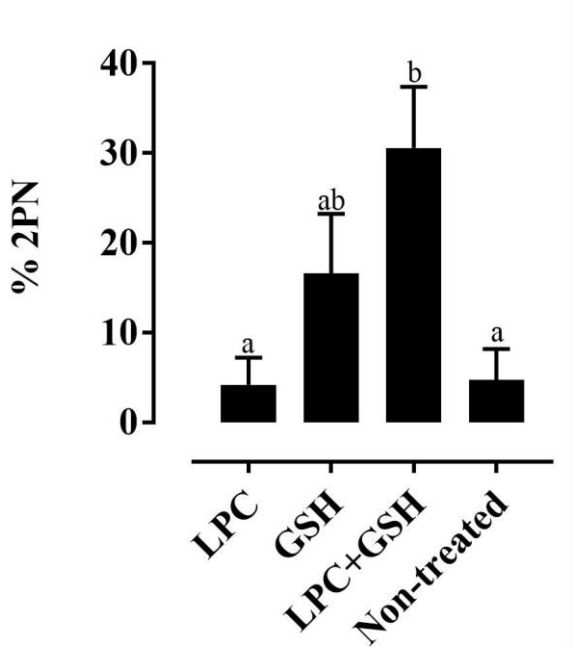


Figure 4.4 Percentage two pronuclei (PN) following normal ICSI with pre-treated spermatozoa (2PN of total microinjected). All treatments included Hep at levels optimised in Chapter 2. (i) LPC (100 µg/ml; 15 min following Hep exposure (80 µg/ml ≡ 4 µM) for 15 min), (ii) 20 h co-incubation in GSH (15 mM) and Hep (80 µM), (iii) LPC+GSH treatments combined, (iv) non-treated sperm (Control). Activation was by 5 min exposure to 5 µM CaIo followed by 20 mM $SrCl_2$ in Ca^{2+}-Mg^{2+}-Free Fert-TALP media supplemented with CHX (10 µg/ml) for 6 h. Pronuclear formation was evaluated at 16 hpa. Experiment was replicated four times (n ~50 microinjected oocytes per treatment). $^{a-b}$**Values with different superscripts differed at P< 0.05**.

4.3.3.2 Expt. 4Cii. Fertility of different bulls following ICSI with spermatozoa pre-treated with LPC+GSH

Given that the best sperm treatment resulted in only around 30% 2PN following ICSI, which was considerably lower than that reported by Canel *et al.* (2017), it was decided to assess the effectiveness of this treatment using semen from other bulls. Data presented in Fig. 4.5 revealed the percentage 2PN for the bull we used in all previous experiment (i.e. Bull 1) and for three other bulls (labelled 2, 3 and 4). Conventional ICSI followed sperm treatment using the optimal protocol described in the preceding section (Section 4.3.3.1), that is LPC/Hep+GSH/Hep. The best results were obtained with semen from Bull 1 (i.e. that used in all previous experiments) following sperm pre-treatment. Bull 4 gave similar, but more variable results to Bull 1. For this reason, Bull 1 continued to be used for the remaining experiments.

4.3.3.3 Expt. 4Ciii. Effect of pre-treating sperm with CaIo, CaIo+GSH compared to LPC+GSH

As the percentage of 2PN was still disappointingly low, so it was decided to try an alternative means of sperm preparation. Other studies in mice had reported encouraging results when sperm were pre-treated with Calcium Ionophore (CaIo) (Tateno *et al.*, 2013). Consequently, different doses of CaIo (10 vs 20 µM) were compared to CaIo (10 µM) + GSH (15 mM) + Hep (80 µM), and LPC (100 µg/mL) + GSH (15 mM) + Hep (80 µM). As a negative control, an additional group consisted parthenogenetically activating oocytes without CB (i.e. leading to haploid zygotes).

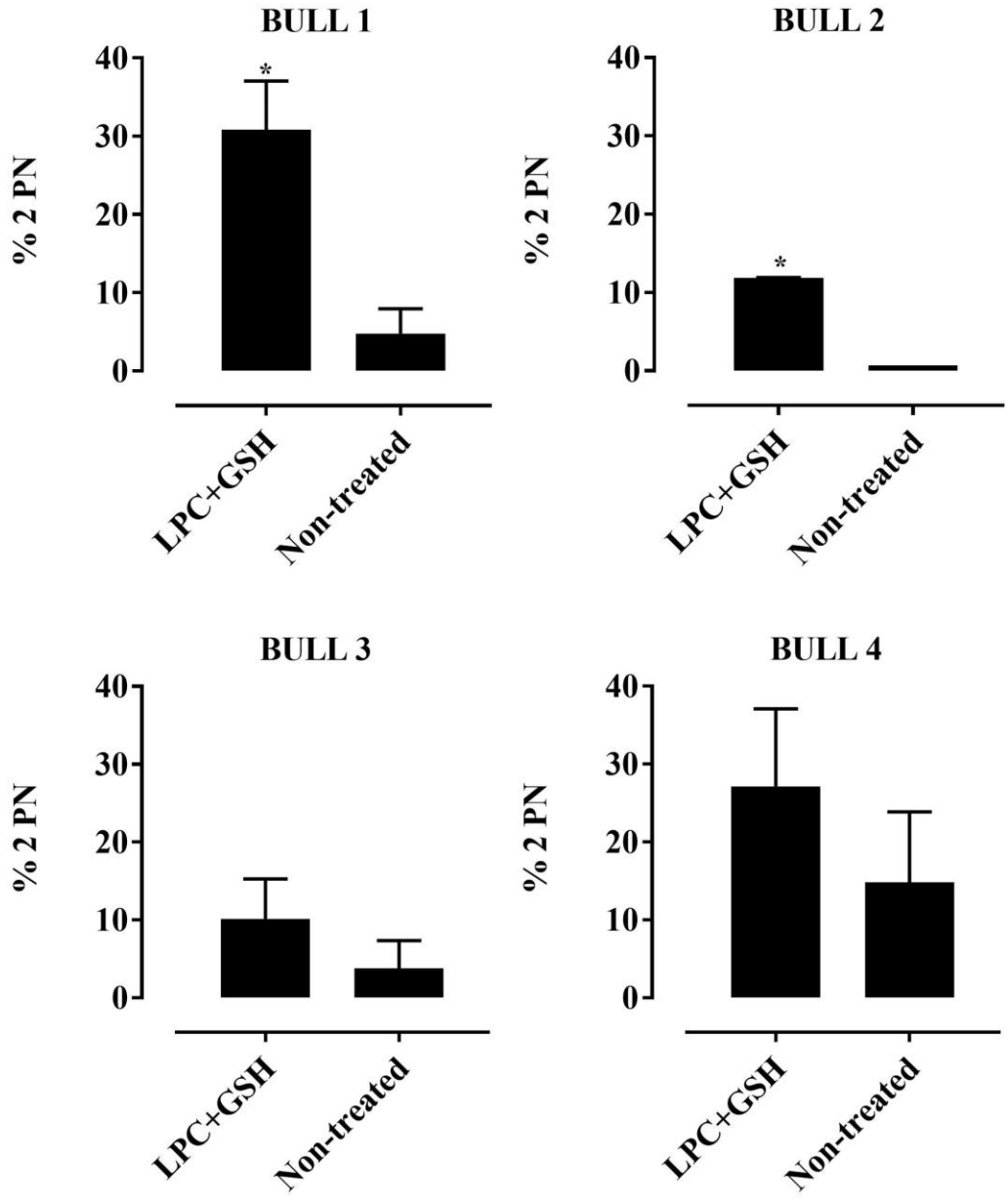


Figure 4.5 Percentages of 2 PN following ICSI with pre-treated spermatozoa from different bulls. Prior to ICSI sperm from each bull were treated with LPC/Hep (100 µg/ml, 15 min; 80 µg/ml, 15 min) + GSH/Hep (15 mM and 80 µM for 20 h) or no sperm pre-treatment (Non-treated). Activation was by 5 min exposure to 5 µM CaIo followed by 20 mM $SrCl_2$ in Ca^{2+}-Mg^{2+}-Free Fert-TALP media supplemented with CHX (10 µg/ml) for 6 h. Pronuclear formation was evaluated at 16 hpa. Experiment was replicated three times (n≈ 30-40 microinjected oocytes per bull).[*]**Values differed at P< 0.05.** Note: Bull 1 was sire used in all previous experiments.

After pre-treated the sperm with different CaIo concentrations or together with GSH/Hep, data from Fig. 4.6 showed that lower dose of CaIo (10 µM) led to a significant improvement (P = 0.009) of the pronuclear formation (32.3 ± 6.60) in a similar way that previous treatment (LPC+GSH); rather than higher concentration or interestingly, combined to GSH/Hep that reduced 2 PN percentages.

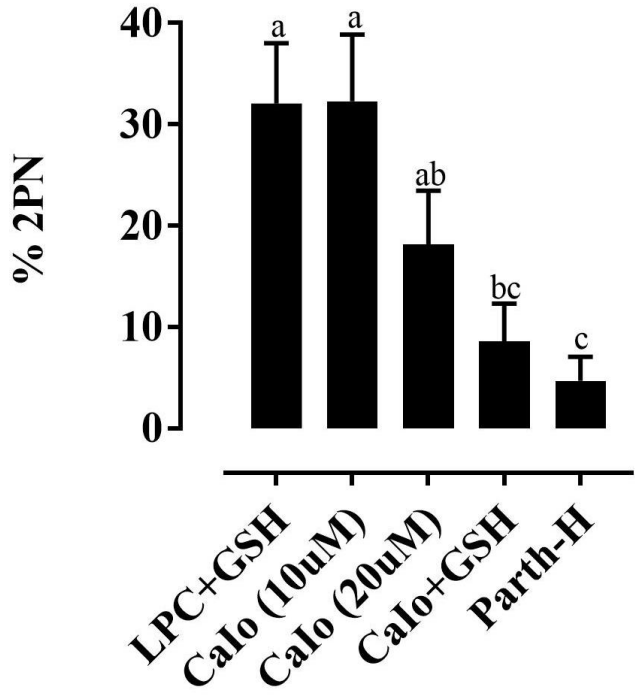


Figure 4.6 Percentage of two pronuclei (PN) following ICSI with pre-treated spermatozoa (from Bull 1, Fig 4.5) by following treatments: (i) LPC/Hep (positive control); (ii) 10 µM CaIo (Calcium Ionophore A23187) or (iii) 20 µM CaIo exposure for 10 min followed by 30 min incubation in fresh modified BWW media containing 3 % BSA, (iv) 10 µM CaIo following 20 h co-incubation GSH/Hep, (v) Haploid parthenogenetic group (negative control). Activation was by 5 min exposure to 5 µM CaIo followed by 20 mM $SrCl_2$ in Ca^{2+}-Mg^{2+}-Free Fert-TALP media supplemented with CHX (10 µg/ml) for 6 h. Pronuclear formation was evaluated at 16 hpa. Experiment was replicated four times (n≈ 50 microinjected oocytes each treatment). **$^{a-c}$Values with different superscripts differed at P< 0.05**.

4.3.4 Study 4D: Embryo production after ICSI using pre-treated sperm

To complete the characterization of sperm pre-treatments considered as optimal in terms of 2PN (Fig. 4.6), their influence on embryo development after ICSI was assessed. Data in Table 4.4 indicated that both sperm treatments (LPC+GSH and CaIo) were similarly effective with regard to the proportion cleaved and blastocyst development following ICSI; leading to numerically superior numbers in groups where sperm were pre-treated. Moreover, embryos from pre-treated sperm were developmentally more competent at 48 hpa ($P<0.001$) than from non-treated sperm.

Table 4.4 Effect of different sperm pre-treatments (using semen from Bull 1, Fig 4.5) on embryo development (Percentage, mean ± s.e.) following ICSI.

Treatments	Treated Oocytes (n)	Cleaved on Day 2 (% of activated)	≥4 Cells on Day 2 (% of cleaved)	Blastocysts on Day 8 (% of activated)
LPC+GSH-ICSI	46	31 **(67.4±5.70)^{ac}**	19 **(61.2±6.05)^{ab}**	3 **(6.4±3.84)^{ac}**
CaIo-ICSI	45	28 **(61.9±6.12)^{a}**	14 **(52.9±6.59)^{b}**	3 **(7.1±4.23)^{ac}**
Non-treat-ICSI	46	28 **(60.8±5.96)^{a}**	6 **(20.3±5.22)^{c}**	0 **(0.0±0.02)^{a}**
Parth (Haploid)	71	46 **(64.7±4.86)^{a}**	15 **(34.3±5.07)^{c}**	7 **(10.3±4.02)^{c}**
Parth (Diploid)	78	73 **(93.6±2.26)^{b}**	55 **(74.9±3.53)^{a}**	28 **(35.6±5.79)^{b}**
IVF	101	82 **(81.3±3.23)^{bc}**	58 **(70.1±3.54)^{a}**	24 **(23.2±4.54)^{bc}**

Experiment replicated four times. Sperm pre-treatments used prior to ICSI: (i) LPC+GSH, (ii) CaIo (10 µM), (iii) non-treat sperm. Additional controls: (iv) haploid parthenogenetic group activated in absence of CB, (v) diploid parthenogenetic group activated in presence of CB (7.5 µg/ml), (vi) IVF. Activation was by 5 min exposure to 5 µM CaIo followed by 20 mM $SrCl_2$ in Ca^{2+}-Mg^{2+}-Free Fert-TALP media supplemented with CHX (10 µg/ml) for 6 h. **^{a}-^{c}Values within columns with different superscripts differed at P< 0.05.**

Finally, data from the blastocyst quality analysis revealed that despite the fact that there were no significant differences between sperm treatments, blastocysts from CaIo (10 μM) sperm pre-treatment reached a more advanced embryo stage (Fig. 4.7-A) with higher total cell numbers (Fig. 4.7-B) than the LPC+GSH group, with values close to IVF control. However, it is difficult to draw firm conclusions due to the low number of blastocysts analysed.

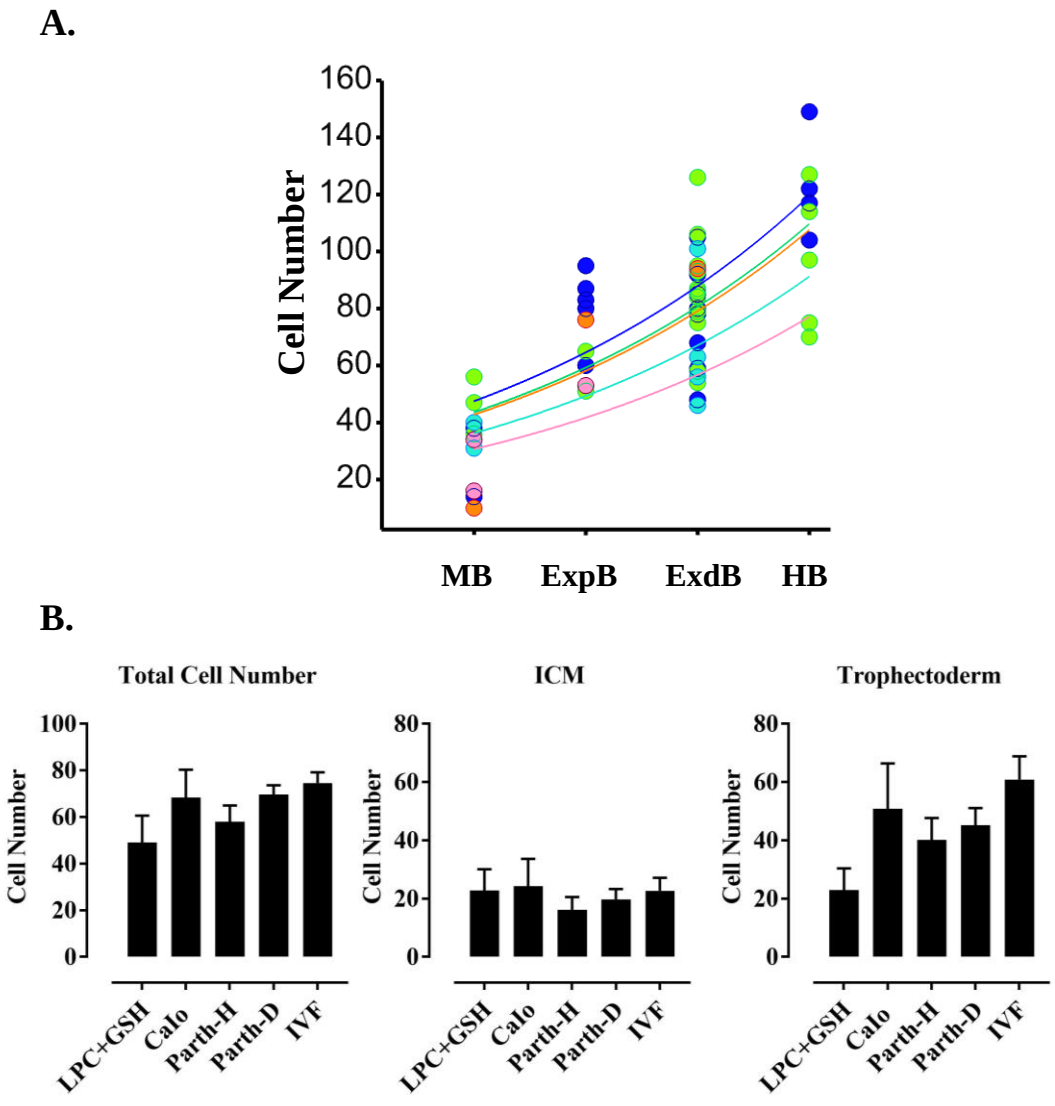


Figure 4.7 Embryo quality assessment after ICSI with pre-treated spermatozoa. A) Total cell number increased (P<0.001) with embryo stage development on Day 8 (MB, mid-blastocyst; ExpB, expanding blastocyst; ExdB, expanded blastocyst; HB, hatched-blastocyst) from blastocysts produced by: (●) LPC-ICSI, (●) CaIo (10μM)-ICSI (●) Haploid parthenos; (●) Diploid parthenos; (●) IVF. B) Average number of total cells, cell in inner-cell mass (ICM) and trophectoderm (TE)

4.3.5 General Discussion

The current study demonstrated that the success of conventional bovine ICSI could be improved by microinjecting spermatozoa in absence of sperm membranes and with decondensed sperm nucleus (Table 4.4 and Figures 4.4 and 4.7). There was a similar increase on 2PN percentages when spermatozoa were pre-treated with either LPC/Hep+GSH/Hep (32%) or CaIo (32%) (Fig. 4.6) compared to non-treated spermatozoa (5%) (Fig. 4.4). Furthermore, this type of sperm pre-treatment (LPC/Hep+GSH/Hep) seems reliable in terms of beneficial effects on pronuclei formation, regardless of bull fertility (Fig. 4.5). Finally, there were no significant differences with regard to cleavage (62-67%) and blastocyst development (6-7%) in ICSI groups (pre-treated spermatozoa) compared to IVF (81% and 23%, respectively) (Table 4.4). However, none of the ICSI blastocysts reached the hatching stage of development (Fig. 4.7A).

4.3.5.1 **Study 4A:** Embryo development following piezo-assisted ICSI

The main concern of this study was to evaluate how the combination of optimal sperm preparations prior to piezo-ICSI and the novel AOA protocol developed in earlier experiments (Sections 2.3.1 and 2.3.2-Chapter 2 and Section 3.3.2-Chapter 3) operated in our hands. The interest to set up the piezo-drill was based on studies that suggested that using this microinjecting system enhances embryo development following ICSI in the cow. This approach offers less traumatic way of puncturing a hole in the ZP and breakage of oolemma through use of ultrasound mechanical pulses (Katayose *et al.*, 1999; Horiuchi *et al.*, 2002; Wei and Fukui, 2002; Galli *et al.*, 2003; Fujinami *et al.*, 2004; Oikawa *et al.*, 2005; Lee *et al.*, 2015; Oikawa *et al.*, 2016).

Initially, sperm pre-treatment (LPC/Hep) focused only on the removal of sperm plasma and acrosomal membranes, and this appeared to increase the percentage of cleaved (69%) and embryo development to the blastocyst stage (14%) compared to non-treated sperm (58% and 4%, respectively) following piezo-assisted ICSI (Table 4.1). However, these rates were still not comparable to the IVF control (86% and 25%) and, blastocyst quality from those embryos

generated by ICSI were developmentally less competent (Fig. 4.3-A) with reduced total cell numbers (Fig. 4.3-B) compared to IVF blastocysts.

This decline in blastocyst development following piezo-assisted ICSI might be associated with cytoskeletal damage induced by the microinjection procedure (Hara *et al.*, 2011). However, the use of the piezo-drill seems to reduce considerably these detrimental effects (Horiuchi *et al.*, 2002). The most likely cause, therefore, could be the absence of normal ploidy as a result of a failure of sperm DNA decondensation (Rho *et al.*, 1998b; Chung *et al.*, 2000; Arias *et al.*, 2015). This could either arise as a consequence of the inability of bull spermatozoa to activate properly the egg or, perhaps unsuccessful oolemma breakage which would prevent the entrance of the bull spermatozoon.

4.3.5.2 **Study 4B:** Piezo-assisted vs conventional ICSI

It follows that, this experiment was focused on investigating the effectiveness of piezo-drill microinjection system in breaking the oolemma of bovine oocytes in order to eliminate this technical factor as the one responsible for the reduced competency of ICSI blastocysts obtained in the previous study (Study 4A). Surprisingly, piezo-assisted ICSI did not work efficiently in our hands, leading to low percentages of oolemma penetration (green fluorescence, 11%) compared to conventional ICSI (77%) (Table 4.2). The cause of this low penetration rate may be largely due to a series of adjustments required to connect the piezo-drill to our rig.

The longer length of micropipettes (~2 times longer than standard size) employed might interfere with the proper transmission of electric pulses, thereby reducing its breaking potential. Moreover, established protocols recommend elemental mercury (Hg^0) to fill the injection pipette as it is a more dense substance (Hg^0 density = 13.53 g/cm^3) than water, and therefore increases the efficacy of drilling (Kimura and Yanagimachi, 1995; Stein and Schultz, 2010). However, in the current series of experiments Fluorinert was used instead (due to legal restrictions with the use of Hg^0). Although Fluorinert FC-770 is considered a suitable alternative due to its high density (1.79 g/cm^3)

(Yoshida and Perry, 2007; Hajarian, 2011) and, certain parameters of the piezo such as speed and number of pulses were adjusted to optimal values, it was not possible get it to work properly. Consequently, conventional ICSI was employed for all subsequent experiments.

4.3.5.3 Study 4C: Alternative factors contributing to ICSI success

4.3.5.3.1 Expt. 4Ci. GSH as an alternative agent for sperm preparation

Experiments performed in this study evaluated the number of pronuclei following conventional ICSI using spermatozoa exposed to different pre-treatments. At this point in the proceedings, a new sperm pre-treatment protocol (GSH//Hep), which helps to unpacked sperm chromatin, appeared to improve significantly ICSI success according to the recent findings reported by Canel *et al.* (2017) who achieved 77% 2PN using this sperm pre-treatment.

Interestingly, it seems that the individual effect of the removal sperm membranes or sperm DNA decondensation prior to ICSI did not enhance pronuclear formation. Similar or slightly higher percentages of 2PN were achieved when spermatozoa were pre-treated by LPC/Hep (4%) or GSH/Hep (17%) compared to non-treated (5%) (Fig. 4.4). Nevertheless, the combination of both treatments (LPC/Hep+GSH/Hep) increased significantly the number of 2PN up to 31%. Similar percentages of 2PN (35%) were achieved by Sekhavati *et al.* (2012) when GSH and Hep used as a sperm pre-treatment in an attempt to improve bovine ICSI. Although, they used exclusively GSH/Hep, focused on decondensing sperm nucleus and did not pay attention to acrosomal status. However, these results were still low and far removed from those obtained by Canel *et al.* (2017).

Initially, it was though that this discrepancy might be related to differences in the AOA protocol employed. But the preliminary experiment performed in the current study (Table 4.3), which reproduced similar conditions that them (sperm pre-treatment = GSH/Hep and AOA = Io+ 6-DMAP), revealed that the AOA protocol was not the main cause of these differences. The 2 PN percentage obtained following activation of microinjected oocytes by Io+6-

DMAP (6%) was even lower than when the novel AOA protocol (CaIo+$SrCl_2$/CHX) was applied (17%, Fig. 4.4). Parthenogenetic embryo development from the same experiment (Table 4.3), however, indicated that there may be other important factors to consider. One is oocyte quality as the proportion blastocysts reported in this study (21%) was low compared to Canel *et al.* (2017) (42%). Nevertheless, other factors such as sire, cattle genotype and/or embryo culture system cannot be excluded (Leroy *et al.*, 2008a; Leroy *et al.*, 2008b; García-Roselló *et al.*, 2009; Leroy *et al.*, 2011).

4.3.5.3.2 Expt. 4Cii. Fertility of different bulls following ICSI with spermatozoa pre-treated with LPC+GSH

This set of experiments demonstrated that the combined action of inducing AR and decondense sperm DNA in bull spermatozoa prior to microinjection enhanced the pronuclear formation compared to non-treated spermatozoa, regardless of the bull used (Fig. 4.5). Although these differences between treated and non-treated spermatozoa were not statistically significant for the four bulls studied, all of them showed increased percentage of 2PN when microinjected spermatozoon was pre-treated with LPC/Hep+GSH/Hep. This is an important finding in terms of standardised sperm pre-treatment protocols for bovine ICSI as, nowadays, there is a great diversity of sperm treatments based mainly on criteria employed in this study, but leading to variable and unreproducible results (Tables 1.3 and 1.4).

4.3.5.3.3 Expt. 4Ciii. Effect of pre-treating sperm with CaIo, CaIo+GSH compared to LPC+GSH

In a final attempt to further increase the percentage of 2PN (~30%) following bovine ICSI, a new treatment (high concentrations of CaIo) focused on triggering AR through an alternative pathway which overcomes certain physiological pathways of capacitation (First and Parrish, 1987; Tateno *et al.*, 2013) was evaluated. It was confirmed that CaIo also promotes the formation of 2 PN (32%) when it is used alone and at low concentrations (10 μM) compared to non-treated sperm but, it was not more effective than LPC/Hep+GSH/Hep (32%) (Fig. 4.6).

Interestingly, a higher dose of CaIo or its association with GSH/Hep seems to be detrimental for pronuclear formation, leading to a decline in the percentage of 2PN which is especially important in the case of CaIo+GSH/Hep (9%). The reason for this effect is not clear, however excessive intracellular levels of Ca^{2+} might inhibit temporary important receptors, channels and ion transporters such as $InsP_3$ receptors (Marshall and Taylor, 1994), Catsper (Ren and Xia, 2010) and secondary Ca^{2+} transporters (Krapf *et al.*, 2010). Consequently, that would prevent the activation of key pathways in capacitation and AR, including the cAMP pathway, phosphorylation of certain substrates like PKA and tyrosine phosphorylation (Tateno *et al.*, 2013) which have essential roles in fertilisation.

With regards to the impact of combined treatment (i.e. CaIo+GSH/Hep), the higher membrane permeability to certain ions caused by the action of CaIo may also promote the membrane transport of other molecules such as GSH or Hep, increasing massively their concentration into cytosol and sperm nucleus. Although that large excess of GSH and Hep seems to be beneficial for unpacking sperm chromatin, a fact which was demonstrated in chapter 2 when sperm were treated by CaIo+GSH/Hep (Expt. 2Bii), at the same time it appears to be detrimental for pronuclear formation (Fig. 4.6). The underlying mechanisms are not known, although alterations to nuclear redox conditions directly affect to the structural stability and packing of chromatin (Markovic *et al.*, 2010). At that point, GSH plays an essential role in providing an appropriate nuclear redox state (reduced conditions). This enables the activation of several nuclear DNA-binding proteins, chromatin decondensation, stability of meiotic spindle and organization of nuclear matrix, needed for the progression of the cell cycle (Dijkwel and Wenink, 1986; Luberda, 2005; Markovic *et al.*, 2010). Once oocytes are fertilised, however, intracellular GSH levels decrease rapidly and continue to decline until the blastocyst stage (Zuelke *et al.*, 2003). Therefore, it was thought that prolonged exposure (20h) to very high levels of GSH (extreme reducing conditions), in this study, probably lead to a deleterious effect on sperm chromatin.

4.3.5.4 **Study 4D:** Embryo production after ICSI using pre-treated sperm

Finally, this study demonstrated that pre-treating bull spermatozoon with either LPC/Hep+GSH/Hep or CaIo also enhances embryo development following bovine ICSI compared to non-treated spermatozoa. These sperm pre-treatments led to similar percentages of cleavage and blastocyst development of similar quality (Table 4.4 and Fig. 4.7). Interestingly, only removal of sperm membranes by CaIo was sufficient to improve pronuclear formation and embryo development following ICSI. In contrast, LPC/Hep required the additional action of GSH/Hep to achieve a similar effect. It was felt that LPC/Hep+GSH/Hep could lead to better results based on its mechanism of action. These agents seemed to act more closely to physiological conditions due to their ability to induce capacitation, AR and sperm DNA decondensation in a sequential manner. However, results did not demonstrate differences to indicate a beneficial effect of one over the other. Therefore, it would be of great interest to perform further molecular studies in order to investigate the pathways that activate each of these sperm pre-treatments.

4.3.5.5 **Conclusions**

In summary, bull sperm preparations based on inducing sperm AR and DNA decondensation improve the success of bovine ICSI. In this chapter, two sperm pre-treatments were developed (i.e. LPC/Hep+GSH/Hep and CaIo), increasing similarly pronuclei formation. There was also an improvement in embryo development when it was compared to the non-treated sperm ICSI group. However, blastocyst yields were low and not comparable to the IVF control. At the same time, this chapter also tested the efficiency of a new AOA protocol (CaIo+$SrCl_2$/CHX) following bovine ICSI. It is concluded, however, that further research is required to develop sperm preparations for bovine ICSI to enhance embryo development in absence of any AOA protocol with outcome similar to conventional IVF.

CHAPTER 5:

General Discussion and Conclusions

5.1 DISCUSSION

This thesis investigated the effect of different sperm preparations together with new combinations of artificial oocyte activation (AOA) agents on the success of bovine ICSI. It was hypothesized that the absence of bull sperm membranes exclusively, or simultaneously with sperm nuclear decondensation, prior to microinjection would enhance pronuclei formation and, therefore, the development of competent embryos. Furthermore, embryo development would be enhanced by the application of an improved AOA protocol following ICSI that more closely mimicked the physiological Ca^{2+} release.

Chapter 2 focused on finding optimal sperm pre-treatment procedures which lead to the highest possible yields of acrosome reacted (AR) spermatozoa whilst retaining high percentages of sperm viability. It also sought to achieve high proportions of decondensed sperm heads. In order to induce AR, two different treatments (LPC/Hep *vs.* CaIo) were studied, differing basically in their mechanism of action. LPC/Hep seemed to act through a more physiological pathway, inducing capacitation and AR whereas CaIo is known to bypass key pathways involved during *in vivo* capacitation (Tateno *et al.*, 2013), although the molecular signaling pathway required to trigger AR is unclear. On the other hand, GSH/Hep was selected as a suitable treatment to promote sperm DNA decondensation because it is already known that GSH acts as a physiological reducing agent in mammals (Calvin and Grosshan, 1986; Perreault *et al.*, 1987; Luberda, 2005; Julianelli *et al.*, 2012).

Initially, the effect of LPC/Hep and CaIo on the induction of AR under different working conditions (incubation times and concentrations) required to reach fully AR spermatozoa was studied. This parameter was determined by an adaptation of a triple fluorescent staining (PNA-FITC/PI/Hoechst 33258) described by Martínez-Pastor *et al.* (2010) and Álvarez *et al.* (2012). Next, it

was corroborated that optimal conditions for GSH/Hep reported by Delgado *et al.* (2001) were also appropriate for our sire, leading to higher percentages of decondensed sperm heads. This was assessed visually using a phase contrast microscope.

After analysing separately the effects of each reagent, either on AR (LPC/Hep, CaIo) or unpacking of sperm chromatin (GSH/Hep), the impact of combined treatments (LPC/Hep+GHS/Hep and CaIo+GSH/Hep) using the same methodology was evaluated. An interesting finding was that when CaIo was associated with GSH/Hep enhanced the proportion of decondensed sperm heads concomitantly with induced AR. This was not observed with the other combined treatment (LPC/Hep+GHS/Hep). This biological phenomenon could be related to the action of CaIo which raises the permeability of cellular membranes to certain ions. Therefore, it is quite likely that GSH and Hep can easily pass across the nuclear envelope, which would increase their levels substantially.

The main interest in Chapter 3 was the development of an efficient AOA protocol which include $SrCl_2$ as a main inducer. Initial experiments focused on assessing the activation potential of $SrCl_2$ alone on bovine parthenogenetic embryo development when optimal conditions were applied (Sr^{+2} concentration, time of exposure and media composition) according to Méo *et al.* (2005).

Unfortunately, $SrCl_2$ alone was not as an efficient at activating bovine oocytes as the results of previous studies appeared to indicate (Méo *et al.*, 2005; Yamazaki *et al.*, 2005; Méo *et al.*, 2007). Several factors could account for these differences, including initial oocyte competency, genetic background of donors, donor age and/or nutritional status (Pontes *et al.*, 2010; Guerreiro *et al.*, 2014; Gimenes *et al.*, 2015; Batista *et al.*, 2016; Sartori *et al.*, 2016).

In such circumstances Méo *et al.* (2007) suggested that “enhanced” AOA protocols are required particularly when IVM is less than 26 h. In particular, the action of Sr^{2+} seems to be highly influenced by oocyte age (Méo *et al.*, 2004). Therefore, the dual effect of combining $SrCl_2$ with either Eth or CaIo

on parthenogenetic embryo development was investigated. The selection of this combination of AOA agents ($SrCl_2$+Eth or $SrCl_2$+CaIo) was based on the premise that the calcium oscillation patterns evoked would be similar to physiological. Eth or CaIo would be responsible for generating the initial Ca^{2+} transient necessary for reducing MPF levels and exit from meiosis arrest (Vitullo and Ozil, 1992; Loi *et al.*, 1998), although there would be differences in the amplitude of Ca^{2+} wave and the period of time to return to baseline levels compared to that induced by the spermatozoon (Nakada and Mizuno, 1998). In contrast, $SrCl_2$ would provoke long-lasting multiple Ca^{2+} spikes, similar to that observed under physiological conditions in mouse studies (Bos-Mikich *et al.*, 1995; Bos-Mikich *et al.*, 1997; Yamazaki *et al.*, 2005).

Results from this experiment showed an increase in both, cleavage and blastocyst development, following bovine oocyte activation by a long incubation period (6h) in $SrCl_2$ after a previous short exposure (5 min) to CaIo. Similar improvements were reported by Yamazaki *et al.* (2005) when bovine oocytes activated under the same optimal conditions. The beneficial effect of combining these two agents (CaIo and $SrCl_2$) for AOA has been also observed during pig and sheep parthenogenetic activation (Che *et al.*, 2007; Choi *et al.*, 2013). Eth followed by $SrCl_2$, however, had the opposite effect, leading to a reduction in percentages of cleaved and parthenogenetic embryos which reach the blastocyst stage. That detrimental effect was not noticed when Eth was combined with CHX. Therefore, it was thought that the Sr^{2+} mechanism of action might interfere through unknown pathways with Eth action, or maybe Sr^{2+} concentration itself would regulate indirectly Eth activation by inhibiting the activity of $InsP_3$ receptors (Marshall and Taylor, 1994).

In spite of the improvement in parthenogenetic embryo development following AOA by $SrCl_2$+CaIo, outcomes were poorer than with conventional protocols (Eth+CHX/CB or CaIo+CHX/CB). Consequently, a new and more comprehensive combination for AOA that included CaIo, $SrCl_2$ and CHX was considered. Although the use of protein synthesis/phosphorylation inhibitors, such as CHX, could lead to detrimental effects on ploidy status and blastocyst development as a result of their low specificity for particular proteins during

the cell-cycle (Szöllösi *et al.*, 1993; Winger *et al.*, 1997; Loi *et al.*, 1998; Fernandes *et al.*, 2014; Lee *et al.*, 2015), the novel AOA protocol (CaIo+$SrCl_2$/CHX/CB) evaluated in the last study undertaken in Chapter 3 led to a substantial increase in percentage cleaved and forming blastocysts. Assessments of blastocyst quality included visual evaluation of embryo stage and total cell number (inner cell mass and trophectoderm) assessed by immunocytochemistry staining (SOX2) on Day 8. However, analysis of chromosome number was not determined, and so full developmental competency could not be assessed.

The final experimental chapter in this thesis, Chapter 4, investigated whether the application of optimal sperm treatments, which considered inducing membrane removal and/or chromatin decondensation (Chapter 2) prior to ICSI and followed by activation using the new developed AOA protocol (Chapter 3) improved ICSI success.

The initial series of experiments were more interested in ensuring how the combination of one of the optimised sperm pre-treatments (LPC/Hep) and the application of the newly developed AOA protocol (CaIo+$SrCl_2$/CHX) worked together following ICSI assisted by the piezo-drill microinjecting system rather than to directly try to improve ICSI success. Initial outcomes from ICSI using pre-treated spermatozoa (LPC/Hep) were promising compared to non-treated sperm-ICSI, except that no blastocysts (LPC/Hep-ICSI) reached an advanced embryo stage such as expanded- or hatched-blastocyst. This limitation on embryo developmental capacity could have been related to their ploidy state, with most being haploid. In turn this could have been due to the inability of bull sperm to decondense its nucleus, which is still the principal challenge of bovine ICSI (Rho *et al.*, 1998b; Chung *et al.*, 2000; Arias *et al.*, 2015; Salamone *et al.*, 2017), oocyte competency to respond adequately to the sperm stimulus (Malcuit *et al.*, 2006a; Swain and Pool, 2008; Julianelli *et al.*, 2012; Vanden-Meerschaut *et al.*, 2014), and/or, technical difficulties to deposit spermatozoon into the oosplasm.

Thus, the following study sought to identify the main factors responsible for the decrease in quality observed in ICSI-blastocysts. Piezo-drill potency to

break the oolemma was evaluated by the microinjection of a fluorescent dye (FITC-Dextran). This dye emits green fluorescence when it is exposed to ultraviolet light. Surprisingly, the percentage penetration (oocytes with green fluorescence) was very low, despite having optimised previously the speed, intensity and number of electrical pulses required for the proper and efficient functioning of the piezo. However, a series of extra adjustments were also needed to be able to use the piezo with the lab micromanipulator, such as the use of longer microinjection needles and filling microinjection pipettes with an alternative heavy fluid instead of mercury (Kimura and Yanagimachi, 1995; Yoshida and Perry, 2007; Stein and Schultz, 2010). It was thought that these adaptations were primarily responsible for the low efficacy of the piezo in our hands.

The principal interest in using the piezo was to reduce cytoskeletal damage induced by the microinjection procedure, enhancing embryo development following ICSI (Katayose *et al.*, 1999; Horiuchi *et al.*, 2002; Wei and Fukui, 2002; Galli *et al.*, 2003; Fujinami *et al.*, 2004; Oikawa *et al.*, 2005; Lee *et al.*, 2015; Oikawa *et al.*, 2016). However, following these findings it was decided to perform conventional ICSI for the remaining experiments.

Pronuclei formation was then assessed at 16 hpa by a fluorescent stain (Hoechst 33342) following oocyte microinjection with spermatozoa. The primary purpose here was to establish the proportion of 2PN (diploid) zygotes. Three different sperm treatments (LPC/Hep, GSH/Hep and LPC/Hep+GSH/Hep) plus the control (non-treated spermatozoa) were evaluated simultaneously. At this stage we became interested in investigating the impact of GSH/Hep on bovine ICSI success as a consequence of the study of Canel *et al.* (2017). The percentage 2PN following oocyte microinjection with pre-treated spermatozoa showed a marked improvement following LPC/Hep+GSH/Hep treatment (i.e. removal of sperm membranes coinciding with chromatin decondensation) compared to LPC/Hep or GSH/Hep applied individually. However, these results were still low compared to those of Canel *et al.* (2017). It was initially felt that these differences might attribute to the use of a different AOA protocol following ICSI (i.e. Io+6-DMAP) compared

to that used in our studies (CaIo+$SrCl_2$/CHX). However, this factor was excluded after we performed a preliminary study that reproduced the same working conditions (sperm preparation = GSH/Hep; AOA protocol = Io+6-DMAP).

Other studies have reported higher percentages of 2PN than in the current study, using similar sperm pre-treatments (LPC and/or GSH) but with slightly different working conditions; including differences in reagent concentrations and incubation times, the use of different AOA protocols and culture media, and/or ICSI assisted by piezo (Lee *et al.*, 2015; Zambrano *et al.*, 2016; Zambrano *et al.*, 2017). Unfortunately, it is difficult to identify a specific factor as the main cause for this discrepancy due to intrinsic variables such as oocyte quality, bull fertility and cattle genotype (Leroy *et al.*, 2008a; Leroy *et al.*, 2008b; García-Roselló *et al.*, 2009; Leroy *et al.*, 2011).

Although LPC/Hep+GSH/Hep led to the maximum proportion of 2PN in our hands, this percentage was still low. Therefore we assessed the performance of different sires. Sire impact on the proportion of 2PN was assessed following treatment with LPC/Hep+GSH/Hep prior to ICSI. It was not possible to achieve a further increase in 2PN percentage. However, this sperm pre-treatment did enhance pronuclear formation following ICSI for all bulls regardless of their fertility. This finding could be of particular relevance to the establishment of standard protocols for preparing bull spermatozoa prior to microinjection.

The final strategy for obtaining a greater number of 2PN focused once again on inducing AR and removing sperm membranes with high concentrations of Ca^{2+} (Schultz & Kopf, 1995; Ducibella *et al.*, 2002; Fissore *et al.*, 2002; Ozil *et al.*, 2006; Wassarman and Litscher, 2008; Ross *et al.*, 2009; Darszon *et al.*, 2011; Gadella., 2013; Armant, 2015; Stival *et al.*, 2016). Bull spermatozoa were exposed briefly to two different doses of CaIo (A23187) prior to ICSI and the percentage 2PN assessed. Furthermore, its effect was also assessed following combination with GSH/Hep based on the benefits observed in earlier studies. In this case, the individual action of CaIo was sufficient to induce an increase in the percentage 2PN similar to that achieved by the

combined action of LPC/Hep with GSH/Hep. In contrast to previous findings from this study, the synergistic effect CaIo+GSH/Hep for pre-treatment of bull spermatozoa prior to ICSI seemed to be detrimental on this occasion. A decrease in pronuclear formation was observed. Similarly, the highest concentration of CaIo also led to a decline in 2PN percentage. It is difficult to explain these results as CaIo uses an alternative signaling pathway, bypassing key physiological pathways which take place during *in vivo* capacitation (First and Parrish, 1987; Tateno *et al.*, 2013).

Finally, the effect of these two optimal sperm pre-treatments (i.e. LPC/Hep+GSH/Hep and CaIo) on embryo development following ICSI and AOA with CaIo+$SrCl_2$/CHX was determined. Both sperm pre-treatments led to similar levels of embryo development which were similar to that achieved following standard IVF. Unfortunately, however, ICSI blastocyst yields and quality following ICSI remained poor.

5.2 CONCLUSIONS

This thesis has proved that appropriate pre-manipulation (i.e. sperm treatments which mimic key physiological processes) of male gamete enhances ICSI success in the cow regardless fertility differences between bulls. However, it was not possible to enhance substantially the success of bovine ICSI. Although recent findings, mainly based on strategies similar to those used in the current thesis (i.e. sperm pre-treatment and AOA), seem to be promising in terms of blastocyst development (Arias *et al.*, 2014; Lee *et al.*, 2015; Oikawa *et al.*, 2016; Zambrano *et al.*, 2016; Águila *et al.*, 2017a; Canel *et al.*, 2017; Zambrano *et al.*, 2017). Nevertheless, bovine ICSI remains a challenge because there are only few reports of heathy calf born to this technique and these very reported many years ago (Goto, 1990; Rho *et al.*, 1998b; Hamano *et al.*, 1999; Hwang *et al.*, 2000; Wei and Fukui, 2002; Horiuchi *et al.*, 2002; Galli *et al.*, 2003; Oikawa *et al.*, 2005).

5.3 FUTURE RESEARCH DIRECTIONS

A number of factors in addition to those investigated in the current study could combine to determine developmental success following ICSI. These include alternative sperm preparations, oocyte source (abattoir vs OPU), IVM of oocytes, and microinjection of the potential sperm factor (PLCζ protein) responsible for physiological activation of mammalian eggs. It would, in future, also be desirable to measure Ca^{2+} oscillations within the egg following microinjection and/or artificial oocyte activation.

With regards to alternative sperm preparations, sperm exposure to bovine oviductal epithelial cells (capacitation) and cumulus-granulosa cells (to induce AR) prior to ICSI would be interesting to perform on this occasion, the attempt to mimic physiological conditions would focus on the recreation of the oviductal microenvironment. On the other hand, it would also be worth trying the encouraging sperm selecting method (thermotaxis) reported in mice by Pérez-Cerezales *et al.* (2018). This seems to improve long-term embryo development (implantation and development to term) following mouse ICSI.

The primary interest of OPU oocytes lies in the potential of this source to provide oocytes of optimal maturational stage (i.e. full oocyte competency) that would lead to an improvement of subsequent embryo development (Blondin *et al.*, 2012). This selection is based on studies that demonstrated the follicular environment to have a crucial role in determining the ability of oocytes to gain full post-insemination developmental potential (Hyttel *et al.*, 1997; Sirard, 2011). Consequently, various parameters such as follicle size, health/atresia, FSH stimulation in combination with specific coasting (hormonal withdrawal) periods, can all be regulated to a greater or lesser extent to determine oocyte quality in a manner that has not yet been equalled using *in vitro* approaches (Blondin *et al.*, 2012). The use of such oocytes for the studies undertaken in the current thesis may have led to improved developmental outcomes following ICSI. These would probably be characterized by the presence of specific mRNAs, proteins and certain molecules that would enable the oocyte to respond properly to sperm entrance

and to complete egg activation thereby allowing oocyte-to-embryo transition and subsequent development (Sirard *et al.*, 2006; Blondin *et al.*, 2012).

Another relevant area that requires further research, independent to that above, is the optimisation of IVM protocols in order to obtain more competent oocytes; characterized for undergoing complete oocyte maturation (nuclear and cytoplasmic). In fact, cytoplasmic incompetent oocytes could be one of main reasons of the low success of bovine ICSI according to recent findings reported by Águila *et al.* (2017b). They observed a consistent fail in bull sperm DNA decondensation following their microinjection into *in vitro* matured bovine and mouse oocytes. However, this problem did not occur when bull sperm were microinjected into *in vivo* matured mouse oocytes. Therefore, it would be desirable to describe a series of cytoplasmic markers which would identify, by non-invasive means, "more developmentally competent oocytes". Improvements would ultimately be assessed in terms of development following ICSI. Suboptimal protocols of IVM are common to all species (Smith, 2001; Sutton *et al.*, 2003; Blondin *et al.*, 2012), improvements to which is the subject of ongoing investigations in our and other laboratories.

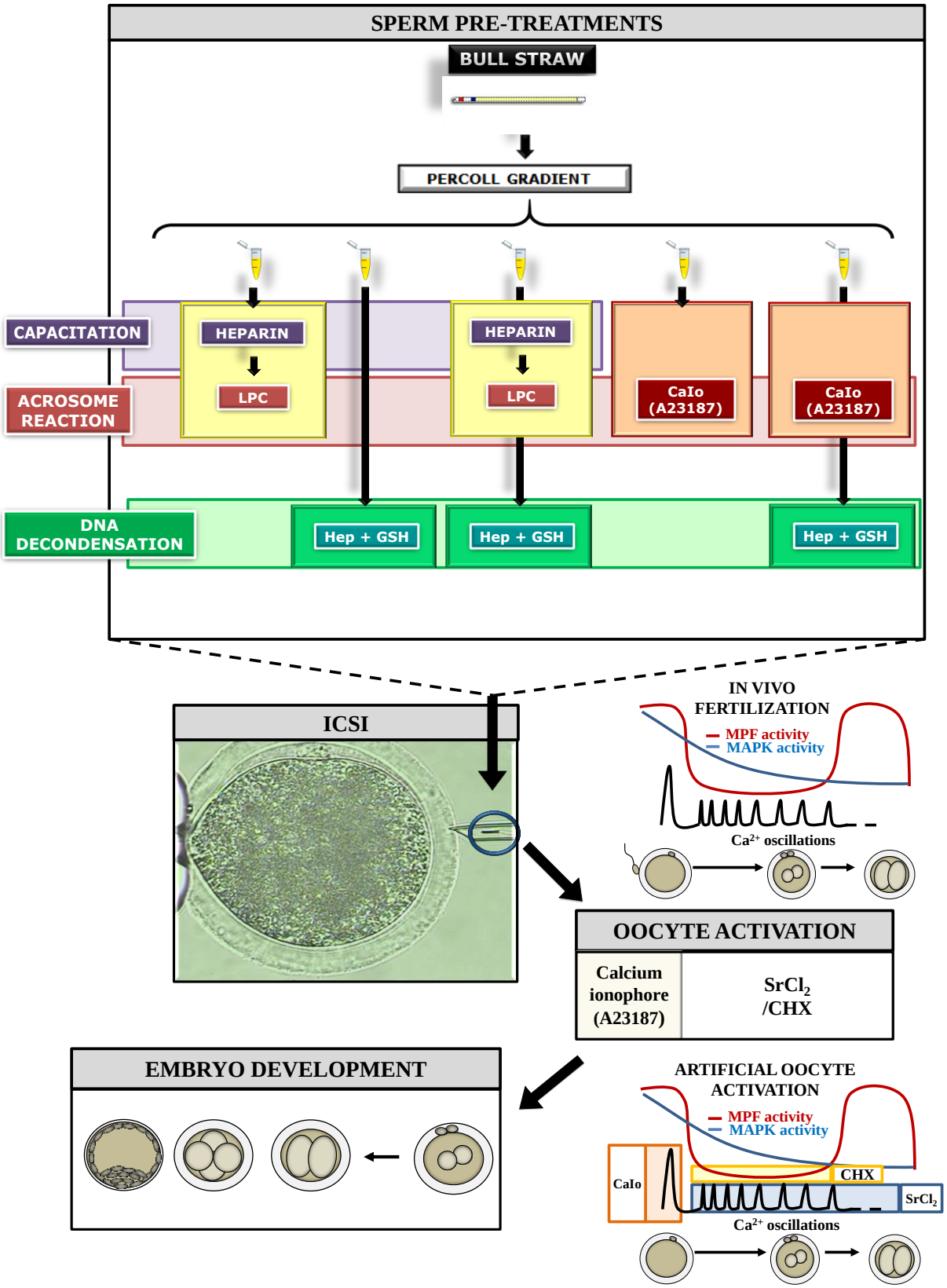
Another strategy that could improve the success of bovine ICSI would involve the direct microinjection of the main physiological sperm factor (PLCζ) responsible of inducing egg activation in several mammalian species. Such a study would involve microinjection of the protein rather than cRNA as it is easier to control the amount of PLCζ protein present into the oocyte cytoplasm (Ross *et al.*, 2008; Yu *et al.*, 2008b; Sanusi *et al.*, 2015). Similarly, it would be also worthy to investigate in depth the pathway and all components involved in the PLCζ-independence mechanism which allows normal sperm-egg fusion and subsequent, embryo development leading to healthy offspring when wild-type mice females are fertilised by PLCζ knockout males (Hachem *et al.*, 2017; Nozawa *et al.*, 2018). It might explain reproductive efficiency differences between IVF and ICSI in cattle.

Finally, as a complementary study, Ca^{2+} oscillations should be recorded following the microinjection of spermatozoon together with the proper amount

of PLCζ protein in order to confirm if this sperm factor (PLCζ) is also responsible for egg activation in the cow, and to compare Ca^{2+} oscillation patterns induced with that following normal fertilisation. Also, the measurement of Ca^{2+} oscillations would provide useful data following the artificial activation of bovine oocytes using the new protocol developed (CaIo+$SrCl_2$/CHX) in this thesis. This would help to elucidate whether the encouraging results (cleaved and blastocyst development) associated with this AOA protocol could be attributed, at least in part, to the action of Sr^{2+}.

All these studies would help to enhance fertilisation success and/or embryo development following ICSI in cattle. However, perhaps the most important contribution would generate basic information on sperm-egg interactions in mammalian species. This could stimulate progress of certain areas that require further research in human assisted reproduction such as IVM or fertility preservation. The existence of several similarities (i.e. folliculogenesis, ovulation, certain features of oocytes and embryo metabolism) between cattle and humans (Langbeen *et al.*, 2015) means the bovine *in vitro* model as very suited for human research.

5.4 SUMMARY


SPERM PRE-TREATMENTS
BULL STRAW
PERCOLL GRADIENT
CAPACITATION
ACROSOME REACTION
DNA DECONDENSATION
HEPARIN
LPC
HEPARIN
LPC
CaIo (A23187)
CaIo (A23187)
Hep + GSH
Hep + GSH
Hep + GSH
ICSI
IN VIVO FERTILIZATION
MPF activity
MAPK activity
Ca2+ oscillations
OOCYTE ACTIVATION
Calcium ionophore (A23187)
SrCl2 /CHX
EMBRYO DEVELOPMENT
ARTIFICIAL OOCYTE ACTIVATION
MPF activity
MAPK activity
CaIo
CHX
SrCl2
Ca2+ oscillations

REFERENCES

Abeydeera LR, Wang WH, Cantley TC, Rieke A, Day BN. 1998. Co-culture with follicular shell pieces can enhance the developmental competence of pig oocytes after in vitro fertilization: Relevance to intracellular glutathione. *Biol. Reprod*. **58**(1):213–18.

Acott TS, Carr DW. 1984. Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. *Biol. Reprod*. **30**(4):926–35.

Adamiak SJ, Mackie K, Watt RG, Webb R, Sinclair KD. 2005. Impact of nutrition on oocyte quality: Cumulative effects of body composition and diet leading to hyperinsulinemia in cattle. *Biol. Reprod.* **73**(5):918–26.

Adamiak SJ, Powell K, Rooke JA, Webb R, Sinclair KD. 2006. Reproduction research body composition, dietary carbohydrates and fatty acids determine post-fertilisation development of bovine oocytes in vitro. *Reproduction.* **131**:247–58.

Adeyemo O, Heath E, Adadevoh BK, Steinbach J, Olaloku EA. 1979. Some physiological and behavioural responses in Bos indicus and Bos taurus heifers acclimatized to the hot humid seasonal equatorial climate. *Int. J. Biometeorol.* **23**(3):231–41.

Águila L, Zambrano F, Arias ME, Felmer R. 2017a. Sperm capacitation pretreatment positively impacts bovine intracytoplasmic sperm injection. *Mol. Reprod. Dev.* **84**(7):649–59.

Águila L, Felmer R, Arias ME, Navarrete F, Martin-Hidalgo D, *et al.* 2017b. Defective sperm head decondensation undermines the success of ICSI in the bovine. *Reproduction.* **154**(3):307–18.

Ajduk A, Małagocki A, Maleszewski M. 2008. Cytoplasmic maturation of mammalian oocytes: Dvelopment of a mechanism responsible for sperm-induced Ca^{2+} oscillations. *Reprod. Biol*. **8**(1):3–22.

Alberio R, Zakhartchenko V, Motlik J, Wolf E. 2001. Mammalian oocyte activation: Lessons from the sperm and implications for nuclear transfer. *Int. J. Dev. Biol.* **45**(7):797–809.

Alexander B, Coppola G, Di Berardino D, Rho GJ, St John E, *et al.* 2006. The effect of 6-dimethylaminopurine (6-DMAP) and cycloheximide (CHX) on the development and chromosomal complement of sheep parthenogenetic and nuclear transfer embryos. *Mol. Reprod. Dev.* **73**(1):20–30.

Álvarez P, Spicer LJ, Chase CC, Payton ME, Hamilton TD, *et al.* 2000. Ovarian and endocrine characteristics during an estrous cycle in Angus, Brahman, and Senepol cows in a subtropical environment. *J. Anim. Sci.* **78**(5):1291–1302.

Álvarez M, Tamayo-Canul J, Anel E, Boixo JC, Mata-Campuzano M, *et al.* 2012. Sperm concentration at freezing affects post-thaw quality and fertility of ram semen. *Theriogenology.* **77**(6):1111–18.

Amaral E, Perdigão A, Souza MH, Mauck C, Waller D, *et al.* 2004. Postcoital testing after the use of a bio-adhesive acid buffering gel (acidform) and a 2% nonoxynol-9 product. *Contraception.* **70**(6):492–97.

Amari S, Yonezawa N, Mitsui S, Katsumata T, Hamano S, *et al.* 2001. Essential role of the nonreducing terminal alpha-mannosyl residues of the N-linked carbohydrate chain of bovine zona pellucida glycoproteins in sperm-egg binding. *Mol. Reprod. Dev.* **59**(2):221–26.

Anderson RA, Lovrien RE. 1981. Erythrocyte membrane sidedness in lectin control of the Ca^{2+}–A23187-mediated diskocyte ⇄ echinocyte conversion. *Nature.* **292**(5819):158–61.

Anzalone DA, Iuso D, Czernik M, Ptak G, Loi P. 2016. Plasma membrane and acrosome loss before ICSI is required for sheep embryonic development**.** *J. Assist Reprod Genet.* **33**: 757-763.

Apichela SA, Valz-Gianinet JN, Schuster S, Jiménez-Díaz MA, Roldán-Olarte EM, *et al.* 2010. Lectin binding patterns and carbohydrate mediation of sperm binding to llama oviductal cells in vitro. *Anim. Reprod. Sci*. **118**(2–4):344–53.

Arias ME, Sánchez R, Risopatrón J, Pérez L, Felmer R. 2014. Effect of sperm pretreatment with sodium hydroxide and dithiothreitol on the efficiency of bovine intracytoplasmic sperm injection. *Reprod. Fertil. Dev.* **26**(6):847–54.

Arias ME, Risopatrón J, Sánchez R, Felmer R. 2015. Intracytoplasmic sperm injection affects embryo developmental potential and gene expression in cattle. *Reprod. Biol.* **15**(1):34–41**.**

Arias ME, Sánchez R, Felmer R. 2016. Effect of anisomycin, a protein synthesis inhibitor, on the in vitro developmental potential, ploidy and embryo quality of bovine icsi embryos. *Zygote.* **24**(5):724–32.

Arlotto T, Schwartz JL, First NL, Leibfried-Rutledge ML. 1996. Aspects of follicle and oocyte stage that affect in vitro maturation and development of bovine oocytes. *Theriogenology.* **45**(5):943–56.

Armant DR. 2015. Intracellular Ca^{2+} signaling and preimplantation development. *Adv Exp Med Biol.* **843**:151–71.

Armstrong DG, McEvoy TG, Baxter G, Robinson JJ, Hogg CO, *et al.* 2001. Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: Associations with the ovarian insulin-like growth factor system. *Biol. Reprod.* **64**(6):1624–32.

Arnoult C, Cardullo RA, Lemos JR, Florman HM. 1996. Activation of mouse sperm T-type Ca^{2+} channels by adhesion to the egg zona pellucida. *Proc. Natl. Acad. Sci.* **93**(23):13004–9.

Ashby MC, Tepikin A V. 2002. Polarized calcium and calmodulin signaling in secretory epithelia. *Physiol. Rev.* **82**(3):701–34.

Avery B, Hay-Schmidt A, Hyttel P, Greve T. 1998. Embryo development, oocyte morphology, and kinetics of meiotic maturation in bovine oocytes exposed to 6-dimethylaminopurine prior to in vitro maturation. *Mol. Reprod. Dev*. **50**(3):334–44.

Bahat A, Caplan SR, Eisenbach M. 2012. Thermotaxis of human sperm cells in extraordinarily shallow temperature gradients over a wide range. *PLoS One*. **7**(7):e41915.

Baillie HS, Pacey AA, Warren MA, Scudamore IW, Barratt CL. 1997. Greater numbers of human spermatozoa associate with endosalpingeal cells derived from the isthmus compared with those from the ampulla. *Hum. Reprod.* **12**(9):1985–92.

Baldrighi J, Sá Filho M, Batista E, Lopes R, Visintin J, *et al.* 2014. Anti-mullerian hormone concentration and antral ovarian follicle population in Murrah heifers compared to Holstein and Gyr kept under the same management. *Reprod. Domest. Anim.* **49**(6):1015–20.

Balhorn R. 1982. A model for the structure of chromatin in mammalian sperm. *J. Cell Biol.* **93**(2):298–305.

Balhorn R. 1989. Mammalian protamines: Structure and molecular interactions. *Molecular Biology of Chromosome Function*, pp. 366–95.

Balhorn R. 2007. The protamine family of sperm nuclear proteins. Genome Biol. **8**(9):227.

Barros CM, Pegorer MF, Vasconcelos JLM, Eberhardt BG, Monteiro FM. 2006. Importance of sperm genotype (indicus versus taurus) for fertility and embryonic development at elevated temperatures. *Theriogenology*. **65**(1):210–18.

Bastos MR, Mattos MCC, Meschiatti MAP, Surjus RS, Guardieiro MM, *et al.* 2010. Ovarian function and circulating hormones in nonlactating Nelore versus Holstein cows. *Acta Sci Vet*, **38**(suppl. 2):776 (abstract).

Batista E, Macedo G, Sala R, Ortolan M, Sá Filho M, *et al.* 2014. Plasma antimullerian hormone as a predictor of ovarian antral follicular population in Bos indicus (Nelore) and Bos taurus (Holstein) heifers. *Reprod. Domest. Anim.* **49**(3):448–52.

Batista EOS, Vieira LM, Sá Filho MF, Dias EAR, Bayeux BM, *et al.* 2016. Ovarian follicular growth suppression by long-term treatment with a GnRH agonist and impact on small follicle number, oocyte yield, and in vitro embryo production in Zebu beef cows. *Theriogenology*. **85**(9):1680–87.

Bedford-Guaus SJ, Yoon SY, Fissore RA, Choi YH, Hinrichs K. 2008. Microinjection of mouse Phospholipase C zeta complementary RNA into mare oocytes induces long-lasting intracellular calcium oscillations and embryonic development. *Reprod. Fertil. Dev.* **20**(8):875–83.

Bedford-Guaus SJ, McPartlin LA, Xie J, Westmiller SL, Buffone MG, *et al.* 2011. Molecular cloning and characterization of Phospholipase C zeta in equine sperm and testis reveals species-specific differences in expression of catalytically active protein. *Biol. Reprod.* **85**(1):78–88.

Beebe SJ, Leyton L, Burks D, Ishikawa M, Fuerst T, *et al.* 1992. Recombinant mouse ZP3 inhibits sperm binding and induces the acrosome reaction. *Dev. Biol.* **151**(1):48–54.

Bennett IL, Finch VA, Holmes CR. 1985. Time spent in shade and its relationship with physiological factors of thermoregulation in three breeds of cattle. *Appl. Anim. Behav. Sci.* **13**(3):227–36.

Bermejo-Álvarez P, Rizos D, Rath D, Lonergan P, Gutiérrez-Adán A. 2008. Can bovine in vitro-matured oocytes selectively process X- or Y-sorted sperm differentially? *Biol. Reprod*. **79**(4):594–97.

Bevacqua RJ, Pereyra-Bonnet F, Fernandez-Martin R, Salamone DF. 2010. High rates of bovine blastocyst development after ICSI-mediated gene transfer assisted by chemical activation. *Theriogenology.* **74**(6):922–31.

Bhak JS, Lee SL, Ock SA, Mohana kumar B, Choe SY, *et al.* 2006. Developmental rate and ploidy of embryos produced by nuclear transfer with different activation treatments in cattle. *Anim. Reprod. Sci.* **92**(1–2):37–49.

Bi M, Hickox JR, Winfrey VP, Olson GE, Hardy DM. 2003. Processing, localization and binding activity of zonadhesin suggest a function in sperm adhesion to the zona pellucida during exocytosis of the acrosome. *Biochem. J.* **375**(2):477–88.

Björndahl L, Söderlund I, Kvist U. 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Hum. Reprod.* **18**(4):813–16.

Bleil JD, Wassarman PM. 1980a. Structure and function of the zona pellucida: Identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev. Biol.* **76**(1):185–202.

Bleil JD, Wassarman PM. 1980b. Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse egg zonae pellucida possessing receptor activity for sperm. *Cell.* **20**(3):873–82.

Bleil JD, Wassarman PM. 1983. Sperm-egg interactions in the mouse: Sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev. Biol.* **95**(2):317–24.

Bleil JD, Greve JM, Wassarman PM. 1988. Identification of a secondary sperm receptor in the mouse egg zona pellucida: Role in maintenance of binding of acrosome-reacted sperm to eggs. *Dev. Biol.* **128**(2):376–85.

Bleil JD, Wassarman PM. 1988. Galactose at the nonreducing terminus of O-linked oligosaccharides of mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity. *Proc. Natl. Acad. Sci.* **85**(18):6778–82.

Blondin P, Vigneault C, Nivet AL, Sirard MA. 2012. Improving oocyte quality in cows and heifers-what have we learned so far? *Anim. Reprod*. **9**(3): 281-289.

Boa-Amponsem K, Minozzi G. 2006. The state of development of biotechnologies as they relate to the managenement of animal genetic resources and their potential application in

developing countries. *Background Study Paper*. **33**.

Bols PE, Vandenheede JM, Van Soom A, de Kruif A. 1995. Transvaginal ovum pick-up (OPU) in the cow: A new disposable needle guidance system. *Theriogenology.* **43**(3):677–87.

Bork P, Sander C. 1992. A large domain common to sperm receptors (ZP2 and ZP3) and TGF-beta type III receptor. *FEBS Lett.* **300**(3):237–40.

Bos-Mikich A, Swann K, Whittingham DG. 1995. Calcium oscillations and protein synthesis inhibition synergistically activate mouse oocytes. *Mol. Reprod. Dev.* **41**(1):84–90.

Bos-Mikich A, Whittingham DG, Jones KT. 1997. Meiotic and mitotic Ca^{2+}oscillations affect cell composition in resulting blastocysts. *Dev. Biol.* **182**(1):172–79.

Brad AM, Bormann CL, Swain JE, Durkin RE, Johnson AE, *et al.* 2003. Glutathione and adenosine triphosphate content of in vivo and in vitro matured porcine oocytes. *Mol. Reprod. Dev.* **64**(4):492–98.

Breitbart H, Spungin B. 1997. The biochemistry of the acrosome reaction. *Mol. Hum. Reprod.* **3**(3):195–202.

Brevini-Gandolfi TAL, Gandolfi F. 2001. The maternal legacy to the embryo: Cytoplasmic components and their effects on early development. *Theriogenology.* **55**(6):1255–76.

Bromfield EG, Nixon B. 2013. The function of chaperone proteins in the assemblage of protein complexes involved in gamete adhesion and fusion processes. *Reproduction.* **145**(2):R31–42.

Bruynzeel AW, Merton JS, Wijst J, Hazeleger W, Kemp B. 1997. Effect of cumulus cells during IVM on bovine early embryonic development. *Theriogenology.* **47**(1):185.

Buffone MG, Rodriguez-Miranda E, Storey BT, Gerton GL. 2009. Acrosomal exocytosis of mouse sperm progresses in a consistent direction in response to zona pellucida. *J. Cell. Physiol.* **220**(3):611–20.

Butler ST, Hutchinson IA, Cromie AR, Shalloo L. 2014. Applications and cost benefits of sexed semen in pasture-based dairy production systems. *Animal.* **8**(s1):165–72.

Caballero JN, Gervasi MG, Veiga MF, Dalvit GC, Perez-Martínez S, *et al.* 2014. Epithelial cadherin is present in bovine oviduct epithelial cells and gametes, and is involved in fertilization-related events. *Theriogenology*. **81**(9):1189–1206.

Calhaz-Jorge C, De Geyter C, Kupka MS, de Mouzon J, Erb K, *et al.* 2017. Assisted reproductive technology in Europe, 2013: Results generated from European registers by ESHRE. *Hum. Reprod.* **32**(10):1957–73.

Calvin HI, Grosshans K, Blake EJ. 1986. Estimation and manipulation of glutathione levels in prepuberal mouse ovaries and ova: Relevance to sperm nucleus transformation in the fertilized egg. *Gamete Res.* **14**(3):265–75.

Campbell AK. 1983. Intracellular calcium: Its universal role as regulator. *John Wiley and Sons, New York.*

Canel NG, Bevacqua RJ, Hiriart MI, Rabelo NC, de Almeida Camargo LS, *et al.* 2017. Sperm pretreatment with heparin and L-glutathione, sex-sorting, and double cryopreservation to improve intracytoplasmic sperm injection in bovine. *Theriogenology.* **93**:62–70.

Canovas S, Coy P, Gomez E. 2007. First steps in the development of a functional assay for human sperm using pig oocytes. *J. Androl.* **28**(2):273–81.

Canovas S, Romar R, Grullon LA, Aviles M, Coy P. 2009. Pre-fertilization zona pellucida hardening by different cross-linkers affects IVF in pigs and cattle and improves embryo production in pigs. *Reproduction.* **137**(5):803–12.

Carr DW, Usselman MC, Acott TS. 1985. Effects of pH, lactate, and viscoelastic drag on sperm motility: a species comparison. *Biol. Reprod.* **33**(3):588–95.

Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, *et al*. 2000. Embryo implantation. *Dev. Biol*. **223**(2):217–37.

Carter SB. 1967. Effects of cytochalasins on mammalian cells. *Nature.* **213**(5073):261–64.

Carvalho JO, Sartori R, Machado GM, Mourão GB, Dode MAN. 2010. Quality assessment of bovine cryopreserved sperm after sexing by flow cytometry and their use in in vitro embryo production. *Theriogenology.* **74**(9):1521–30.

Catt JW, Rhodes SL. 1995. Comparative intracytoplasmic sperm injection (ICSI) in human and domestic species. *Reprod. Fertil. Dev.* **7**(2):161–6.

Catt SL, Catt JW, Gomez MC, Maxwell WM, Evans G. 1996. Birth of a male lamb derived from an in vitro matured oocyte fertilised by intracytoplasmic injection of a single presumptive male sperm. *Vet. Rec.* **139**(20):494–95.

Cha SK, Kim NH, Lee SM, Baik CS, Lee HT, *et al.* 1997. Effect of cytochalasin B and cycloheximide on the activation rate, chromosome constituent and in vitro development of porcine oocytes following parthenogenetic stimulation. *Reprod. Fertil. Dev.* **9**(4):441–46.

Chan AWS, Luetjens CM, Dominko T, Ramalho-Santos J, Simerly CR, *et al.* 2000. Foreign DNA transmission by ICSI: Injection of spermatozoa bound with exogenous DNA results in embryonic GFP expression and live rhesus monkey births. *Mol. Hum. Reprod.* **6**(1):26–33.

Che L, Lalonde A, Bordignon V. 2007. Chemical activation of parthenogenetic and nuclear transfer porcine oocytes using ionomycin and strontium chloride. *Theriogenology.* **67**(7):1297–1304.

Cheek TR, McGuinness OM, Vincent C, Moreton RB, Berridge MJ, *et al.* 1993. Fertilisation and thimerosal stimulate similar calcium spiking patterns in mouse oocytes but by separate mechanisms. *Development.* **119**(1):179–89.

Chebel RC, Santos JEP, Reynolds JP, Cerri RLA, Juchem SO, *et al.* 2004. Factors affecting conception rate after artificial insemination and pregnancy loss in lactating dairy

cows. *Anim. Reprod. Sci.* **84**(3–4):239–55.

Cheng W-M, An L, Wu Z-H, Zhu Y-B, Liu J-H, *et al.* 2009. Effects of disulfide bond reducing agents on sperm chromatin structural integrity and developmental competence of in vitro matured oocytes after intracytoplasmic sperm injection in pigs. *Reproduction*. **137**(4):633–43.

Chiu PCN, Wong BST, Chung M-K, Lam KKW, Pang RTK, *et al.* 2008. Effects of native human zona pellucida glycoproteins 3 and 4 on acrosome reaction and zona pellucida binding of human spermatozoa. *Biol. Reprod.* **79**(5):869–77.

Choi I, Zhu J, Campbell KHS. 2013. The combined treatment of calcium ionophore with strontium improves the quality of ovine SCNT embryo development. *Zygote.* **21**(2):139–50.

Choi YH, Love CC, Love LB, Varner DD, Brinsko S, *et al.* 2002. Developmental competence in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen-thawed spermatozoa. *Reproduction.* **123**(3):455–65.

Choi YH, Velez IC, Macías-García B, Riera FL, Ballard CS, *et al.* 2016. Effect of clinically-related factors on in vitro blastocyst development after equine ICSI. *Theriogenology*. **85**(7):1289–96.

Chung JT, Keefer CL, Downey BR. 2000. Activation of bovine oocytes following intracytoplasmic sperm injection (ICSI). *Theriogenology*. **53**(6):1273–84.

Cochran R, Meintjes M, Reggio B, Hylan D, Carter J, *et al.* 1998. Live foals produced from sperm-injected oocytes derived from pregnant mares. *J. Equine Vet. Sci.* **18**(11):736–40.

Cohen-Dayag A, Tur-Kaspa I, Dor J, Mashiach S, Eisenbach M. 1995. Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. *Proc. Natl. Acad. Sci*. **92**(24):11039–43.

Cooney MA, Malcuit C, Cheon B, Holland MK, Fissore RA, et al. 2010. Species-specific differences in the activity and nuclear localization of murine and bovine Phospholipase C zeta. *Biol. Reprod.* **83**(1):92–101.

Cornwall GA, Tulsiani DR, Orgebin-Crist MC. 1991. Inhibition of the mouse sperm surface alpha-d-mannosidase inhibits sperm-egg binding in vitro. *Biol. Reprod.* **44**(5):913–21.

Corzett M, Mazrimas J, Balhorn R. 2002. Protamine 1: Protamine 2 stoichiometry in the sperm of eutherian mammals. *Mol. Reprod. Dev.* **61**(4):519–27.

Costa MZ, Oliveira LZ, Resende MV, Lucio AC, Perini AP, *et al.* 2010. Induction of the acrosome reaction test to in vitro estimate embryo production in nelore cattle. *Arq. Bras. Med. Veterinária e Zootec.* **62**(4):771–77.

Courvalin JC, Dumontier M, Bornens M. 1982. Solubilization of nuclear structures by the polyanion heparin. *J. Biol. Chem.* **257**(1):456–63.

Coward K, Ponting CP, Chang HY, Hibbitt O, Savolainen P, *et al.* 2005. Phospholipase C,

the trigger of egg activation in mammals, is present in a non-mammalian species. *Reproduction*. **130**(2):157–63.

Coy P, Canovas S, Mondejar I, Saavedra MD, Romar R, *et al.* 2008. Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proc. Natl. Acad. Sci.* **105**(41):15809–14.

Coy P, Garcia-Vazquez FA, Visconti PE, Aviles M. 2012. Roles of the oviduct in mammalian fertilization. *Reproduction*. **144**(6):649–60.

Darszon A, Liévano A, Beltrán C. 1996. Ion channels: Key elements in gamete signalling. *Curr. Top. Dev. Biol.* **34**:117–67.

Darszon A, Nishigaki T, Beltran C, Treviño CL. 2011. Calcium channels in the development, maturation, and function of spermatozoa. *Physiol. Rev*. **91**(4):1305–55.

Day ML, McGuinness OM, Berridge MJ, Johnson MH. 2000. Regulation of fertilization-induced Ca^{2+} spiking in the mouse zygote. *Cell Calcium.* **28**(1):47–54.

De Jonge CJ, Barratt CLR. 2013. Methods for the assessment of sperm capacitation and acrosome reaction excluding the sperm penetration assay. *In Methods in molecular biology*. **927**:113–18.

De La Fuente R, King WA. 1998. Developmental consequences of karyokinesis without cytokinesis during the first mitotic cell cycle of bovine parthenotes. *Biol. Reprod.* **58**(4):952–62.

De Lamirande E, Leclerc P, Gagnon C. 1997. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol. Hum. Reprod*. **3**(3):175–94.

Delgado NM, Flores-Alonso JC, Rodríguez-Hernández HM, Merchant-Larios H, Reyes R. 2001. Heparin and glutathione II: correlation between decondensation of bull sperm cells and its nucleons. *Arch. Androl.* **47**(1):47–58.

Dell'Aquila ME, Albrizio M, Maritato F, Minoia P, Hinrichs K. 2003. Meiotic competence of equine oocytes and pronucleus formation after intracytoplasmic sperm injection (ICSI) as related to granulosa cell apoptosis. *Biol. Reprod.* **68**(6):2065–72.

DeMott RP, Lefebvre R, Suarez SS. 1995. Carbohydrates mediate the adherence of hamster sperm to oviductal epithelium. *Biol. Reprod.* **52**(6):1395–1403.

Dijkwel PA, Wenink PW. 1986. Structural integrity of the nuclear matrix: Differential effects of thiol agents and metal chelators. *J. Cell Sci.* **84**:53–67.

Dobrinski I, Ignotz GG, Thomas PG, Ball BA. 1996. Role of carbohydrates in the attachment of equine spermatozoa to uterine tubal (oviductal) epithelial cells in vitro. *Am. J. Vet. Res.* **57**(11):1635–39.

Dostál J, Veselský L, Marounek M, Zelezná B, Jonáková V. 1997. Inhibition of bacterial and boar epididymal sperm immunogenicity by boar seminal immunosuppressive component in mice. *J. Reprod. Fertil.* **111**(1):135–41.

Dozortsev D, Rybouchkin A, De Sutter P, Dhont M. 1995. Sperm plasma membrane

damage prior to intracytoplasmic sperm injection: A necessary condition for sperm nucleus decondensation. *Hum. Reprod.* **10**(11):2960–64.

Drobyshev A. 1999. Massive parallel analysis of DNA-Hoechst 33258 binding specificity with a generic oligodeoxyribonucleotide Microchip. *Nucleic Acids Res.* **27**(20):4100–4105.

Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, *et al*. 2002. Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. *Dev. Biol.* **250**(2):280–91.

Dyer S, Chambers GM, de Mouzon J, Nygren KG, Zegers-Hochschild F, *et al.* 2016. International committee for monitoring assisted reproductive technologies world report: Assisted reproductive technology 2008, 2009 and 2010. *Hum. Reprod.* **31**(7):1588–1609.

Ebner T, Montag M. 2016. Artificial oocyte activation: Evidence for clinical readiness. *Reprod. Biomed. Online.* **32**(3):271–73.

Edwards JL, Hansen PJ. 1997. Differential responses of bovine oocytes and preimplantation embryos to heat shock. *Mol. Reprod. Dev.* **46**(2):138–45.

Eisenbach M, Tur-Kaspa I. 1999. Do human eggs attract spermatozoa? *BioEssays*. **21**(3):203–10.

Ekhlasi-Hundrieser M, Gohr K, Wagner A, Tsolova M, Petrunkina A, *et al.* 2005. Spermadhesin AQN1 is a candidate receptor molecule involved in the formation of the oviductal sperm reservoir in the pig1. *Biol. Reprod.* **73**(3):536–45.

Escoffier J, Yassine S, Lee HC, Martinez G, Delaroche J, *et al.* 2015. Subcellular localization of phospholipase Cζ in human sperm and its absence in DPY19L2-deficient sperm are consistent with its role in oocyte activation. *Mol. Hum. Reprod.* **21**(2):157–68.

Espinosa-Cervantes R, Córdova-Izquierdo A. 2013. Sexing sperm of domestic animals. *Tropical Animal Health and Production.* **45**(1):1–8.

Evans G, Hollinshead FK, Maxwell WMC. 2004. Preservation and artificial insemination of sexed semen in sheep. *Reprod. Fertil. Dev.* **16**(4):455–64.

Fair T, Hyttel P, Greve T. 1995. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Mol. Reprod. Dev.* **42**(4):437–42.

Felmer R, Arias ME. 2015. Activation treatment of recipient oocytes affects the subsequent development and ploidy of bovine parthenogenetic and somatic cell nuclear transfer (SCNT) embryos. *Mol. Reprod. Dev.* **82**(6):441–49.

Fernandes CB, Devito LG, Martins LR, Blanco IDP, de Lima Neto JF, *et al.* 2014. Artificial activation of bovine and equine oocytes with cycloheximide, roscovitine, strontium, or 6-dimethylaminopurine in low or high calcium concentrations. *Zygote.* **22**(03):387–94.

Ferraretti AP, Nygren K, Andersen AN, de Mouzon J, Kupka M, *et al.* 2017. Trends over 15 years in art in Europe: An analysis of 6 million cycles. *Hum. Reprod. Open*. **2017**(2):1-10.

First NL, Parrish JJ. 1987. In-vitro fertilization of ruminants. *J. Reprod. Fertil.* Suppl. **34**:151–65.

Fissore RA, Pinto-Correia C, Robl JM. 1995. Inositol trisphosphate-induced calcium release in the generation of calcium oscillations in bovine eggs. *Biol. Reprod.* **53**(4):766–74.

Fissore RA, Smyth J, Kurokawa M, Collas P. 2002. Chapter 2 – Activation of mammalian oocytes. *Principles of Cloning*, 1st edition, Academic Press. pp. 21–45.

Florman HM. 1994. Sequential focal and global elevations of sperm intracellular Ca^{2+} are initiated by the zona pellucida during acrosomal exocytosis. *Dev. Biol.* **165**(1):152–64.

Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CMB. 1998. A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm:A tale of two channels. *Biol. Reprod.* **59**(1):12–16.

Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CMB. 1999. An intimate biochemistry: Egg-regulated acrosome reactions of mammalian sperm. *Adv. Dev. Biochem.* **5**:199–233.

Fraser LR. 1998. Sperm capacitation and the acrosome reaction. *Hum. Reprod.* **13**(suppl 1):9–19.

Fujinami N, Hosoi Y, Kato H, Matsumoto K, Saeki K, *et al.* 2004. Activation with ethanol improves embryo development of ICSI-derived oocytes by regulation of kinetics of MPF activity. *J. Reprod. Dev*. **50**(2):171–78.

Furnus CC, de Matos DG, Moses DF. 1998. Cumulus expansion during in vitro maturation of bovine oocytes: Relationship with intracellular glutathione level and its role on subsequent embryo development. *Mol. Reprod. Dev.* **51**(1):76–83.

Gadella BM. 2010. Interaction of sperm with the zona pellucida during fertilization. *Soc. Reprod. Fertil*. Suppl. **67**:267–87.

Gadella BM, Evans JP. 2011. Membrane fusions during mammalian fertilization. *Adv. Exp. Med. Biol.* **713**:65–80.

Gadella BM, 2013. Dynamic regulation of sperm interactions with the zona pellucida prior to and after fertilisation. *Reprod. Fertil.Dev.* **25**: 26-37.

Galantino-Homer HL, Visconti PE, Kopf GS. 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3'5'-monophosphate-dependent pathway. *Biol. Reprod.* **56**(3):707–19.

Galli C, Crotti G, Notari C, Turini P, Duchi R, *et al.* 2001. Embryo production by ovum pick up from live donors. *Theriogenology.* **55**(6):1341–57.

Galli C, Vassiliev I, Lagutina I, Galli A, Lazzari G. 2003. Bovine embryo development following ICSI: Effect of activation, sperm capacitation and pre-treatment with dithiothreitol. *Theriogenology*. **60**(8):1467–80.

Galli C, Duchi R, Colleoni S, Lagutina I, Lazzari G. 2014. Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. *Theriogenology.* **81**(1):138–51.

Ganguly A, Sharma RK, Gupta SK. 2008. Bonnet monkey (Macaca radiata) ovaries, like human oocytes, express four zona pellucida glycoproteins. *Mol. Reprod. Dev.* **75**(1):156–66.

Ganguly A, Bukovsky A, Sharma RK, Bansal P, Bhandari B, *et al.* 2010. In humans, zona pellucida glycoprotein-1 binds to spermatozoa and induces acrosomal exocytosis. *Hum. Reprod.* **25**(7):1643–56.

García-Roselló E, García-Mengual E, Coy P, Alfonso J, Silvestre MA. 2009. Intracytoplasmic sperm injection in livestock species: An update. *Reprod. Domest. Anim.* **44**(1):143–51.

Garcia-Vazquez FA, Ruiz S, Matas C, Izquierdo-Rico MJ, Grullon LA, *et al*. 2010. Production of transgenic piglets using ICSI-sperm-mediated gene transfer in combination with recombinase RecA. *Reproduction.* **140**(2):259–72.

Garner DL. 2006. Flow cytometric sexing of mammalian sperm. *Theriogenology.* **65**(5):943–57.

Garner DL, Seidel GE. 2008. History of commercializing sexed semen for cattle. *Theriogenology*. **69**(7):886–95.

Gervasi MG, Osycka-Salut C, Caballero J, Vazquez-Levin M, Pereyra E, *et al.* 2011. Anandamide capacitates bull spermatozoa through CB1 and TRPV1 activation. *PLoS One.* **6**(2):e16993.

Gibbons JR, Beal WE, Krisher RL, Faber EG, Pearson RE, *et al.* 1994. Effects of once versus twice weekly transvaginal follicular aspiration on bovine oocyte recovery and embryo development. *Theriogenology*. **42**: 405-419.

Gilbert SF, Barresi MJF. 2016. Chapter 7-Fertilization. *Developmental biology*, 11th edition. Sunderland, Massachusetts Press. pp 217-250.

Gillan L, Evans G, Maxwell WMC. 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology.* **63**(2):445–57.

Gimenes LU, Sá Filho MF, Carvalho NAT, Torres-Júnior JRS, Souza AH, *et al*. 2008. Follicle deviation and ovulatory capacity in Bos indicus heifers. *Theriogenology.* **69**(7):852–58.

Gimenes LU, Ferraz ML, Fantinato-Neto P, Chiaratti MR, Mesquita LG, *et al.* 2015. The interval between the emergence of pharmacologically synchronized ovarian follicular waves and ovum pickup does not significantly affect in vitro embryo production in Bos indicus, Bos taurus, and Bubalus bubalis. *Theriogenology.* **83**(3):385–93.

Gioia L, Barboni B, Turriani M, Capacchietti G, Pistilli MG, *et al.* 2005. The capability of reprogramming the male chromatin after fertilization is dependent on the quality of oocyte maturation. *Reproduction.* **130**(1):29–39.

Goissis MD, Cibelli JB. 2014. Functional characterization of SOX2 in bovine preimplantation embryos. *Biol. Reprod.* **90**(2):30–30.

Gómez MC, Catt JW, Evans G, Maxwell WM. 1998a. Cleavage, development and competence of sheep embryos fertilized by intracytoplasmic sperm injection and in vitro fertilization. *Theriogenology*. **49**(6):1143–54

Gómez MC, Catt JW, Evans G, Maxwell WM. 1998b. Sheep oocyte activation after intracytoplasmic sperm injection (ICSI). *Reprod. Fertil. Dev.* **10**(2):197–205.

Gonçalves FS, Barretto LSS, Arruda RP, Perri SH V, Mingoti GZ. 2014. Heparin and penicillamine-hypotaurine-epinephrine (PHE) solution during bovine in vitro fertilization procedures impair the quality of spermatozoa but improve normal oocyte fecundation and early embryonic development. *In Vitro Cell. Dev. Biol. Anim.* **50**(1):39–47.

Gong JG. 2002. Influence of metabolic hormones and nutrition on ovarian follicle development in cattle: Practical implications. *Domest. Anim. Endocrinol*. **23**(1–2):229–41.

Gonzalez-Garcia J, Bradley J, Nomikos M, Paul L, Machaty Z, *et al.* 2014. The dynamics of MAPK inactivation at fertilization in mouse eggs. *J. Cell Sci*. **127**: 2749-2760.

Goto K. 1990. Bovine microfertilisation, and embryo transfer. *Mol Reprod Dev*. **36**: 288–90.

Goud PT, Goud AP, Rybouchkin AV, De Sutter P, Dhont M. 1998. Chromatin decondensation, pronucleus formation, metaphase entry and chromosome complements of human spermatozoa after intracytoplasmic sperm injection into hamster oocytes. *Hum. Reprod*. **13**(5):1336–45.

Goud PT, Goud AP, Van Oostveldt P, Dhont M. 1999. Presence and dynamic redistribution of Type I inositol 1,4,5-trisphosphate receptors in human oocytes and embryos during in-vitro maturation, fertilization and early cleavage divisions. *Mol. Hum. Reprod.* **5**(5):441–51.

Goud PT, Goud AP, Leybaert L, Oostveldt PV, Mikoshiba K, *et al.* 2002. Inositol 1,4,5-trisphosphate receptor function in human oocytes: calcium responses and oocyte activation-related phenomena induced by photolytic release of $InsP_3$ are blocked by a specific antibody to the Type I receptor. *Mol. Hum. Reprod.* **8**(10):912–18.

Goudet G, Bezard J, Duchamp G, Gerard N, Palmer E. 1997. Equine oocyte competence for nuclear and cytoplasmic in vitro maturation: Effect of follicle size and hormonal environment. *Biol Reprod.* **57**:232-245.

Goudet G, Mugnier S, Callebaut I, Monget P. 2008. Phylogenetic analysis and identification of pseudogenes reveal a progressive loss of zona pellucida genes during evolution of vertebrates. *Biol. Reprod.* **78**(5):796–806.

Griffin J, Emery BR, Huang I, Peterson CM, Carrell DT. 2006. Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). *J. Exp. Clin. Assist. Reprod*. **3**:2.

Guerreiro BM, Rodrigues CA, Castro Neto A, Silveira CRA, Vieira LM, *et al.* 2014. Prepubertal Holstein heifers have low efficiency when submitted to ovum pick-up and in

vitro embryo production. *Anim. Reprod.* **11**(3): 405 (abstract).

Gupta SK, Bansal P, Ganguly A, Bhandari B, Chakrabarti K. 2009. Human zona pellucida glycoproteins: Functional relevance during fertilization. *J. Reprod. Immunol*. **83**(1–2):50–55.

Gupta SK, Bhandari B, Shrestha A, Biswal BK, Palaniappan C, *et al.* 2012. Mammalian zona pellucida glycoproteins: structure and function during fertilization. *Cell Tissue Res.* **349**(3):665–78.

Haccard O, Sarcevic B, Lewellyn A, Hartley R, Roy L, *et al.* 1993. Induction of metaphase arrest in cleaving Xenopus embryos by MAP kinase. *Science* **262**:1262–1265.

Hachem A, Godwin J, Ruas M, Lee HC, Ferrer Buitrago M, *et al.* 2017. PLCζ is the physiological trigger of the Ca 2+ oscillations that induce embryogenesis in mammals but conception can occur in its absence. *Development.* **144**(16):2914–24.

Hagemann LJ, Beaumont SE, Berg M, Donnison MJ, Ledgard A, et al. 1999. Development during single IVP of bovine oocytes from dissected follicles: Interactive effects of estrous cycle stage, follicle size and atresia. *Mol. Reprod.* Dev. **53**(4):451–58.

Hajarian, 2011. Piezo-actuated Mouse ICSI. Eppendorf Userguide Nº: 037. www.eppendorf.com.

Hamano KI, Li X, Qian XQ, Funauchi K, Furudate M. 1999. Gender preselection in cattle with intracytoplasmically injected, flow cytometrically sorted heads. *Biol Reprod*. **60**:1194–7.

Hara H, Abdalla H, Morita H, Kuwayama M, Hirabayashi M, Hochi S. 2011. Procedure for bovine ICSI, not sperm freeze-drying, impairs the function of the microtubule-organizing center. *J. Reprod. Dev.* **57**(3):428–32.

Harper MJ. 1973. Relationship between sperm transport and penetration of eggs in the rabbit oviduct. *Biol. Reprod.* **8**(4):441–50.

Hashimoto S, Takakura R, Kishi M, Sudo T, Minami N, *et al.* 1999. Ultrasound-guided follicle aspiration: The collection of bovine cumulus-oocyte complexes from ovaries of slaughtered or live cows. *Theriogenology.* **51**(4):757–65.

Hatanaka Y, Nagai T, Tobita T, Nakano M. 1992. Changes in the properties and composition of zona pellucida of pigs during fertilization in vitro. *J. Reprod. Fertil*. **95**(2):431–40.

Hedrick JL, Wardrip NJ. 1987. On the macromolecular composition of the zona pellucida from porcine oocytes. *Dev. Biol.* **121**(2):478–88.

Heuwieser W, Yang X, Jiang S, Foote RH. 1992. Fertilization of bovine oocytes after microsurgical injection of spermatozoa. *Theriogenology.* **38**(1):1–9.

Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, *et al.* 2000. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biol. Reprod.* **63**(6):1787–94.

Hinrichs K. 2005. Update on equine ICSI and cloning. *Theriogenology.* **64**(3):535–41.

Hinrichs K. 2013. Assisted reproduction techniques in the horse. *Reprod. Fertil. Dev*. **25**(1):80.

Hiramoto Y. 1962. Microinjection of the live spermatozoa into sea urchin eggs. *Exp. Cell Res*. **27**(3):416–26.

Hirao Y, Nagai T, Kubo M, Miyano T, Miyake M, *et al.* 1994. In vitro growth and maturation of pig oocytes. *J. Reprod. Fertil.* **100**(2):333–39.

Hong CY, Chiang BN, Ku J, Wei YH, Fong JC. 1985. Calcium antagonists stimulate sperm motility in ejaculated human semen. *Br. J. Clin. Pharmacol.* **19**(1):45–49.

Hoodbhoy T, Talbot P. 1994. Mammalian cortical granules: Contents, fate, and function. *Mol. Reprod. Dev*. **39**(4):439–48.

Hoodbhoy T, Joshi S, Boja ES, Williams SA, Stanley P, *et al.* 2005. Human sperm do not bind to rat zonae pellucida despite the presence of four homologous glycoproteins. *J. Biol. Chem.* **280**(13):12721–31.

Hook SJ, Hafez ESE. 1968. A comparative anatomical study of the mammalian uterotubal junction. *J. Morphol.* **125**(2):159–84.

Horan B, Mee JF, O'Connor P, Rath M, Dillon P. 2005. The effect of strain of Holstein-friesian cow and feeding system on postpartum ovarian function, animal production and conception rate to first service. *Theriogenology.* **63**(3):950–71.

Horiuchi T, Emuta C, Yamauchi Y, Oikawa T, Numabe T, *et al.* 2002. Birth of normal calves after intracytoplasmic sperm injection of bovine oocytes: A methodological approach. *Theriogenology.* **57**(3):1013–24.

Hosoi Y, Miyake M, Utsumi K, Iritani A, 1988. Development of rabbit oocytes after microinjection of spermatozoa. In: Proceedings of 11th International Congress on Animal Reproduction and Artificial Insemination, June 26–30 1988, Dublin, Ireland, Vol. **1**, p. 331 (abstract).

Hou YP, Liu Y, Dai YP, Li R, Shi WQ, *et al.* 2009. Improved parthenogenetic development of vitrified-warmed bovine oocytes activated with 9% ethanol plus 6-DMAP. *Theriogenology.* **72**(5):643–49.

Howes E, Pascall JC, Engel W, Jones R. 2001. Interactions between mouse ZP2 glycoprotein and proacrosin: A mechanism for secondary binding of sperm to the zona pellucida during fertilization. *J. Cell Sci*. **114**(22):4127–36.

Hunter RHF. 1973. Polyspermic fertilization in pigs after tubal deposition of excessive numbers of spermatozoa. *J. Exp. Zool*. **183**(1):57–62.

Hunter RH. 1990. Fertilization of pig eggs in vivo and in vitro. *J. Reprod. Fertil.* Suppl. **40**:211–26.

Hunter RH. 1981. Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *J. Reprod. Fertil*. **63**(1):109–17.

Hunter RHF, Nichol R. 1983. Transport of spermatozoa in the sheep oviduct: Preovulatory sequestering of cells in the caudal isthmus. *J. Exp. Zool.* **228**(1):121–28.

Hunter RH, Wilmut I. 1984. Sperm transport in the cow: Peri-ovulatory redistribution of viable cells within the oviduct. *Reprod. Nutr. Dev.* **24**(5A):597–608.

Hunter RH, Vajta G, Hyttel P. 1998. Long-term stability of the bovine block to polyspermy. *J. Exp. Zool.* **280**(2):182–88.

Hunter RHF. 2012. Components of oviduct physiology in eutherian mammals. *Biol. Rev.* **87**(1):244–55.

Hwang S, Lee E, Yoon J, Yoon BK, Lee JH, *et al.* 2000. Effects of electric stimulation on bovine oocyte activation and embryo development in intracytoplasmic sperm injection procedure. *J. Assist. Reprod. Genet.* **17**(6):310–14.

Hynes RO. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell.* **110**(6):673–87.

Hyttel P, Fair T, Callesen H, Greve T. 1997. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology.* **47**(1):23–32.

Ignotz GG, Lo MC, Perez CL, Gwathmey TM, Suarez SS. 2001. Characterization of a fucose-binding protein from bull sperm and seminal plasma that may be responsible for formation of the oviductal sperm reservoir. *Biol. Reprod.* **64**(6):1806–11.

Inoue N, Ikawa M, Okabe M. 2011. The mechanism of sperm–egg interaction and the involvement of IZUMO1 in fusion. *Asian J. Androl.* **13**(1):81–87.

Iritani A, Utsumi K, Miyake M, Hosoi Y, Saeki K. 1988. In vitro fertilization by a routine method and by micromanipulation. *Annals of the New York Academy of Sciences.* **541**:583–90.

Ito M, Shikano T, Oda S, Horiguchi T, Tanimoto S, *et al.* 2008. Difference in Ca^{2+} oscillation-inducing activity and nuclear translocation ability of PLCζ1, an egg-activating sperm factor candidate, between mouse, rat, human, and medaka fish. *Biol. Reprod.* **78**(6):1081–90.

Izquierdo-Rico MJ, Jiménez-Movilla M, Llop E, Pérez-Oliva AB, Ballesta J, *et al.* 2009. Hamster zona pellucida is formed by four glycoproteins: ZP1, ZP2, ZP3, and ZP4. *J. Proteome Res.* **8**(2):926–41.

Jamil K, White IG. 1981. Induction of acrosomal reaction in sperm with ionophore A23187 and calcium. *Arch. Androl.* **7**(4):283–92.

Jansen RP. 1978. Fallopian tube isthmic mucus and ovum transport. *Science.* **201**(4353):349–51.

Jansen RP, Bajpai VK. 1982. Oviduct acid mucus glycoproteins in the estrous rabbit: Ultrastructure and histochemistry. *Biol. Reprod.* **26**(1):155–68.

Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, *et al.* 2011. Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proc. Natl. Acad. Sci.* **108**(12):4892–96.

Jo HT, Bang JI, Kim SS, Choi BH, Jin JI, *et al.* 2014. Production of female bovine embryos with sex-sorted sperm using intracytoplasmic sperm injection: Efficiency and in vitro developmental competence. *Theriogenology.* **81**(5):675–82.

Johnson LA, Flook JP, Hawk HW. 1989. Sex preselection in rabbits: Live births from X and Y sperm separated by DNA and cell sorting. *Biol. Reprod*. **41**(2):199–203.

Jones KT, Cruttwell C, Parrington J, Swann KA. 1998. Mammalian sperm cytosolic phospholipase C. Activity generates inositol trisphosphate and causes Ca^{2+} release in sea urchin egg homogenates. *FEBS Lett.* **437**: 297–300.

Jovine L, Qi H, Williams Z, Litscher E, Wassarman PM. 2002. The ZP domain is a conserved module for polymerization of extracellular proteins. *Nat. Cell Biol.* **4**(6):457–61.

Julianelli V, Farrando B, Alvarez Sedo C, Calvo L, Romanato M, *et al.* 2012. Heparin enhances protamine disulfide bond reduction during in vitro decondensation of human spermatozoa. *Hum. Reprod.* **27**(7):1930–38.

Kadzere C., Murphy M., Silanikove N, Maltz E. 2002. Heat stress in lactating dairy cows: A review. *Livest. Prod. Sci.* **77**(1):59–91.

Kaimio I, Mikkola M, Lindeberg H, Heikkinen J, Hasler JF, Taponen J. 2013. Embryo production with sex-sorted semen in superovulated dairy heifers and cows. *Theriogenology*. **80**(8):950–54.

Kanai S, Kitayama T, Yonezawa N, Sawano Y, Tanokura M, *et al.* 2008. Disulfide linkage patterns of pig zona pellucida glycoproteins ZP3 and ZP4. *Mol. Reprod. Dev.* **75**(5):847–56.

Karadjole Martina Karadjole M, Getz I, Samardžija M, Maćešić N, Matković Matković M, *et al.* 2010. The developmental competence of bovine immature oocytes and the developmental competence of bovine immature oocytes and quality of embryos derived from slaughterhouse ovaries or live quality of embryos derived from slaughterhouse ovaries or live donors by ovum pick up donors by ovum pick up. *Vet. Arh.* **80**(4):445–54.

Kashir J, Nomikos M, Lai FA, Swann K. 2014. Sperm-induced Ca^{2+} release during egg activation in mammals. *Biochem. Biophys. Res. Commun.* **450**(3):1204–11.

Kastrop PMM, Hulshof SCJ, Bevers MM, Destrée OHJ, Kruip TAM. 1991. The effects of α-amanitin and cycloheximide on nuclear progression, protein synthesis, and phosphorylation during bovine oocyte maturation in vitro. *Mol. Reprod. Dev.* **28**(3):249–54.

Katayama M, Sutovsky P, Yang BS, Cantley T, Rieke A, *et al.* 2005. Increased disruption of sperm plasma membrane at sperm immobilization promotes dissociation of perinuclear theca from sperm chromatin after intracytoplasmic sperm injection in pigs. *Reproduction.* **130**(6):907–16.

Katayose H, Yanagida K, Shinoki T, Kawahara T, Horiuchi T, *et al.* 1999. Efficient

injection of bull spermatozoa into oocytes using a piezo-driven pipette. *Theriogenology*. **52**(7):1215–24.

Kaufman MH, Sachs L. 1976. Complete preimplantation development in culture of parthenogenetic mouse embryos. *J. Embryol. Exp. Morphol.* **35**(1):179–90.

Kaufman MH, Barton SC, Surani MAH. 1977. Normal postimplantation development of mouse parthenogenetic embryos to the forelimb bud stage. *Nature.* **265**(5589):53–55.

Keefer CL. 1989. Fertilization by sperm injection in the rabbit. *Gamete Res.* **22**(1):59–69.

Keefer CL, Younis AI, Brackett BG. 1990. Cleavage development of bovine oocytes fertilized by sperm injection. *Mol. Reprod. Dev.* **25**(3):281–85.

Kharche SD, Birade HS. 2013. Parthenogenesis and activation of mammalian oocytes for in vitro embryo production: A review. *Adv. Biosci. Biotechnol.* **4**(2):170–82.

Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, *et al.* 2002. Successful piglet production after transfer of blastocysts produced by a modified in vitro system. *Biol. Reprod.* **66**(4):1033–41.

Kim HJ, Yoon HJ, Jang JM, Lee WD, Yoon SH, *et al.* 2017. Evaluation of human embryo development in in vitro fertilization- and intracytoplasmic sperm injection-fertilized oocytes: A time-lapse study. *Clin. Exp. Reprod. Med.* **44**(2):90.

Kim JW, Choi JL, Yang SH, Yoon SH, Jung JH, *et al.* 2012. Live birth after $SrCl_2$ oocyte activation in previous repeated failed or low fertilization rates after ICSI of frozen-thawed testicular spermatozoa: Case report. *J. Assist. Reprod. Genet.* **29**(12):1393–96.

Kimura Y, Yanagimachi R. 1995. Intracytoplasmic sperm injection in the mouse. *Biol. Reprod.* **52**(4):709–20.

Kline D, Kline JT. 1992. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149**(1):80–89.

Kohda T, Ogonuki N, Inoue K, Furuse T, Kaneda H, *et al.* 2011. Intracytoplasmic sperm injection induces transcriptome perturbation without any transgenerational effect. *Biochem. Biophys. Res. Commun.* **410**(2):282–88.

Komar A. 1982. Fertilization of parthenogenetically activated mouse eggs: Behaviour of sperm nuclei in the cytoplasm of parthenogenetically activated eggs. *Exp. Cell Res.* **139**(2):361–67.

Korkmaz Ö, Küplülü Ş, Ağca Y, Polat İM. 2013. Effect of oocyte quality and activation protocols on bovine embryo development following intracytoplasmic sperm injection. *Turk. J. Vet. Anim. Sci*. **37**: 26-30.

Kouchi Z, Fukami K, Shikano T, Oda S, Nakamura Y, *et al.* 2004. Recombinant Phospholipase Cζ has high Ca^{2+} sensitivity and induces Ca^{2+} oscillations in mouse eggs. *J. Biol. Chem.* **279**(11):10408–12.

Krapf D, Arcelay E, Wertheimer E V., Sanjay A, Pilder SH, *et al.* 2010. Inhibition of Ser/Thr phosphatases induces capacitation-associated signalling in the presence of Src

kinase inhibitors. *J. Biol. Chem.* **285**(11):7977–85.

Kubiak JZ, Weber M, de Pennart H, Winston NJ, Maro B. 1993. The metaphase II arrest in mouse oocytes is controlled through microtubule-dependent destruction of cyclin b in the presence of CSF. *EMBO J.* **12**(10):3773–78.

Kumar N, Singh AK. 2015. Trends of male factor infertility, an important cause of infertility: A review of literature. *Hum. Reprod. Sci*. **8**(4):191–96.

Kyono K, Kumagai S, Nishinaka C, Nakajo Y, Uto H, *et al.* 2008. Birth and follow-up of babies born following ICSI using $SrCl_2$ oocyte activation. *Reprod. Biomed. Online.* **17**(1):53–58.

Lagutina I, Lazzari G, Duchi R, Galli C. 2004. Developmental potential of bovine androgenetic and parthenogenetic embryos: A comparative study. *Biol. Reprod.* **70**(2):400–405.

Langbeen A, De porte HFM, Bartholomeus E, Leroy JLMR, Bols PEJ. 2015. Bovine in vitro reproduction models can contribute to the development of (female) fertility preservation strategies. *Theriogenology.* **84**(4):477–89.

Langley M, Doody K. 2014. Blastocyst formation: 16 year review of IVF and ICSI cycles. *Fertil. Steril.* **e343** (Abstract).

Latham KE, Akutsu H, Patel B, Yanagimachi R. 2002. Comparison of gene expression during preimplantation development between diploid and haploid mouse embryos. *Biol. Reprod*. **67**(2):386–92.

Lee CH, Cho YH. 1999. Aspects of mammalian spermatogenesis: Electrophoretical analysis of protamines in mammalian species. *Mol. Cells*. **9**(5):556–59.

Lee JW, Chang HC, Wu HY, Liu SS, Wang CH, *et al.* 2015. Effects of sperm pretreatment and embryo activation methods on the development of bovine embryos produced by intracytoplasmic sperm injection. *Reprod. Biol.* **15**(3):154–62.

Lefievre L, Conner SJ, Salpekar A, Olufowobi O, Ashton P, *et al.* 2004. Four zona pellucida glycoproteins are expressed in the human. *Hum. Reprod.* **19**(7):1580–86.

Leroy J, Opsomer G, Van Soom A, Goovaerts I, Bols P. 2008a. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? PART I-The importance of negative energy balance and altered corpus luteum function to the reduction of oocyte and embryo quality in high-yielding dairy cows*. *Reprod. Domest. Anim.* **43**(5):612–22.

Leroy J, Van Soom A, Opsomer G, Goovaerts I, Bols P. 2008b. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? PART II -Mechanisms linking nutrition and reduced oocyte and embryo quality in high-yielding dairy cows*. *Reprod. Domest. Anim.* **43**(5):623–32.

Leroy JLMR, Rizos D, Sturmey R, Bossaert P, Gutierrez-Adan A, *et al.* 2011. Intrafollicular conditions as a major link between maternal metabolism and oocyte quality: A focus on dairy cow fertility. *Reprod. Fertil. Dev.* **24**(1):1.

Li GP, Seidel GE, Squires EL. 2004. Improved cleavage of bovine ICSI ova cultured in heparin-containing medium. *Theriogenology.* **61**(6):1077–84.

Li XX, Lee D-S, Kim KJ, Lee JH, Kim EY, *et al.* 2013. Leptin and nonessential amino acids enhance porcine preimplantation embryo development in vitro by intracytoplasmic sperm injection. *Theriogenology*. **79**(2):291–98.

Lindsey AC, Schenk JL, Graham JK, Bruemmer JE, Squires EL. 2002. Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa. *Equine Vet. J.* **34**(2):121–27.

Liu L, Ju JC, Yang X. 1998. Parthenogenetic development and protein patterns of newly matured bovine oocytes after chemical activation. *Mol. Reprod. Dev.* **49**(3):298–307.

Liu L, Trimarchi JR, Keefe DL. 2002. Haploidy but not parthenogenetic activation leads to increased incidence of apoptosis in mouse embryos. *Biol. Reprod.* **66**(1):204–10.

Loi P, Ledda S, Fulka J, Cappai P, Moor RM. 1998. Development of parthenogenetic and cloned ovine embryos: Effect of activation protocols. *Biol. Reprod.* **58**(5):1177–87.

Lopes A, Martinussen T, Greve T, Callesen H. 2006. Effect of days post-partum, breed and ovum pick-up scheme on bovine oocyte recovery and embryo development. *Reprod. Domest. Anim.* **41**(3):196–203.

Luberda Z. 2005. The role of glutathione in mammalian gametes. *Reprod. Biol.* **5**(1):5–17.

Lucy JA. 1978. Mechanism of chemically induced cell fusion. *Membrane fusion Cell Surface Rev*. **5**: 267-304.

Lybaert P, Danguy A, Leleux F, Meuris S, Lebrun P. 2009. Improved methodology for the detection and quantification of the acrosome reaction in mouse spermatozoa. *Histol. Histopathol.* **24**(8):999–1007.

Lyu QF, Deng L, Xue SG, Cao SF, Liu XY, *et al.* 2010. New technique for mouse oocyte injection via a modified holding pipette. *Reprod. Biomed. Online.* **21**(5):663–66.

Ma SF, Liu XY, Miao DQ, Han ZB, Zhang X, *et al.* 2005. Parthenogenetic activation of mouse oocytes by strontium chloride: A search for the best conditions. *Theriogenology.* **64**(5):1142–57.

Malcuit C, Knott JG, He C, Wainwright T, Parys JB, *et al.* 2005. Fertilization and inositol 1,4,5-trisphosphate (IP_3)-induced calcium release in Type-1 inositol 1,4,5-trisphosphate receptor down-regulated bovine eggs. *Biol. Reprod.* **73**(1):2–13.

Malcuit C, Kurokawa M, Fissore RA. 2006a. Calcium oscillations and mammalian egg activation. *J. Cell. Physiol.* **206**(3):565–73.

Malcuit C, Maserati M, Takahashi Y, Page R, Fissore RA. 2006b. Intracytoplasmic sperm injection in the bovine induces abnormal $[Ca^{2+}]_i$ responses and oocyte activation. *Reprod. Fertil. Dev.* **18**(1–2):39–51.

Mann GE, Lamming GE. 2001. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction.*

121(1):175–80.

Markoulaki S, Matson S, Ducibella T. 2004. Fertilization stimulates long-lasting oscillations of CAMKII activity in mouse eggs. *Dev. Biol.* **272**(1):15–25.

Markovic J, García-Gimenez JL, Gimeno A, Viña J, Pallardó FV. 2010. Role of glutathione in cell nucleus. *Free Radic. Res.* **44**(7):721–33.

Marquez B, Suarez SS. 2004. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. *Biol. Reprod.* **70**(6):1626–33.

Marshall IC, Taylor CW. 1994. Two calcium-binding sites mediate the interconversion of liver inositol 1,4,5-trisphosphate receptors between three conformational states. *Biochem. J.* **301** (2):591–98.

Martin MJ. 2000. Development of in vivo-matured porcine oocytes following intracytoplasmic sperm injection. *Biol. Reprod.* **63**(1):109–12.

Martínez-Pastor F, Mata-Campuzano M, Álvarez-Rodríguez M, Álvarez M, Anel L, *et al.* 2010. Probes and techniques for sperm evaluation by flow cytometry. *Reprod. Domest.* Anim. **45**:67–78.

McEvoy TG, Robinson JJ, Aitken RP, Findlay PA, Palmer RM, *et al.* 1995. Dietary-induced suppression of pre-ovulatory progesterone concentrations in superovulated ewes impairs the subsequent in vivo and in vitro development of their ova. *Anim. Reprod. Sci.* **39**(2):89–107.

McLay DW, Carroll J, Clarke HJ. 2002. The ability to develop an activity that transfers histones onto sperm chromatin is acquired with meiotic competence during oocyte growth. *Dev. Biol.* **241**(1):195–206.

Menge AC, Edwards RP. 1993. Mucosal immunity of the reproductive tract and infertility. In Zaz RK (ed.), Immunology of Reproduction. CRC Press, Boca Raton, FL, pp 19-36.

Méo SC, Leal CLV, Garcia JM. 2004. Activation and early parthenogenesis of bovine oocytes treated with ethanol and strontium. *Anim. Reprod. Sci.* **81**(1–2):35–46.

Méo SC, Yamazaki W, Leal CLV, Oliveira JA de, Garcia JM. 2005. Use of strontium for bovine oocyte activation. *Theriogenology.* **63**(8):2089–2102.

Méo SC, Yamazaki W, Ferreira CR, Perecin F, Saraiva NZ, *et al.* 2007. Parthenogenetic activation of bovine oocytes using single and combined strontium, ionomycin and 6-dimethylaminopurine treatments. *Zygote.* **15**(04):295–306.

Merton JS, de Roos APW, Mullaart E, de Ruigh L, Kaal L, *et al.* 2003. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology.* **59**(2):651–74.

Merton JS, Ask B, Onkundi DC, Mullaart E, Colenbrander B, Nielen M. 2009. Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up–in vitro production embryo-production program. *Theriogenology.* **72**(7):885–93.

Mikkola M, Andersson M, Taponen J. 2015. Transfer of cattle embryos produced with sex-

sorted semen results in impaired pregnancy rate and increased male calf mortality. *Theriogenology.* **84**(7):1118–22.

Miranda P V, Gonzalez-Echeverría F, Marín-Briggiler CI, Brandelli A, Blaquier JA, *et al.* 1997. Glycosidic residues involved in human sperm-zona pellucida binding in vitro. *Mol. Hum. Reprod.* **3**(5):399–404.

Miyamoto H, Ishibashi T. 1975. The role of calcium ions in fertilization of mouse and rat eggs in vitro. J. *Reprod. Fertil*. **45**(3):523–26.

Mizuno K, Hoshi K, Huang T. 2002. Fertilization and embryo development in a mouse ICSI model using human and mouse sperm after immobilization in polyvinylpyrrolidone. *Hum. Reprod.* **17**(9):2350–55.

Mocé E, Graham JK, Schenk JL. 2006. Effect of sex-sorting on the ability of fresh and cryopreserved bull sperm to undergo an acrosome reaction. *Theriogenology.* **66**(4):929–36.

Molnarova Z, Machatkova M, Machal L, Horakova J, Hanzalova K. 2006. A potential relationship between the acrosome response characteristics of bovine spermatozoa and their in vitro fertilizing ability. *Zygote.* **14**(1):63–69.

Moore K, Thatcher WW. 2006. Major advances associated with reproduction in dairy cattle. *J. Dairy Sci.* **89**(4):1254–66.

Moreira F, Risco CA, Pires MFA, Ambrose JD, Drost M, *et al.* 2000. Use of bovine somatotropin in lactating dairy cows receiving timed artificial insemination. *J. Dairy Sci.* **83**(6):1237–47.

Moreno RD, Hoshi M, Barros C. 1999. Functional interactions between sulphated polysaccharides and proacrosin: Implications in sperm binding and digestion of zona pellucida. *Zygote.* **7**(2):105–11.

Morozumi K, Yanagimachi R. 2005. Incorporation of the acrosome into the oocyte during intracytoplasmic sperm injection could be potentially hazardous to embryo development. *Proceedings of National Academy of Sciences USA.* **102**(40):14209–14.

Morozumi K, Shikano T, Miyazaki S, Yanagimachi R. 2006. Simultaneous removal of sperm plasma membrane and acrosome before intracytoplasmic sperm injection improves oocyte activation/embryonic development. *Proc. Natl. Acad. Sci.* **103**(47):17661–66.

Morrell JM. 2011. Chapter-Artificial Insemination: Current and Future Trends. *Artificial Insemination in Farm Animals.* IntechOpen. DOI: 10.5772/17943.

Mortimer D. 1985. The male factor in infertility. Part I: Semen analysis. *In Current problems in Obstetrics, Gynecology and Fertility.* **7**: 75-76.

Mortimer, D. 1994. Practical Laboratory Andrology. *Oxford University Press, New York, USA.* pp 66-69.

Mullaart E, Verbrugge A, Aerts B, Merton JS. 1999. Optimization of OPU procedure.

Proceedings of the 15th Scientific Meeting of European Embryo Transfer Association, 10-11 September. Lyon, France. pp. 216.

Nagai T. 1987. Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol. *Gamete Res.* **16**(3):243–49.

Nagy S, Jansen J, Topper EK, Gadella BM. 2003. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biol. Reprod*. **68**(5):1828–35.

Nakada K, Mizuno J, Shiraishi K, Endo K, Miyazaki S. 1995. Initiation, persistence, and cessation of the series of intracellular Ca^{2+} responses during fertilisation of bovine eggs. *J.Reprod.Dev*. **41**(1): 77-84.

Nakada K, Mizuno J. 1998. Intracellular calcium responses in bovine oocytes induced by spermatozoa and by reagents. *Theriogenology.* **50**(2):269–82.

Nakai M, Ito J, Sato KI, Noguchi J, Kaneko H, *et al.* 2011. Pre-treatment of sperm reduces success of ICSI in the pig. *Reproduction*. **142**(2):285–93.

Nakai M, Suzuki S, Ito J, Fuchimoto D, Sembon S, *et al.* 2016. Efficient pig ICSI using percoll-selected spermatozoa; Evidence for the essential role of phospholipase C-ζ in ICSI success. *J. Reprod. Dev.* **62**(6):639–43.

Niemann H, Kues W, Carnwath JW. 2005. Transgenic farm animals: Present and future. *Rev. Sci. Tech.* **24**(1):285–98.

Noguchi S, Yonezawa N, Katsumata T, Hashizume K, Kuwayama M, *et al.* 1994. Characterization of the zona pellucida glycoproteins from bovine ovarian and fertilized eggs. *Biochim. Biophys. Acta - Gen. Subj.* **1201**(1):7–14.

Nomikos M, Blayney LM, Larman MG, Campbell K, Rossbach A, *et al.* 2005. Role of phospholipase C-ζ domains in Ca^{2+}-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca^{2+} oscillations. *J. Biol. Chem.* **280**(35):31011–18.

Nomikos M, Kashir J, Swann K, Lai FA. 2013. Sperm PLCζ: From structure to Ca^{2+} oscillations, egg activation and therapeutic potential. *FEBS Lett.* **587**(22):3609–16.

Nozawa K, Satouh Y, Fujimoto T, Oji A, Ikawa M. 2018. Sperm-borne phospholipase C zeta-1 ensures monospermic fertilization in mice. *Sci. Rep.* **8**(1):1315.

Oikawa T, Takada N, Kikuchi T, Numabe T, Takenaka M, *et al.* 2005. Evaluation of activation treatments for blastocyst production and birth of viable calves following bovine intracytoplasmic sperm injection. *Anim. Reprod. Sci*. **86**(3-4):187–94.

Oikawa T, Itahashi T, Numabe T. 2016. Improved embryo development in japanese black cattle by in vitro fertilization using ovum pick-up plus intracytoplasmic sperm injection with dithiothreitol. *J. Reprod. Dev.* **62**(1):11–16.

O'Neill C. 1997. Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro. *Biol. Reprod.* **56**(1):229–37.

O'Neill C. 1998. Autocrine mediators are required to act on the embryo by the 2-cell stage to promote normal development and survival of mouse preimplantation embryos in vitro. *Biol. Reprod.* **58**(5):1303–9.

Otoi T, Yamamoto K, Koyama N, Tachikawa S, Suzuki T. 1997. Bovine oocyte diameter in relation to developmental competence. *Theriogenology.* **48**(5):769–74.

O'Toole CM, Arnoult C, Darszon A, Steinhardt RA, Florman HM. 2000. Ca^{2+} entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. *Mol. Biol. Cell.* **11**(5):1571–84.

Ozil JP, Markoulaki S, Toth S, Matson S, Banrezes B, *et al.* 2005. Egg activation events are regulated by the duration of a sustained $[Ca^{2+}]$cyt signal in the mouse. *Dev. Biol.* **282**(1):39–54.

Ozil JP, Banrezes B, Tóth S, Pan H, Schultz RM. 2006. Ca^{2+} oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. *Dev. Biol*. **300**(2):534–44.

Palermo G, Joris H, Devroey P, Van Steirteghem AC. 1992. Pregnancies after cytoplasmic injection of single spermatozoon into an oocyte. *Lancet.* **340**:17–8.

Palermo GD, Schlegel PN, Colombero LT, Zaninovic N, Moy F, *et al.* 1996. Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. *Hum. Reprod.* **11**(5):1023–29.

Palermo GD, Kocent J, Monahan D, Neri Q V., Rosenwaks Z. 2014. Treatment of male infertility. *In Methods in molecular biology*. **1154**:385–405.

Palermo G, Neri Q, Rosenwaks Z. 2015. To ICSI or not to ICSI. *Semin. Reprod. Med.* **33**(02):092-102.

Palermo GD, O'Neill CL, Chow S, Cheung S, Parrella A, *et al.* 2017. Intracytoplasmic sperm injection: State of the art in humans. *Reproduction*. **154**(6):F93–110.

Palmer E, Bézard J, Magistrini M, Duchamp G. 1991. In vitro fertilization in the horse. A retrospective study. *J. Reprod. Fertil.* Suppl. **44**:375–84.

Parrish JJ, Susko-Parrish J, Winer MA, First NL. 1988. Capacitation of bovine sperm by heparin. *Biol. Reprod.* **38**(5):1171–80.

Parrish JJ, Susko-Parrish JL, Handrow RR, Sims MM, First NL. 1989. Capacitation of bovine spermatozoa by oviduct fluid. *Biol. Reprod.* **40**(5):1020–25.

Patrat C, Auer J, Fauque P, Leandri RL, Jouannet P, *et al.* 2006. Zona pellucida from fertilised human oocytes induces a voltage-dependent calcium influx and the acrosome reaction in spermatozoa, but cannot be penetrated by sperm. *BMC Dev. Biol.* **6**(1):59.

Pereyra-Bonnet F, Fernández-Martín R, Olivera R, Jarazo J, Vichera G, *et al.* 2008. A unique method to produce transgenic embryos in ovine, porcine, feline, bovine and equine species. *Reprod. Fertil. Dev.* **20**(7):741–49.

Pérez-Aguirreburualde MS, Fernández S, Córdoba M. 2012. Acrosin activity regulation by protein kinase c and tyrosine kinase in bovine sperm acrosome exocytosis induced by

lysophosphatidylcholine. *Reprod. Domest. Anim.* **47**(6):915–20.

Pérez-Cerezales S, Laguna-Barraza R, de Castro AC, Sánchez-Calabuig MJ, Cano-Oliva E, *et al.* 2018. Sperm selection by thermotaxis improves ICSI outcome in mice. *Sci. Rep.* **8**(1):2902.

Perreault SD, Wolff RA, Zirkin BR. 1984. The role of disulfide bond reduction during mammalian sperm nuclear decondensation in vivo. *Dev. Biol.* **101**(1):160–67.

Perreault SD, Naish SJ, Zirkin BR. 1987. The timing of hamster sperm nuclear decondensation and male pronucleus formation is related to sperm nuclear disulfide bond content1. *Biol. Reprod.* **36**(1):239–44.

Perreault SD, Barbee RR, Elstein KH, Zucker RM, Keefer CL. 1988. Interspecies differences in the stability of mammalian sperm nuclei assessed in vivo by sperm microinjection and in vitro by flow cytometry. *Biol. Reprod.* **39**(1):157–67.

Perry G. 2018. 2016 Statistics of embryo collection and transfer in domestic farm animals. *IETS-Data-Retrieval Report-2016*. DOI: 10.13140/RG.2.2.24793.42087.

Pey R, Vial C, Schatten G, Hafner M. 1998. Increase of intracellular Ca^{2+} and relocation of e-cadherin during experimental decompaction of mouse embryos. *Proc. Natl. Acad. Sci.* **95**(22):12977–82.

Phillips DM, Shalgi RM. 1980. Surface properties of the zona pellucida. *J. Exp. Zool.* **213**(1):1–8.

Phillips SV, Yu Y, Rossbach A, Nomikos M, Vassilakopoulou V, *et al.* 2011. Divergent effect of mammalian PLCζ in generating Ca^{2+} oscillations in somatic cells compared with eggs. *Biochem. J.* **438**(3):545–53.

Pieterse MC, Vos PLAM, Kruip ThAM, Wurth YA, Van Benden ThT, *et al.* 1992. Repeated transvaginal ultrasound-guided ovum pick-up in ECG treated cows. *Theriogenology*. **37**:273.

Piotrowska-Nitsche K, Chan AWS. 2013. Effect of sperm entry on blastocyst development after in vitro fertilization and intracytoplasmic sperm injection — mouse model. *J. Assist. Reprod. Genet.* **30**(1):81–89.

Pontes JHF, Silva KCF, Basso AC, Rigo AG, Ferreira CR, *et al.* 2010. Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm. *Theriogenology.* **74**(8):1349–55.

Pope CE, Johnson CA, McRae MA, Keller GL, Dresser BL. 1998. Development of embryos produced by intracytoplasmic sperm injection of cat oocytes. *Anim. Reprod. Sci.* **53**(1–4):221–36.

Presicce GA, Yang X. 1994. Parthenogenetic development of bovine oocytes matured in vitro for 24 hr and activated by ethanol and cycloheximide. *Mol. Reprod. Dev.* **38**(4):380–85.

Purdy PH, Graham JK. 2004. Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility. *Biol. Reprod.* **71**(2):522–27.

Quill TA, Sugden SA, Rossi KL, Doolittle LK, Hammer RE, et al. 2003. Hyperactivated sperm motility driven by CatSper2 is required for fertilization. *Proc. Natl. Acad. Sci.* **100**(25):14869–74.

Rader K, Choi YH, Hinrichs K. 2016. Intracytoplasmic sperm injection, embryo culture, and transfer of in vitro–produced blastocysts. *Vet. Clin. North Am. Equine Pract.* **32**(3):401–13.

Ralt D, Goldenberg M, Fetterolf P, Thompson D, Dor J, *et al.* 1991. Sperm attraction to a follicular factor(s) correlates with human egg fertilizability. *Proc. Natl. Acad. Sci.* **88**(7):2840–44.

Rath D, Johnson LA, Dobrinsky JR, Welch GR, Niemann H. 1997. Production of piglets preselected for sex following in vitro fertilization with X and Y chromosome-bearing spermatozoa sorted by flow cytometry. *Theriogenology.* **47**(4):795–800.

Rath D, Barcikowski S, de Graaf S, Garrels W, Grossfeld R, *et al.* 2013. Sex selection of sperm in farm animals: status report and developmental prospects. *Reproduction*. **145**(1):R15-30.

Ren D, Xia J. 2010. Calcium signaling through CatSper channels in mammalian fertilization. *Physiology*. **25**(3):165–75.

Reyes R, Rosado A, Hernández O, Delgado NM. 1989. Heparin and glutathione: Physiological decondensing agents of human sperm nuclei. *Gamete Res.* **23**(1):39–47.

Reyes R, Sánchez-Vazquez ML, Merchant-Larios H, Rosado A, Delgado NM. 1996. Effect of heparin-reduced glutathione on hamster sperm DNA unpacking and nuclear swelling. *Arch. Androl.* **37**(1):33–45.

Rho GJ, Wu B, Kawarsky S, Leibo SP, Betteridge KJ. 1998a. Activation regimens to prepare bovine oocytes for intracytoplasmic sperm injection. *Mol. Reprod. Dev.* **50**(4):485–92.

Rho GJ, Kawarsky S, Johnson WH, Kochhar K, Betteridge KJ. 1998b. Sperm and oocyte treatments to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. *Biol. Reprod.* **59**(4):918–24.

Rho GJ, Lee SL, Kim YS, Yeo HJ, Ock SA, *et al.* 2004. Intracytoplasmic sperm injection of frozen-thawed bovine oocytes and subsequent embryo development. *Mol. Reprod. Dev.* **68**(4):449–55.

Rice A, Parrington J, Jones KT, Swann K. 2000. Mammalian sperm contain a Ca^{2+}-sensitive Phospholipase C activity that can generate $InsP_3$ from PIP_2 associated with intracellular organelles. *Dev. Biol.* **228**(1):125–35.

Rocha A, Randel RD, Broussard JR, Lim JM, Blair RM, *et al.* 1998. High environmental temperature and humidity decrease oocyte quality in Bos taurus but not in Bos indicus cows. *Theriogenology.* **49**(3):657–65.

Rogers NT, Hobson E, Pickering S, Lai FA, Braude P, *et al.* 2004. Phospholipase C causes Ca^{2+} oscillations and parthenogenetic activation of human oocytes. *Reproduction*. **128**(6):697–702.

Rogers NT, Halet G, Piao Y, Carroll J, Ko MSH, Swann K.. 2006. The absence of a Ca^{2+} signal during mouse egg activation can affect parthenogenetic preimplantation development, gene expression patterns, and blastocyst quality. *Reproduction.* **132**(1):45–57.

Romanato M, Cameo MS, Bertolesi G, Baldini C, Calvo JC, *et al*. 2003. Heparan sulphate: A putative decondensing agent for human spermatozoa in vivo. *Hum. Reprod.* **18**(9):1868–73.

Romanato M, Regueira E, Cameo MS, Baldini C, Calvo L, Calvo JC. 2005. Further evidence on the role of heparan sulfate as protamine acceptor during the decondensation of human spermatozoa. *Hum. Reprod.* **20**(10):2784–89.

Romanato M, Julianelli V, Zappi M, Calvo L, Calvo JC. 2008. The presence of heparan sulfate in the mammalian oocyte provides a clue to human sperm nuclear decondensation in vivo. *Hum. Reprod.* **23**(5):1145–50.

Rosenwaks Z, Pereira N. 2017. The pioneering of intracytoplasmic sperm injection: Historical perspectives. *Reproduction.* **154**(6):F71–77.

Ross PJ, Beyhan Z, Iager AE, Yoon SY, Malcuit C, *et al.* 2008. Parthenogenetic activation of bovine oocytes using bovine and murine Phospholipase C zeta. *BMC Dev. Biol.* **8**:16.

Ross PJ, Rodriguez RM, Iager AE, Beyhan Z, Wang K, *et al.* 2009. Activation of bovine somatic cell nuclear transfer embryos by plcz crna injection. *Reproduction.* **137**(3):427–37.

Rubinstein E, Ziyyat A, Wolf J, Lenaour F, Boucheix C. 2006. The molecular players of sperm–egg fusion in mammals. *Semin. Cell Dev.* Biol. **17**(2):254–63.

Saeki K, Nagao Y, Kishi M, Nagai M. 1997. Developmental capacity of bovine oocytes following inhibition of meiotic resumption by cycloheximide or 6-Dimethylaminopurine. *Theriogenology*. **48**(7):1161–72.

Sagata N, Watanabe N, Vande Woude GF, Ikawa Y. 1989. The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature.* **342**(6249):512–18.

Salamone DF, Canel NG, Rodríguez MB. 2017. Intracytoplasmic sperm injection in domestic and wild mammals. *Reproduction.* **154**(6):F111–24.

Sales JNS, Iguma LT, Batista RITP, Quintão CCR, Gama MAS, *et al.* 2015. Effects of a high-energy diet on oocyte quality and in vitro embryo production in Bos indicus and Bos taurus cows. *J. Dairy Sci.* **98**(5):3086–99.

Sánchez-Vázquez ML, Reyes R, Delgado NM, Merchant-Larios H, Rosado A. 1996. Differential decondensation of class I (rat) and class II (mouse) spermatozoa nuclei by

physiological concentrations of heparin and glutathione. *Arch. Androl.* **36**(3):161–76.

Sánchez-Vázquez ML, Reyes R, Ramírez G, Merchant-Larios H, Rosado A, *et al.* 1998. DNA unpacking in guinea pig sperm chromatin by heparin and reduced glutathione. *Arch. Androl.* **40**(1):15–28.

Sánchez-Vázquez ML, Flores-Alonso JC, Merchant-Larios H, Reyes R. 2008. Presence and release of bovine sperm histone h1 during chromatin decondensation by heparin-glutathione. *Syst. Biol. Reprod. Med.* **54**(6):221–30.

Sanders JR, Swann K. 2016. Molecular triggers of egg activation at fertilization in mammals. *Reproduction.* **152**(2):R41–50.

Sanders JR, Ashley B, Moon A, Woolley TE, Swann K. 2018. PLCζ induced Ca^{2+} oscillations in mouse eggs involve a positive feedback cycle of Ca^{2+} induced $InsP_3$ formation from cytoplasmic PIP_2. *Front. Cell Dev. Biol.* **6**:36.

Santos P, Chaveiro A, Simões N, Moreira da Silva F. 2008. Bovine oocyte quality in relation to ultrastructural characteristics of zona pellucida, polyspermic penetration and developmental competence. *Reprod. Domest. Anim.* **43**(6):685–89.

Santos RR, Schoevers EJ, Roelen BA. 2014. Usefulness of bovine and porcine IVM/IVF models for reproductive toxicology. *Reprod. Biol. Endocrinol.* **12**(1):117.

Sanusi R, Yu Y, Nomikos M, Lai FA, Swann K. 2015. Rescue of failed oocyte activation after ICSI in a mouse model of male factor infertility by recombinant Phospholipase Cζ. *Mol. Hum. Reprod.* **21**(10):783–91.

Sarkadi B, Szász I, Gárdos G. 1976. The use of ionophores of rapid loading of human red cells with radioactive cations for cation-pump studies. *J. Membr. Biol.* **26**(4):357–70.

Sartori R, Souza AH, Guenther JN, Caraviello DZ, Geiger LN, *et al.* 2004. Fertilization rate and embryo quality in superovulated Holstein heifers artificially inseminated with X-sorted or unsorted sperm. *Anim Reprod.* **1** (1):86–90.

Sartori R, Bastos MR, Baruselli PS, Gimenes LU, Ereno RL, *et al.* 2010. Physiological differences and implications to reproductive management of Bos taurus and Bos indicus cattle in a tropical environment. *Soc. Reprod. Fertil.* Suppl. **67**:357–75.

Sartori R, Barros CM. 2011. Reproductive cycles in Bos indicus cattle. *Anim. Reprod. Sci*. **124**(3–4):244–50.

Sartori R, Guardieiro MM, Surjus RS, Melo LF, Prata AB, *et al.* 2013. Metabolic hormones and reproductive function in cattle. *Anim. Reprod.* **10**(3):199-205.

Sartori R, Gimenes LU, Monteiro PLJ, Melo LF, Baruselli PS, *et al.* 2016. Metabolic and endocrine differences between Bos taurus and Bos indicus females that impact the interaction of nutrition with reproduction. *Theriogenology.* **86**(1):32–40.

Sato K, Wakai T, Seita Y, Takizawa A, Fissore RA, *et al.* 2013. Molecular characteristics of horse Phospholipase C zeta (PLCζ). *Anim. Sci. J.* **84**(4):359–68.

Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, *et al.* 2002. PLC zeta: A

sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Development.* **129**(15):3533–44.

Scaramuzzi RJ, Baird DT, Campbell BK, Driancourt MA, Dupont J, *et al.* 2011. Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reprod. Fertil. Dev.* **23**(3):444.

Schultz RM, Kopf GS. 1995. Molecular basis of mammalian egg activation. Curr. *Top. Dev. Biol.* **30**:21–62.

Scott CA, Robertson L, de Moura RT, Paterson C, Boyd JS. 1994. Technical aspects of transvaginal ultrasound-guided follicular aspiration in cows. *Vet. Rec.* **134**(17):440–43.

Seidel GE, Schenk JL, Herickhoff LA, Doyle SP, Brink Z, et al. 1999. Insemination of heifers with sexed sperm. *Theriogenology.* **52**(8):1407–20.

Sekhavati MH, Shadanloo F, Hosseini MS, Tahmoorespur M, Nasiri MR, *et al.* 2012. Improved bovine ICSI outcomes by sperm selected after combined heparin-glutathione treatment. *Cell. Reprogram.* **14**(4):295–304.

Sengoku K, Tamate K, Horikawa M, Takaoka Y, Ishikawa M, *et al.* 1995. Plasma membrane block to polyspermy in human oocytes and preimplantation embryos. *J. Reprod. Fertil.* **105**(1):85–90.

Shams-Borhan G, Harrison RAP. 1981. Production, characterization, and use of ionophore-induced, calcium-dependent acrosome reaction in ram spermatozoa. *Gamete Res.* **4**(5):407–32.

Shen PC, Lee SN, Liu BT, Chu FH, Wang CH, *et al.* 2008. The effect of activation treatments on the development of reconstructed bovine oocytes. *Anim. Reprod. Sci.* **106**(1–2):1–12.

Shirazi A, Derakhshan-Horeh M, Pilvarian A, Ahmadi E, Nazari H, *et al.* 2011. Effect of pre-treatment of ovine sperm on male pronuclear formation and subsequent embryo development following intracytoplasmic sperm injection. *Reprod. Domest. Anim.* **46**(1):87–94.

Signorelli J, Diaz ES, Morales P. 2012. Kinases, phosphatases and proteases during sperm capacitation. *Cell Tissue Res*. **349**(3):765–82.

Sirard MA, Richard F, Blondin P, Robert C. 2006. Contribution of the oocyte to embryo quality. *Theriogenology.* **65**(1):126–36.

Sirard MA. 2011. Follicle environment and quality of in vitro matured oocytes. *J. Assist. Reprod. Genet.* **28**(6):483–88.

Slimane-Bureau WC, King WA. 2002. Chromosomal abnormalities: A potential quality issue for cloned cattle embryos. *Cloning Stem Cells.* **4**(4):319–29.

Smith GD. 2001. In vitro maturation of oocytes. *Curr. Womens. Health Rep*. **1**(2):143–51.

Snijders SEM, Dillon P, O'Callaghan D, Boland MP. 2000. Effect of genetic merit, milk yield, body condition and lactation number on in vitro oocyte development in dairy cows.

Theriogenology. **53**(4):981–89.

Squires E, Wilson J, Kato H, Blaszczyk A. 1996. A pregnancy after intracytoplasmic sperm injection into equine oocytes matured in vitro. *Theriogenology*. **45**(1):306.

Srivastava N, Jerome A, Srivastava SK, Ghosh SK, Kumar A. 2013. Bovine seminal PDC-109 protein: An overview of biochemical and functional properties. *Anim. Reprod. Sci*. **138**(1–2):1–13.

Stachecki JJ, Armant DR. 1996. Transient release of calcium from inositol 1,4,5-trisphosphate-specific stores regulates mouse preimplantation development. *Development.* **122**(8):2485–96.

Stein P, Schneider I. Piezo-actuated Mouse ICSI. Eppendorf Userguide Nº: 032. www.eppendorf.com.

Stein P, Schultz M. 2010. ICSI in the mouse. Methods in Enzymology. **476**: 251-262.

Stival C, Puga Molina L del C, Paudel B, Buffone MG, Visconti PE, *et al.* 2016. Sperm capacitation and acrosome reaction in mammalian sperm. *Adv Anat Embryol Cell Biol*. **220**:93–106.

Stricker SA. 1999. Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol.* **211**(2):157–76.

Sturmey R, Reis A, Leese H, McEvoy T. 2009. Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reprod. Domest. Anim.* **44**:50–58.

Suarez SS, Oliphant G. 1982. Interaction of rabbit spermatozoa and serum complement components. *Biol. Reprod.* **27**(2):473–83.

Suarez SS. 1987. Sperm transport and motility in the mouse oviduct: Observations in situ. *Biol. Reprod*. **36**(1):203–10.

Suarez SS, Vincenti L, Ceglia MW. 1987. Hyperactivated motility induced in mouse sperm by calcium ionophore A23187 is reversible. *J. Exp. Zool.* **244**(2):331–36.

Suarez SS, Dai X. 1992. Hyperactivation enhances mouse sperm capacity for penetrating viscoelastic media. *Biol. Reprod.* **46**(4):686–91.

Suarez SS, Brockman K, Lefebvre R. 1997. Distribution of mucus and sperm in bovine oviducts after artificial insemination: The physical environment of the oviductal sperm reservoir. *Biol. Reprod.* **56**(2):447–53.

Suarez SS, Pacey AA. 2006. Sperm transport in the female reproductive tract. *Hum. Reprod. Update*. **12**(1):23–37.

Suarez SS. 2016. Mammalian sperm interactions with the female reproductive tract. *Cell Tissue Res.* **363**(1):185–94.

Sudano MJ, Caixeta ES, Paschoal DM, Martins A, Machado R, *et al.* 2014. Cryotolerance and global gene-expression patterns of Bos taurus indicus and Bos taurus taurus in vitro- and in vivo-produced blastocysts. *Reprod. Fertil. Dev.* **26**(8):1129.

Susko-Parrish JL, Leibfried-Rutledge ML, Northey DL, Schutzkus V, First NL. 1994.

Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. *Dev. Biol.* **166**(2):729–39.

Suttner R, Zakhartchenko V, Stojkovic P, Müller S, Alberio R, *et al.* 2000. Intracytoplasmic sperm injection in bovine: Effects of oocyte activation, sperm pretreatment and injection technique. *Theriogenology.* **54**(6):935–48.

Sutton ML, Gilchrist RB, Thompson JG. 2003. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum. Reprod. Update.* **9**(1):35–48.

Swain JE, Pool TB. 2008. ART failure: Oocyte contributions to unsuccessful fertilization. *Hum. Reprod. Update.* **14**(5):431–46.

Swann K, Saunders C, Rogers N, Lai F. 2006. PLCζ(zeta): A sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals. Semin. *Cell Dev. Biol.* **17**(2):264–73.

Swann K, Lai FA. 2016. Egg activation at fertilization by a soluble sperm protein. *Physiol. Rev.* **96**(1):127–49.

Szöllösi MS, Kubiak JZ, Debey P, de Pennart H, Szöllösi D, *et al.* 1993. Inhibition of protein kinases by 6-dimethylaminopurine accelerates the transition to interphase in activated mouse oocytes. *J. Cell Sci.* **104** (3):861–72.

Talbot P, Summers RG, Hylander BL, Keough EM, Franklin LE. 1976. The role of calcium in the acrosome reaction: An analysis using ionophore A23187. *J. Exp. Zool.* **198**(3):383–92.

Talevi R, Gualtieri R. 2010. Molecules involved in sperm-oviduct adhesion and release. *Theriogenology*. **73**(6):796–801.

Tateno H, Mikamo K. 1987. A chromosomal method to distinguish between X- and Y-bearing spermatozoa of the bull in zona-free hamster ova. *J. Reprod. Fertil.* **81**(1):119–25.

Tateno H, Krapf D, Hino T, Sánchez-Cárdenas C, Darszon A, *et al.* 2013. Ca^{2+} ionophore A23187 can make mouse spermatozoa capable of fertilizing in vitro without activation of camp-dependent phosphorylation pathways. *Proc. Natl. Acad. Sci. U. S. A.* **110**(46):18543–48.

Tatone C, Delle Monache S, Iorio R, Caserta D, Di Cola M, *et al.* 2002. Possible role for Ca^{2+} calmodulin-dependent protein kinase II as an effector of the fertilization Ca^{2+} signal in mouse oocyte activation. *Mol. Hum. Reprod.* **8**(8):750–57.

Tomashov-Matar R, Tchetchik D, Eldar A, Kaplan-Kraicer R, Oron Y, *et al.* 2005. Strontium-induced rat egg activation. *Reproduction.* **130**(4):467–74.

Tosti E, Boni R, Cuomo A. 2002. Fertilization and activation currents in bovine oocytes. *Reproduction.* **124**(6):835–46.

Tung CK, Ardon F, Fiore AG, Suarez SS, Wu M. 2014. Cooperative roles of biological flow and surface topography in guiding sperm migration revealed by a microfluidic model. *Lab*

Chip. **14**(7):1348–56.

Uehara T, Yanagimachi R. 1976. Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. *Biol Reprod.* **15**: 467–70.

Urnov FD, Wolffe AP. 2002. Chapter 3 – The nucleus. *Principles of Cloning*, 1st edition, Academic Press. pp. 47–83.

Urrego R, Rodriguez-Osorio N, Niemann H. 2014. Epigenetic disorders and altered gene expression after use of assisted reproductive technologies in domestic cattle. *Epigenetics.* **9**(6):803–15.

Usui N, Yanagimachi R. 1976. Behavior of hamster sperm nuclei incorporated into eggs at various stages of maturation, fertilization, and early development. the appearance and disappearance of factors involved in sperm chromatin decondensation in egg cytoplasm. *J. Ultrastruct. Res.* **57**(3):276–88.

Vadnais ML, Galantino-Homer HL, Althouse GC. 2007. Current concepts of molecular events during bovine and porcine spermatozoa capacitation. *Arch. Androl*. **53**(3):109–23.

Van Knegsel ATM, Van den Brand H, Dijkstra J, Tamminga S, Kemp B. 2005. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. *Reprod. Nutr. Dev.* **45**(6):665–88.

Vanden-Meerschaut F, Nikiforaki D, Heindryckx B, De Sutter P. 2014. Assisted oocyte activation following ICSI fertilization failure. *Reprod. Biomed. Online*. **28**(5):560–71.

Vanderheyden V, Wakai T, Bultynck G, De Smedt H, Parys JB, *et al.* 2009. Regulation of inositol 1,4,5-trisphosphate receptor Type 1 function during oocyte maturation by MPM-2 phosphorylation. *Cell Calcium.* **46**(1):56–64.

Varea-Sánchez M, Bastir M, Roldan ERS. 2013. Geometric morphometrics of rodent sperm head shape. *PLoS One*. **8**(11):e80607.

Veerkamp RF, Beerda B, van der Lende T. 2003. Effects of genetic selection for milk yield on energy balance, levels of hormones, and metabolites in lactating cattle, and possible links to reduced fertility. *Livest. Prod. Sci.* **83**(2–3):257–75.

Velásquez JG, Canovas S, Barajas P, Marcos J, Jiménez-Movilla M, *et al.* 2007. Role of sialic acid in bovine sperm–zona pellucida binding. *Mol. Reprod. Dev.* **74**(5):617–28.

Verma OP, Kumar R, Kumar A, Chand S. 2012. Assisted reproductive techniques in farm animal - from artificial insemination to nanobiotechnology. *Vet. World.* **5** (5):301-310.

Vichera G, Alfonso J, Duque C, Silvestre M, Pereyra-Bonnet F, *et al.* 2010. Chemical activation with a combination of ionomycin and dehydroleucodine for production of parthenogenetic, ICSI and cloned bovine embryos. *Reprod. Domest. Anim.* **45**(6):e306–12.

Visconti PE, Johnson LR, Oyaski M, Fornés M, Moss SB, *et al.* 1997. Regulation, localization, and anchoring of protein kinase a subunits during mouse sperm capacitation.

Dev. Biol. **192**(2):351–63.

Vitullo AD, Ozil JP. 1992. Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. *Dev. Biol.* **151**(1):128–36.

Wakai T, Vanderheyden V, Fissore RA. 2011. Ca^{2+} signaling during mammalian fertilization: Requirements, players, and adaptations. Cold Spring Harb. *Perspect. Biol.* **3**(4):a006767–a006767.

Wakai T, Fissore RA. 2013. Ca^{2+} homeostasis and regulation of ER Ca^{2+} in mammalian oocytes/eggs. *Cell Calcium*. **53**(1):63–67.

Wakayama T, Perry ACF, Zuccotti M, Johnson KR, Yanagimachi R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature.* **394**(6691):369–74.

Wang J, Mayernik L, Armant DR. 2002. Integrin signaling regulates blastocyst adhesion to fibronectin at implantation: Intracellular calcium transients and vesicle trafficking in primary trophoblast cells. *Dev. Biol.* **245**(2):270–79.

Wang Z, Wang W, Yu S, Xu Z. 2008. Effects of different activation protocols on preimplantation development, apoptosis and ploidy of bovine parthenogenetic embryos. *Anim. Reprod. Sci.* **105**(3–4):292–301.

Wassarman PM. 1987. The biology and chemistry of fertilization. *Science.* **235**(4788):553–60.

Wassarman PM. 1988. Zona pellucida glycoproteins. *Annu. Rev. Biochem.* **57**(1):415–42.

Wassarman PM. 1999. Mammalian fertilization: Molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell.* **96**(2):175–83.

Wassarman PM, Jovine L, Litscher ES. 2001. A profile of fertilization in mammals. *Nat. Cell Biol.* **3**(2):E59–64.

Wassarman PM. 2008. Zona pellucida glycoproteins. *J. Biol. Chem.* **283**(36):24285–89.

Wassarman PM, Litscher ES. 2008. Mammalian fertilization:the eggs multifunctional zona pellucida. *Int. J. Dev. Biol.* **52**(5–6):665–76.

Wei H, Fukui Y. 1999. Effects of bull, sperm type and sperm pretreatment on male pronuclear formation after intracytoplasmic sperm injection in cattle. *Reprod. Fertil. Dev.* **11**(1):59–65.

Wei H, Fukui Y. 2002. Births of calves derived from embryos produced by intracytoplasmic sperm injection without exogenous oocyte activation. *Zygote.* **10**(2):149–53.

Whitaker M, Patel R. 1990. Calcium and cell cycle control. *Development.* **108**(4):525–42.

Whittingham DG, Siracusa G. 1978. The involvement of calcium in the activation of mammalian oocytes. *Exp. Cell Res.* **113**(2):311–17.

Winger QA, De La Fuente R, King WA, Armstrong DT, Watson AJ. 1997. Bovine parthenogenesis is characterized by abnormal chromosomal complements: Implications for maternal and paternal co-dependence during early bovine development. *Dev. Genet.*

21(2):160–66.

Wrobel KH, Kujat R, Fehle G. 1993. The bovine tubouterine junction: General organization and surface morphology. *Cell Tissue Res.* **271**(2):227–39.

Yadav VK, Saraswat M, Chhikara N, Singh S, Yadav S. 2013. Heparin and heparin binding proteins: Potential relevance to reproductive physiology. *Current Protein and Peptide Science.* **14**(1):61–69.

Yamauchi N, Nagai T. 1999. Male pronuclear formation in denuded porcine oocytes after in vitro maturation in the presence of cysteamine. *Biol. Reprod.* **61**(3):828–33.

Yamazaki W, Ferreira CR, Méo SC, Leal CLV, Meirelles FV, *et al.* 2005. Use of strontium in the activation of bovine oocytes reconstructed by somatic cell nuclear transfer. *Zygote.* **13**(4):295–302.

Yanagida K, Yanagimachi R, Perreault SD, Kleinfeld RG. 1991. Thermostability of sperm nuclei assessed by microinjection into hamster oocytes. *Biol. Reprod.* **44**(3):440–47.

Yanagimachi R, Chang MC. 1963. Sperm ascent through the oviduct of the hamster and rabbit in relation to the time of ovulation. *J. Reprod. Fertil.* **6**:413–20.

Yang X, Presicce GA, Moraghan L, Jiang SE, Foote RH. 1994. Synergistic effect of ethanol and cycloheximide on activation of freshly matured bovine oocytes. *Theriogenology.* **41**(2):395–403.

Yoneda A, Watanabe T. 2015. Involvement of mouse and porcine PLCζ-induced calcium oscillations in preimplantation development of mouse embryos. *Biochem. Biophys. Res. Commun*. **460**(2):476–81.

Yonezawa N, Amari S, Takahashi K, Ikeda K, Imai FL, *et al.* 2005. Participation of the nonreducing terminal beta-galactosyl residues of the neutral N-linked carbohydrate chains of porcine zona pellucida glycoproteins in sperm-egg binding. *Mol. Reprod. Dev.* **70**(2):222–27.

Yong HY, Hao Y, Lai L, Li R, Murphy CN, *et al.* 2006. Production of a transgenic piglet by a sperm injection technique in which no chemical or physical treatments were used for oocytes or sperm. *Mol. Reprod. Dev.* **73**(5):595–99.

Yoon S-Y, Fissore RA. 2007. Release of Phospholipase C zeta and $[Ca^{2+}]_i$ oscillation-inducing activity during mammalian fertilization. *Reproduction.* **134**(5):695–704.

Yoshida N, Perry AC. 2007. Piezo-actuated mouse intracytoplasmic sperm injection (ICSI). *Nat. Protoc.* **2**(2):296–304.

Yu Y, Halet G, Lai FA, Swann K. 2008a. Regulation of diacylglycerol production and Protein kinase C stimulation during sperm- and PLCζ-mediated mouse egg activation. *Biol. Cell.* **100**(11):633–43.

Yu Y, Saunders CM, Lai FA, Swann K. 2008b. Preimplantation development of mouse oocytes activated by different levels of human Phospholipase C zeta. *Hum. Reprod*. **23**(2):365–73.

Yu Y, Nomikos M, Theodoridou M, Nounesis G, Lai FA, Swann K. 2012. PLCζ causes Ca^{2+} oscillations in mouse eggs by targeting intracellular and not plasma membrane PI(4,5)P(2). *Mol. Biol. Cell.* **23**(2):371–80.

Xiao Y, Zhang H, Ahmad S, Bai L, Wang X, *et al.* 2013. Sperm capacitation combined with removal of the sperm acrosome and plasma membrane enhances paternal nucleus remodelling and early development of bovine androgenetic embryos. *Reprod. Fertil. Dev.* **25**(4):624.

Xu J, Chaubal SA, Du F. 2009. Optimizing ivf with sexed sperm in cattle. *Theriogenology*. **71**(1):39–47.

Xu ZZ. 2014. Application of liquid semen technology improves conception rate of sex-sorted semen in lactating dairy cows. *J. Dairy Sci.* **97**(11):7298–7304.

Zambrano F, Aguila L, Arias ME, Sánchez R, Felmer R. 2016. Improved preimplantation development of bovine ICSI embryos generated with spermatozoa pretreated with membrane-destabilizing agents lysolecithin and triton X-100. *Theriogenology*. **86**(6):1489–97.

Zambrano F, Aguila L, Arias M, Sanchez R, Felmer R. 2017. Effect of sperm pretreatment with glutathione and membrane destabilizing agents lysolecithin and triton X-100, on the efficiency of bovine intracytoplasmic sperm injection. *Reprod. Domest. Anim.* **52**(2):305–11.

Zhang D, Pan L, Yang LH, He XK, Huang XY, *et al.* 2005. Strontium promotes calcium oscillations in mouse meiotic oocytes and early embryos through $InsP_3$ receptors, and requires activation of phospholipase and the synergistic action of $InsP_3$. *Hum. Reprod.* **20**(11):3053–61.

Zirkin BR, Soucek DA, Chang TSK, Perreault SD. 1985. In vitro and in vivo studies of mammalian sperm nuclear decondensation. *Gamete Res.* **11**(4):349–65.

Zuelke KA, Jeffay SC, Zucker RM, Perreault SD. 2003. Glutathione (GSH) concentrations vary with the cell cycle in maturing hamster oocytes, zygotes, and pre-implantation stage embryos. *Mol. Reprod. Dev.* **64**(1):106–12.

APPENDICES:

Media and Stock solutions

Chemicals and reagents were purchased from Sigma-Aldrich Company Ltd. (Dorset, England), unless otherwise indicated. Media composition of solutions not mentioned here are present in the text.

I. MEDIA FOR BOVINE OOCTYE RETRIEVAL AND *IN VITRO* MATURATION (IVM)

a. Ovary collection Medium- Phosphate buffered saline (PBS)

Component	g/ 1 l	M (n/l)
NaCl	8	0.1369
KCl	0.2	0.0027
Na_2HPO_4	1.44	0.0101
KH_2PO_4	0.24	0.0018

pH adjusted to 7.4 with 5M NaOH. Sterilised by autoclaving for 20 min at 15 Ib/in^2.

b. Oocyte maturation Medium

Component	Search media	Maturation media
Hepes-TCM199	9.5 ml	--
TCM199	--	9 ml
FCS	0.5 ml	1 ml
L-Glutamine	1 mg	1 mg
FSH (1 mg/ml Stock)	--	100 µl
LH (1 mg/ml Stock)	--	100 µl
Pen/Strep	50 µl	50 µl
Osmolarity	290-300 mOsm	

pH adjusted to 7.3-7.4 with NaOH and HCl. Sterilised by filtration through a 0.22 µm Millipore filter and equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h prior to use.

II. MEDIA FOR BOVINE *IN VITRO* FERTILISATION (IVF) OR ICSI

a. *In vitro* Fertilisation Medium- TALP media (Fert-TALP)

Component	mg/ 100 ml	mM (n/ml)
NaCl	544	93.09
KCl	23	3.09
$NaHCO_3$	220	26.19
$Na_2HPO_4 \cdot 2H_2O$	4	0.22
$MgCl_2 \cdot 6H_2O$	10	0.492
$CaCl_2 \cdot 2H_2O$	78	5.31
Caffeine	27	1.39
Na-Pyruvate	100	9.1
Na-Lactate	186 µl	9.95
BSA	600	6 mg/ml
Pen/Strep	500 µl	50units/0.05mg/ml
Heparin	*	*
Hypotaurine (1 ml/mg)	50 µl Stock	0.5 µl/ml
Epinephrine (1 ml/mg)	50 µl Stock	0.5 µl/ml
Osmolarity	270-290 mOsm	

pH adjusted to 7.8 with NaOH and HCl. Sterilised by filtration through a 0.22 µm Millipore filter and equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h prior to use. * Heparin dose adjusted according to experimental requirements. Standard Hep concentration: 10 µg/ml.

b. Modified Ca^{2+} and Mg^{2+} Free *In vitro* Fertilisation Medium- TALP media (Ca^{2+}-Mg^{2+}-Free Fert-TALP)

Component	mg/ 100 ml	mM (n/ml)
NaCl	544	93.09
KCl	23	3.09
$NaHCO_3$	220	26.19
$Na_2HPO_4 \cdot 2H_2O$	4	0.22
Caffeine	27	1.39
Na Pyruvate	100	9.1
Na Lactate	186 µl	9.95
BSA	600	6 mg/ml
Pen/Strep	500 µl	50units/0.05mg/ml
Osmolarity	270-290 mOsm	

pH adjusted to 7.8 with NaOH and HCl. Sterilised by filtration through a 0.22 µm Millipore filter and equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h prior to use.

c. Modified Biggers, Whitten and Whittingham (BWW) Medium (modified BWW media)

Component	mg/ 100 ml	mM (n/ml)
NaCl	595.38	101.88
KCl	35.6	4.78
$NaHCO_3$	150	17.86
$MgSO_4 \cdot 7H_2O$	29.4	1.19
$CaCl_2 \cdot 2H_2O$	25	1.70
Glucose	100	5.55
Phenol Red (0.5%)	100 µl	0.014
Na-Pyruvate	2.8	0.25
Na-Lactate	370 µl	19.79
BSA	300	3 mg/ml
Pen/Strep	500 µl	50units/0.05mg/ml
Osmolarity	300 mOsm	

pH adjusted to 7.8 with NaOH and HCl. Sterilised by filtration through a 0.22 µm Millipore filter and equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h prior to use.

d. Oocyte washing Medium. Hepes-TALP media (Hepes-TALP)

Component	mg/ 100 ml	mM (n/ml)
NaCl	666	113.96
KCl	23.8	3.19
$NaHCO_3$	16.8	2
Na_2HPO_4	4.77	0.4
$MgCl_2 \cdot 6H_2O$	10	0.49
$CaCl_2 \cdot 2H_2O$	29.4	2
Hepes	240	10.07
Phenol Red (0.5%)	200 µl	0.028
Na-Pyruvate	5.5	0.5
Na-Lactate	300 µl	16.05
BSA	300	3 mg/ml
Pen/Strep	500 µl	50units/0.05mg/ml
Osmolarity	270-290 mOsm	

pH adjusted to 7.3-7.4 with NaOH and HCl. Sterilised by filtration through a 0.22 µm Millipore filter and equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h prior to use.

III. MEDIA FOR BOVINE EMBRYO *IN VITRO* CULTURE

a. Modified Synthetic Oviduct Fluid medium (mSOFaaci)

Component	mg/ 100 ml	mM (n/ml)
NaCl	629	107.63
KCl	53.4	7.16
$NaHCO_3$	210	25
KH_2PO_4	16.2	1.19
$MgSO_4$	18.2	1.51
$CaCl_2 \cdot 2H_2O$	26.2	1.78
L-Glutamine	2.9	0.20
Tri-sodium citrate	10	0.34
Na-Pyruvate	80	7.27
Na-Lactate	75.7 µl	4.05
BSA	300	3 mg/ml
Phenol red (0.5%)	200 µl	0.028
Myo-Inositol	49.9	2.77
BME amino acids (50x)	4.5 ml	--
MEM amino acids (100x)	500 µl	--
Pen/Strep	500 µl	50units/0.05mg/ml
Osmolarity	270-290 mOsm	

pH adjusted to 7.4 with NaOH and HCl. Sterilised by filtration through a 0.22 µm Millipore filter and equilibrated in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at a temperature of 38.5ºC for at least 2 h prior to use.

b. Hepes-buffered Synthetic Oviduct Fluid medium (Hepes-SOF)

Component	mg/ 100 ml	mM (n/ml)
NaCl	629	107.63
KCl	53.4	7.16
$NaHCO_3$	42	25
KH_2PO_4	16.2	1.19
$MgSO_4$	18.2	1.51
$CaCl_2 \cdot 2H_2O$	26.2	1.78
L-Glutamine	2.9	0.20
Tri-sodium citrate	10	0.34
Na-Pyruvate	80	7.27
Na-Lactate	75.7 µl	4.05
BSA	300	3 mg/ml
Phenol red (0.5%)	200 µl	0.028
Hepes	47.66	2
MEM amino acids (100x)	500 µl	--
Pen/Strep	500 µl	50units/0.05mg/ml
Osmolarity	270-290 mOsm	

Adjust pH to 7.4 with NaOH and HCl. Sterilised by filtration through a 0.22 µm Millipore filter and equilibrated in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at a temperature of 38.5ºC for at least 2 hours prior to use.

IV. STOCK SOLUTIONS

a. Lysophosphatidylcholine Stock ($[LPC]_0$ = 10 mg/ml)

21 mg L-α-Lysophosphatidylcholine from egg yolk (L4129)
2.1 ml ddH_20

Stock solution was dispensed in 20 µl aliquots, and stored at -20ºC. LPC stock was diluted in Fert-TALP media at a final working dilution of 100 µg/ml.

b. Lectin-FITC Conjugate Stock ($[Lectin\text{-}FITC]_0$ = 1 mg/ml)

1 mg Lectin from *Arachis hypogaea* FITC Conjugate (L7381)
1 ml Ca^{2+}-, Mg^{2+}- free Dulbecco's phosphate-buffered saline (DPBS-)

Stock solution was dispensed in 20 µl aliquots, and stored at -20ºC in dark. Lectin-FITC stock was diluted in PBS at a final working dilution of 3.3 µg/ml.

c. Hoechst 33258 Stock ($[Hoechst\ 33258]_0$ = 10 mg/ml)

20 mg Hoechst 33258 (B2883)
2 ml ddH_2O

Stock solution was dispensed in 10 µl aliquots, and stored at -20ºC. Hoechst 33258 stock was diluted in PBS at a final working dilution of 83 µg/ml.

d. Hoechst 33342 Stock ($[Hoechst\ 33342]_0$ = 5 mg/ml)

25 mg Hoechst 33352 (B2261)
5 ml ddH_2O

Stock solution was dispensed in 10 µl aliquots, and stored at -20ºC. Hoechst 33342 stock was diluted in PBS at a final working dilution of 10 µg/ml.

e. Heparin Stock ([Heparin]$_0$ = 80 mg/ml)

80 mg Heparin from porcine intestinal mucose as a sodium salt (H3149-100KU)

8 m ddH$_2$O

Stock solution was dispensed in 20 µl aliquots, and stored at -20ºC. Heparin stock was diluted in Fert-TALP media at different final working dilutions of 10 µg/ml, 40 µg/ml, 80 µg/ml (≈ 4.4 µM), 100 µg/ml, 685 µg/ml (≈ 40 µM) and 1370 (≈ 80 µM). It was sterilised by filtration through a 0.22 µm Millipore filter and equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 hours.

f. Glutathione solution ([GSH]$_0$ = 40 mg/ml)

40 mg L-Glutathione reduced (G4251)

1 ml Fert-TALP media

GSH solution was made fresh in each replicate. Final working dilution was 15 mM. It was also sterilised by filtration through a 0.22 µm Millipore filter and prior to use the medium was equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 hours.

g. Hyaluronidase stock (300 IU/mg)

100 mg Hyaluronidase (H-3506)

1.1 g Polyvinylpyrrolidone (PVP40)

1 ml Ca^{2+}-, Mg^{2+}- free Dulbecco's phosphate-buffered saline (DPBS-)

Stock solution was dispensed in 1 ml aliquots, and stored at -20ºC. Hyaluronidase stock was diluted in Hepes-TALP, was sterilised by sterilised by filtration through a 0.22 µm Millipore filter and prior to use the medium was warmed at a temperature of 38.5ºC for at least 2 hours.

h. Cytochalasin B stock ($[CB]_0$ = 10 mg/ml)

Cytochalasin B solution from *Drechslera dematioidea* (C2743) was already made and sterilised by 0.20 µm filters. It was dispensed in 10 µl aliquots, and stored at -20ºC. CB stock was diluted in Fert-TALP or Ca^{2+}-Mg^{2+}-Free Fert-TALP at a final working dilution of 7.5 µg/ml.

i. Cycloheximide stock ($[CHX]_0$ = 5 mg/ml)

5 mg	Cycloheximide (C7698)
1 ml	Ca^{2+}-, Mg^{2+}- free Dulbecco's phosphate-buffered saline (DPBS-)

Stock solution was dispensed in 20 µl aliquots, and stored at -20ºC. CHX stock was diluted in Fert-TALP or Ca^{2+}-Mg^{2+}-Free Fert-TALP at a final working dilution of 10 µg/ml. It was sterilised by filtration through a 0.22 µm Millipore filter and prior to use the medium was equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h.

j. Strontium chloride solution ($[SrCl_2]_0$ = 1 M)

266.62 mg	Strontium chloride hexahydrate (255521)
1 ml	Ca^{2+}-Mg^{2+}-Free Fert-TALP

$SrCl_2$ solution was made fresh in each replicate. Final working dilution was 20 mM. It was also sterilised by filtration through a 0.22 µm Millipore filter and prior to use the medium was equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h.

k. Calcium ionophore stock ($[CaIo]_0$ = 20 mM)

1 mg	Calcium Ionophore A23187 (C7522)
0.096 ml	DMSO (final concentration= 0.1%)

From initial stock solution (20 mM) was made other CaIo stocks (15, 10 and 5 mM) and, were dispensed in10 µl aliquots and stored at -20ºC. CaIo stocks were diluted in Hepes-TALP or modified BWW media at different final

working dilutions of 5 µM or 10, 15 and 20 µM, respectively. All solutions were sterilised by filtration through a 0.22 µm Millipore filter and prior to use the medium was warmed at a temperature of 38.5ºC for at least 2 h.

l. Ionomycin stock ($[Io]_0$ = 5 mM)

1 mg	Ionomycin calcium salt from *Streptomyces conglobatus* (IO634)
0.38 ml	DMSO (final concentration= 0.1%)

Stock solution was dispensed in10 µl aliquots, and stored at -20ºC. Io stock was diluted in Hepes-TALP at a final working dilution of 5 µM. It was sterilised by filtration through a 0.22 µm Millipore filter and prior to use the medium was warmed at a temperature of 38.5ºC for at least 2 h.

m. 6-DMAP Stock ($[6\text{-DMAP}]_0$ = 200 mM)

65.27 mg	6-Dimethylamino-purine (D2629)
2 ml	ddH_2O

Stock solution was dispensed in 20 µl aliquots, and stored at -20ºC. Io stock was diluted in Fert-TALP at a final working dilution of 1.9 mM. It was sterilised by filtration through a 0.22 µm Millipore and was equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h.

n. PVP Solution ($[PVP]_0$ = 10% w/v)

35 µl	Polyvinylpyrrolidone (PVP) 10% in Hepes-HTF (Origion-Sage media, 4006-A)
15 µl	Hepes-TALP

2.5 µl dropets PVP (7%) were made in microinjection dishes and were equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 hours.

o. Hypotaurine stock ($[Hypo]_0$ = 1 mg/ml)

20 mg Hypotaurine (H1384)
20 ml ddH_2O

Stock solution was dispensed in 20 µl aliquots, and stored at -20ºC. Hypotaurin stock was diluted in Fert-TALP at a final working dilution of 0.5 µg/ml. It was sterilised by filtration through a 0.22 µm Millipore filter and prior to use the medium was equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h.

p. Epinephrine stock ($[Epi]_0$ = 1 mg/ml)

20 mg Epinephrine (H1384)
20 ml ddH_2O

Stock solution was dispensed in 20 µl aliquots, and stored at -20ºC in dark. Epinephrine stock was diluted in Fert-TALP at a final working dilution of 0.5 µg/ml. It was sterilised by filtration through a 0.22 µm Millipore filter and prior to use the medium was equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h.

q. Luteinizing hormone stock ($[LH]_0$ = 25 units/vial)

1 vl LH from sheep pituitary (L5269)
12.5 ml TCM199

Stock solution was dispensed in 110 µl aliquots, and stored at -20ºC. LH stock was diluted in TCM199 at a final working dilution of 0.01 mg/ml (2 units/ml). It was sterilised by filtration through a 0.22 µm Millipore filter and prior to use the medium was equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h.

r. Follicular stimulating hormone stock ($[FSH]_0$ = 50 units/vial)

1 vl FSH from porcine pituitary (F2293)

25 ml TCM199

Stock solution was dispensed in 110 µl aliquots, and stored at -20ºC. FSH stock was diluted in TCM199 at a final working dilution of 0.01 mg/ml (2 units/ml). It was sterilised by filtration through a 0.22 µm Millipore filter and prior to use the medium was equilibrated in an atmosphere of 5% CO_2 in air with maximum humidity at a temperature of 38.5ºC for at least 2 h