FACULTY OF VETERINARY MEDICINE

Department of Reproduction, Obstetrics and Herd Health

NOVEL INSIGHTS IN THE ROLE OF PROTEASES DURING PORCINE FERTILIZATION *IN VITRO*

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Knowledge is proud that he has learned so much,

Wisdom is humble that he knows no more.

- William Cowper

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LIST OF ABBREVIATIONS

A2M	alpha$_2$-macroglobulin
AA	amino acids
ADAM	a disintegrin and metalloprotease
ADAMTS	a disintegrin and metalloprotease with thrombospondin motifs
AEBSF	4-(2-Aminoethyl) benzene sulfonyl fluoride hydrochloride
AI	artificial insemination
ANOVA	analysis of variance
ART	assisted reproductive technology
BSA	bovine serum albumin
CASA	computer-assisted sperm analysis
CD	cluster of differentiation
CF	cumulus-free
CI	cumulus-intact
COC	cumulus-oocyte-complex
DABCO	1,4-diazabicyclo (2.2.2) octane
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor
(p)FF	(porcine) follicular fluid
FITC	fluorescein isothyiocyanate-conjugated
FSH	follicle stimulating hormone
GSH	glutathione
GV(BD)	germinal vesicle (breakdown)
HA	hyaluronan
hCG	human chorionic gonadotropin
IAM	inner acrosomal membrane
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* production
kDa	kilodalton

LH	luteinizing hormone
MI/II	metaphase I/II
MMP	matrix metalloproteases
mMP	mitochondrial membrane potential
mTALP	modified Tyrode's albumin lactate pyruvate medium
mTBM	modified tris-buffered medium
MW	molecular weight
NCSU23	North Carolina State University 23
PBS	phosphate buffered saline
PHEN	phenanthroline
PSA	pisum sativum agglutinin
PVP	poly-vinyl pyrrolidone
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
SOAF	sperm-borne oocyte-activating factor
SPI	sperm penetration index
STI	soybean trypsin inhibitor
TCM199	tissue culture medium 199
TIMP	tissue inhibitor of metalloproteases
TLCK	Nα-tosyl-L-lysinyl-chloromethyl ketone
TPCK	N_p-Tosyl-L phenylalanine-chloromethyl ketone
VAP	average path velocity
VSL	straight line velocity
ZF	zona-free
ZP	zona pellucida

CHAPTER 1

GENERAL INTRODUCTION

1.1 Assisted reproductive technologies and their applications in the pig

1.1.1 Assisted reproductive technologies

Assisted reproductive technologies (ART) are methods used to achieve fertilization and/or pregnancy by artificial or partly artificial procedures. In human, assisted reproductive technologies are primarily used to help infertile couples to fulfill their wish to have a child (Dondorp and de Wert 2011). Similarly, ART can be used in animals to improve reproductive results. Next to that, ART may be part of animal breeding programs to improve production traits or to preserve threatened or endangered species (Gordon 2004). Assisted reproductive technologies are also part of fundamental and biomedical research. The term ART refers to much more than *in vitro* fertilization and covers different methods by which either oocytes, spermatozoa or both gametes are handled. Several ARTs are successfully implemented in animal reproduction such as collection of oocytes by ovum pick up (cattle, horse), artificial insemination (e.g. cattle, horse, pig) and transfer of embryos from donor to recipient animals (cattle, horse) (Gordon 2004; Gerrits *et al.* 2005; Hinrichs 2012; Rodriguez-Martinez 2012). Other ARTs such as *in vitro* production of embryos, the production of cloned embryos by nuclear transfer or the production of transgenic embryos, are now mainly performed under research conditions but hold interesting opportunities for the future (Niemann and Kues 2007; Gil *et al.* 2010).

1.1.2 Assisted reproductive technologies in modern pig production

Among the assisted reproductive technologies (ART) applied in pig production, artificial insemination (AI) is most commonly practiced. In countries with intensive pig production, more than 90% of the sows are bred by AI (Gerrits *et al.* 2005). Artificial insemination allows for introducing new genetics into a herd while at the same time minimizing the risk for introduction of pathogens. Especially for exchange of valuable genetics between countries worldwide, transport of frozen semen or embryos is preferred above movement of live animals because of the lower risk of disease transmission, animal welfare issues and transport costs. Up to now, the utilization of frozen-thawed semen for AI on pig farms is limited because fertility results are lower compared to AI with fresh-diluted semen. The interest in porcine embryo transfer has increased especially in recent years because of new developments such as the establishment of minimally invasive and non-surgical techniques for embryo collection and embryo transfer (Besenfelder *et al.* 1997; Martinez *et al.* 2004) as well as a

vitrification procedure for porcine embryos (Cuello *et al.* 2004; Cuello *et al.* 2005). These developments give rise to new perspectives for the use of embryo transfer in pig production (Cuello *et al.* 2005).

In the light of the current discussion on piglet castration, the use of sex-sorted semen could be a noteworthy alternative to produce offspring of the desired sex (Rath *et al.* 1999; Johnson *et al.* 2005). Yet boar spermatozoa are very sensitive to manipulation. At this time, the process of semen sorting leads to a substantial degree of membrane damaged spermatozoa which results in a lower fertilizing potential of sex-sorted semen. For that reason, the use of sex-sorted semen currently implies the use of additional reproductive technologies such as deep intra-uterine insemination (Rath *et al.* 2003; Vazquez *et al.* 2003) or *in vitro* production of embryos followed by embryo transfer (Rath *et al.* 1999; Probst and Rath 2003).

The technology of transgenesis may find its way into pig production as it has the potential to improve the quantity and quality of food production, reduce production costs and limit environmental pollution (Niemann and Kues 2003; Whyte and Prather 2011). Research in this area requires the availability of large numbers of zygotes, which are mostly produced by *in vitro* maturation and fertilization of oocytes. There are examples of transgenic pigs with improvement of economically important traits. Weight gain was significantly higher in litters of lactating transgenic gilts expressing bovine alpha-lactalbumin in their milk compared to control gilts (Noble *et al.* 2002). The expression of a bacterial phytase gene in the salivary glands of pigs was shown to result in a better usage of dietary phosphorus which reduced the feed requirements and the level of fecal shedding of phosphorus (Golovan *et al.* 2001). This transgenic pig is named “Enviropig®” and is an example of a genetically modified animal that can reduce the environmental footprint of food production (Forsberg *et al.* 2003).

Another interesting application of transgenesis in animal production is the improvement of disease resistance in animals. Müller *et al.* (1992) reported on gene transfer to pigs of cDNA encoding the mouse Mx1 protein which is associated with resistance to influenza virus. The gene transfer resulted in expression of mouse *Mx1*mRNA but no difference in Mx1 protein level between control and transgenic pigs was recorded.

1.1.3 Assisted reproductive technologies in biomedical research

The domestic pig, *Sus scrofa domestica*, is considered to be a major animal model in human biomedical research because of similarities in anatomy and physiology. There are many human diseases which can be studied using the pig as a model, including infections with *Staphylococcus aureus* (Nielsen *et al.* 2009; Jensen *et al.* 2010), influenza viruses (Van Reeth *et al.* 1998; Khatri *et al.* 2010) and *Helicobacter pylori* (Nedrud 1999), as well as different human metabolic disorders (Litten-Brown *et al.* 2010). Depending on breed and age of the pig, the size of organs in the digestive and cardiovascular system is to a large extent similar to these of human. Interestingly, the anatomy of pulmonary vascularization and coronary arteries is also very similar. With respect to physiology, pigs and human share several characteristics such as similar respiratory rates, cardiac output, blood pressure and renal function parameters (Sachs 1994; Ibrahim *et al.* 2006). For that reason, pigs are used as a model to perform surgical and non-surgical procedures typically used in human medicine (Meurens *et al.* 2012). Furthermore, there is also an increasing interest to use pigs as non-rodent species in toxicity testing of pharmaceutical products (Swindle *et al.* 2012).

The pig has received much attention as a potential organ donor for human patients (Ibrahim *et al.* 2006). Porcine xenografts have been put forward as the solution of choice to overcome the shortage of human donor organs for organ transplantation (Niemann and Kues 2007). By expression of certain human proteins in transgenic pigs, some of the immunologic and physiologic incompatibilities between humans and pigs can be overcome (Ibrahim *et al.* 2006). Preclinical studies with xenografts of transgenic pigs lacking alpha-1,3-galactosyl-transferase or expressing human complement regulatory proteins CD46, CD55 and CD59 have demonstrated that a hyper acute rejection response after xenotransplantation to non-human primates was reduced with prolonged survival of the pig organ (Sachs and Galli 2009; Klymiuk *et al.* 2010).

1.2 *In vitro* production of porcine embryos

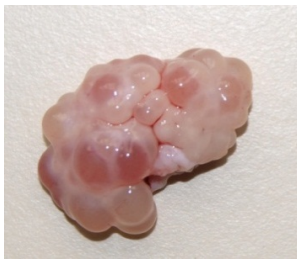
In vitro production of pig embryos is a prerequisite for techniques such as cloning and transgenesis. Although procedures of cloning and transgenesis are relatively well described, efficiency is limited and large numbers of mature oocytes or zygotes need to be used to obtain live born cloned or transgenic piglets, respectively. Surgical collection of oocytes or embryos from donor animals is time consuming, expensive, and therefore not feasible for large scale implementations (Abeydeera 2002). For that reason, ovaries from prepubertal gilts collected after slaughter are the main source of (immature) oocytes for *in vitro* technologies.
The competence of *in vitro* maturation (IVM) of immature oocytes, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of embryos has been shown by the birth of live piglets after transfer of *in vitro* produced blastocysts to recipients (Mattioli *et al.* 1989; Yoshida *et al.* 1993b; Kikuchi *et al.* 1999; Kikuchi *et al.* 2002). However, the efficiency of IVP is still suboptimal and high numbers of blastocysts need to be transferred to have embryos that develop to term. The IVP procedure of porcine embryos needs further optimization in order to facilitate biomedical research, biotechnical techniques and appliance in commercial pig production. A standard IVP procedure comprises three major consecutive steps: *in vitro* maturation (IVM) of immature oocytes, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of zygotes (Fig. 1).

1.2.1 *In vitro* maturation of porcine oocytes

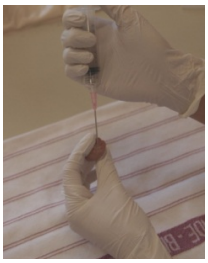
Nuclear and cytoplasmic maturation

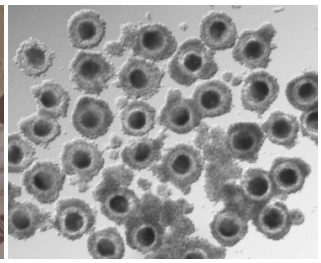
In vitro maturation of porcine oocytes includes two aspects: nuclear and cytoplasmic maturation. Immature oocytes derived from antral follicles are in the germinal vesicle stage at the time of collection and resume meiosis shortly after removal from the follicle (Funahashi *et al.* 1997b). Nuclear maturation is completed when the oocyte has extruded the first polar body and arrests at the metaphase II stage. In parallel with reorganization of the oocyte DNA, there is relocation of organelles in the cytoplasm, which is supported by the cytoskeleton. For example, cortical granules migrate towards the area just beneath the oocyte membrane to form a monolayer (Wang *et al.* 1997). There are also changes in the oocyte metabolism and mRNAs and proteins are accumulated in the cytoplasm. Together these cytoplasmic changes are called “cytoplasmic maturation” (Krisher *et al.* 2007). Incomplete cytoplasmic maturation leads to a lower developmental competence of oocytes, despite completion of nuclear maturation. Thus, both nuclear and cytoplasmic maturation are essential for successful fertilization and subsequent embryo development.

Collection of cumulus-oocyte-complexes from tertiary follicles

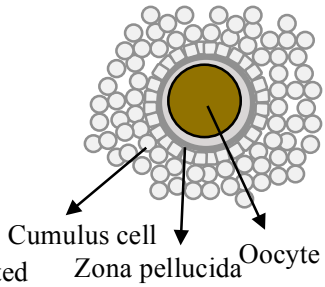


Ovaries from prepubertal gilts are collected at a local slaughterhouse.

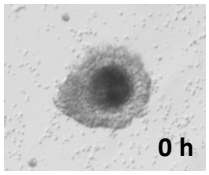


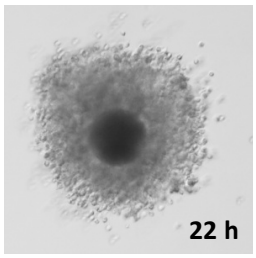


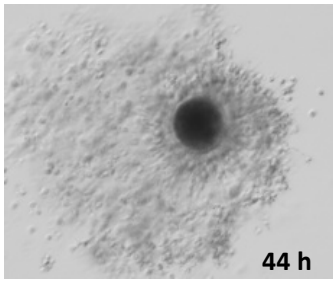
Cumulus-oocyte complexes (COCs) are aspirated from follicles with a diameter ranging from 3 to 6 mm. Only COCs with a homogenous ooplasm and a multilayered cumulus are selected.



***In vitro* maturation**

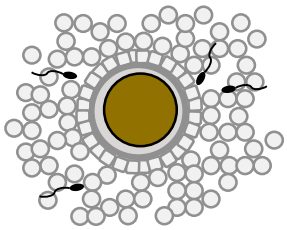






Groups of 100 COCs are cultured for the first 22h in maturation medium with eCG and hCG (39°C, 5% CO_2). Subsequently, COCs are cultured in hormone-free maturation medium for another 22h.

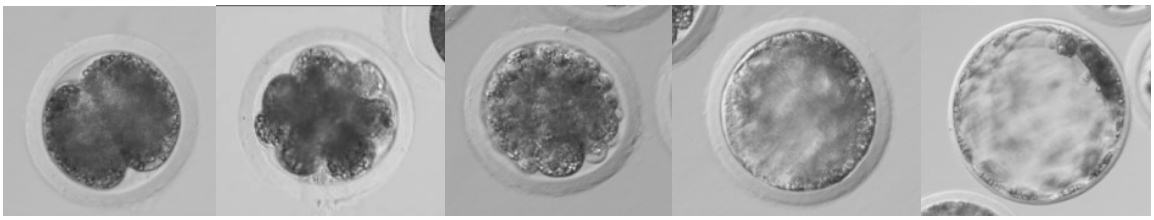
***In vitro* fertilization**



D0

Gamete co-incubation during 6h (39°C, 5% CO_2)

***In vitro* culture**



D1 **D4** **D5** **D6** **D7**

Presumed zygotes are vortexed to remove loosely bound spermatozoa, washed and cultured in embryo culture medium (modular incubator chamber, 39°C, 5% CO_2).

Figure 1: Schematic overview of the major steps of *in vitro* production of porcine embryos (Photos: J. Beek)

In vivo versus *in vitro* maturation

In vivo, the growth of tertiary (antral) follicles towards the pre-ovulatory stage is stimulated by gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Follicle growth is associated with an increase in estradiol level that, in turn, induces the LH surge followed by ovulation. The time-span between the onset of the LH-surge and ovulation is approximately 44 h (Soede *et al.* 1994). For *in vitro* maturation of oocytes, cumulus-oocyte-complexes (COCs) derived from antral follicles are cultured between 36 and 48 h, with some preference given to 40-44 h (Prather and Day 1998).

During *in vivo* maturation, COCs are surrounded by follicular fluid until the time of ovulation. The composition of follicular fluid, including steroid hormone concentrations, undergoes significant changes over time coinciding with progression of oocyte maturation (Chang *et al.* 1976; Ainsworth *et al.* 1980; Bertoldo *et al.* 2013). In contrast, *in vitro* maturation of COCs is mostly performed in a static system with abrupt changes when oocytes are transferred from one medium to another. For example, gonadotropins are mostly added to the maturation medium only during the first half of the maturation period, after which the COCs are transferred to hormone-free medium (Funahashi and Day 1993).

In vitro matured oocytes display a lower developmental competence when compared to their *in vivo* counterparts (Petters and Wells 1993). Many studies have been undertaken to elucidate reasons for this difference and to find biochemical markers that reflect oocyte developmental competence. The intracellular glutathione (GSH) content has been shown to be an important oocyte factor associated with developmental competence (Abeydeera *et al.* 1998; Sun and Nagai 2003). Oocytes show a significant higher GSH content after maturation *in vivo* than *in vitro* (Yoshida *et al.* 1993a; Brad *et al.* 2003). Several adjustments to the maturation medium promoting oocyte developmental competence have been published using GSH levels as a biochemical marker.

Oocytes from gilts versus sows

Surgical collection of oocytes or embryos from donor animals is time-consuming, expensive, and therefore not feasible for large scale implementation (Abeydeera 2002). Oocytes derived from slaughtered animals are readily available in large numbers, with low costs and no need for surgical procedures. In many laboratories, the main source of oocytes used for *in vitro* production of porcine embryos is ovaries collected from prepubertal gilts. When compared to oocytes derived from mature gilts or sows, oocytes from prepubertal gilts display a lower sensitivity to hormones and growth factors, a higher degree of polyspermic fertilization after

IVF and a lower developmental capacity (Marchal *et al.* 2001). In the study of Marchal *et al.* (2001), FSH increased the proportion of oocytes reaching the metaphase II stage at a lower dose in sow oocytes (40 ng/ml) than in gilt oocytes (400 ng/ml). EGF was effective at 1 ng/ml in sow oocytes, whereas gilt oocytes needed at least 10 ng/ml to obtain the maximum nuclear maturation rate. Sperm penetration was significantly higher in gilt oocytes than in sow oocytes, with polyspermy rates of 31 ± 17% and 17 ± 7%, respectively. In contrast, Sherrer et al (2004) did not find a difference in polyspermy between gilt and sow oocytes. Nevertheless, a consistent finding is that oocytes from prepubertal female pigs yield a lower blastocyst rate (-7 to -30%) in comparison with oocytes from sows (Marchal *et al.* 2001; Sherrer *et al.* 2004; Bagg *et al.* 2006). Yet there are unique advantages to the use of ovaries from prepubertal female pigs (6-7 months of age) such as the similar physiological state of all ovaries (non-cyclic), the high comparability of ovaries in the size of follicles as well as the availability of many tertiary follicles on the ovarian surface in the absence of pre-ovulatory follicles and corpora lutea. Furthermore, advances in the IVM system via the use of hormones, growth factors and other supplements in the medium have improved the maturation rate and developmental competence of oocytes from prepubertal pigs. Addition of FSH (400 ng/ml) and EGF (10 ng/ml) to the IVM medium increased the percentage of gilt oocytes reaching metaphase II from 32% to 85% (Marchal *et al.* 2001). Treatment with 1 mM dbcAMP for the first 22 h of IVM improved the blastocyst rate of gilt oocytes from 18.6 ± 5.4% to 35.9 ± 6.9% (Bagg *et al.* 2006). The suitability of oocytes from prepubertal pigs for *in vitro* embryo production has further been confirmed by the birth of liveborn piglets (Mattioli *et al.* 1989; Yoshida *et al.* 1993b; Kikuchi *et al.* 1999; Marchal *et al.* 2001).

Effect of follicle size

Oocytes for pig IVP are generally collected from antral follicles ranging 3 to 6 mm in diameter and then selected according to the morphology of the cumulus-oocyte-complex (COC). Only oocytes with a uniform ooplasm surrounded by several dense layers of cumulus cells are selected for IVM (Abeydeera 2002; Marchal *et al.* 2002).

Pig oocytes reach full meiotic competence in follicles with a diameter of 3 mm or more, whereas the developmental competence coincides with follicular size (Marchal *et al.* 2002). Oocytes derived from medium (3-5 mm) and large (>5 mm) follicles have higher sperm penetration rates and higher blastocyst development than oocytes from small follicles <3 mm (Sun *et al.* 2001; Marchal *et al.* 2002). There is also heterogeneity in the stage of nuclear maturation at the time of collection (Funahashi *et al.* 1997a).

Role of cumulus cells during *in vitro* maturation

Cumulus cells have beneficial effects on nuclear and cytoplasmic maturation (Mattioli *et al.* 1988; Sun *et al.* 2001; Wongsrikeao *et al.* 2005; Maedomari *et al.* 2007). Moreover, the degree of cumulus expansion has been related to oocyte developmental competence in different species, including the pig (Qian *et al.* 2003). Therefore, maturation of cumulus enclosed oocytes is preferred above maturation of cumulus denuded oocytes with only few exceptions. Several receptors for hormones and growth factors are found on cumulus cells, such as LH receptors (Shimada *et al.* 2003), progesterone receptors (Shimada and Terada 2002a; Shimada *et al.* 2004b) and EGF receptors (Prochazka *et al.* 2003).

Communication and transfer of small molecules, such as glutathione and cAMP, between the cumulus cells and the oocyte take place via gap junctions (Mori *et al.* 2000; Van Soom *et al.* 2002). Cumulus cells enhance the amount of glutathione (GSH) in oocytes and thereby protect oocytes against oxidative stress (Tatemoto *et al.* 2000). The intracellular content of GSH has also been associated with the successful formation of the male pronucleus after fertilization (Yamauchi and Nagai 1999; Maedomari *et al.* 2007).

Cumulus cells play an important role in the regulation of nuclear maturation by keeping the cAMP level in the oocyte high (Shimada and Terada 2002b). Although IVM is usually performed with cumulus-enclosed oocytes, loss of gap junctions between cumulus cells and oocyte during IVM is described (Motlik *et al.* 1986). The uncoupling of cumulus cells and oocyte may lead to a premature drop in cAMP level and progression to the MII stage before cytoplasmic maturation is completed. This is one of the hypotheses for the inferior developmental competence of oocytes matured *in vitro* compared to *in vivo* (Funahashi *et al.* 1997b). It has been shown that by keeping the cAMP level artificially high in the first part of IVM, nuclear maturation of oocytes is synchronized and developmental capacity is improved (Funahashi *et al.* 1997b; Somfai *et al.* 2003).

Effect of gonadotropins on cumulus expansion during *in vitro* maturation

The use of gonadotropins such as FSH and LH is widely adopted by laboratories performing porcine embryo production *in vitro* with only minor differences between IVM protocols. In general, COCs are exposed to gonadotropins for only the first half of the maturation period which is based on a study by Funahashi and Day (1993). The addition of compounds with FSH- and LH-activity mimics the stimulation by gonadotropins during the follicular phase *in vivo*. Similar to the changes induced by the pre-ovulatory LH surge *in vivo*, the morphology of the COC changes after exposure to gonadotropins *in vitro*. The volume of the cumulus matrix

enlarges by deposition of hyaluronan (HA) and other matrix components produced by the cumulus cells, a process called cumulus expansion (Mattioli 1994). The organization and restriction of HA in the extracellular matrix of porcine COCs is mediated by HA binding proteins such as versican (Russell *et al.* 2003; Shimada *et al.* 2004a), tumor necrosis factor-stimulated gene-6 protein (Nagyova *et al.* 2008) and serum-derived members of the inter-α-trypsin inhibitor family (Nagyova *et al.* 2004).

The stimulatory role of FSH and LH on cumulus expansion is well established (Mattioli *et al.* 1991). In addition to FSH and LH, cumulus expansion is stimulated *in vitro* by the presence of epidermal growth factor and/or follicular fluid in the maturation medium (Mattioli *et al.* 1991; Procházka *et al.* 2000; Bijttebier *et al.* 2008; Grupen and Armstrong 2010).

Development of chemically defined *in vitro* maturation media

Successful maturation of pig oocytes can be achieved in medium supplemented with porcine follicular fluid or fetal calf serum, in conjunction with gonadotropins and/or epidermal growth factor (Yoshida *et al.* 1993b; Rath *et al.* 1995; Singh and Armstrong 1997; Vatzias and Hagen 1999; Abeydeera 2002; Bijttebier *et al.* 2008). Supplementation of follicular fluid or serum introduces many unknown factors and makes it difficult to identify key factors responsible for normal oocyte maturation (Abeydeera 2002). In order to evaluate the effect of medium composition and to minimize variability between laboratories, effort has been directed to the development of a chemically defined medium for oocyte maturation (Abeydeera *et al.* 2000; Yoshioka *et al.* 2008; Mito *et al.* 2009). However, the beneficial effects of follicular fluid supplementation during IVM are well described, which explains its use in the majority of laboratories (Gil *et al.* 2010). Clearly, new insights in the composition of follicular fluid and the importance of specific components would contribute to further improvement of a defined IVM system for porcine oocytes.

1.2.2 *In vitro* fertilization of porcine oocytes

Fertilization results after porcine IVF largely depend on the type of medium used, the fertilizing capacity of spermatozoa (boar influence), sperm concentration, duration of gamete co-incubation and the presence of caffeine in the fertilization medium (Abeydeera 2002). Successful *in vitro* fertilization of porcine oocytes has been realized with fresh ejaculated semen, fresh epididymal semen, frozen-thawed ejaculated semen and frozen-thawed epididymal semen.

Normal versus polyspermic fertilization

Normal (monospermic) fertilization is defined as the penetration of one sperm cell into the oocyte, followed by the formation and fusion of the male and female pronucleus. Polyspermic fertilization occurs when more than one sperm cell enters the oocyte (Fig. 2). In the latter, the fusion of pronuclei is hampered because of the presence of an unequal number of each chromosome (polyploidy). In porcine IVF, polyspermy is still a major issue to be solved. Although polyploid zygotes are able to develop to the blastocyst stage, their developmental competence to term is inferior to normal fertilized zygotes (Funahashi 2003). Polyspermic fertilization also occurs *in vivo*, however, to a far lesser extent when compared with *in vitro* conditions (Hunter 1973). Interestingly, the degree of polyspermy *in vivo* could be increased by artificially increasing the number of spermatozoa at the site of fertilization. The ratio between spermatozoa and oocytes at fertilization thus seems one of the crucial factors for normal fertilization *in vivo* (Hunter 1973; Hunter 1996).

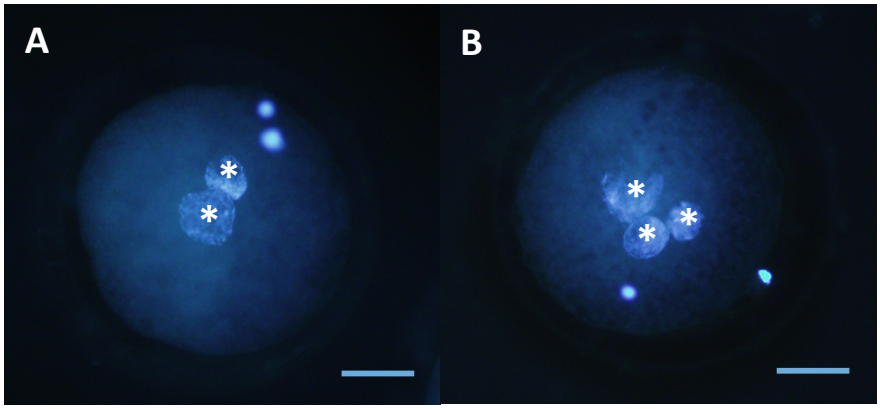


Figure 2: Normal versus polyspermic fertilization. Cumulus-denuded porcine oocyte, stained with Hoechst 33342 ± 20h after fertilization. Pronuclei are marked with *. **A:** normal fertilization, **B:** polyspermic fertilization (Original Magnification x400; Bar = 50 µm)

Type of spermatozoa: fresh versus frozen-thawed, ejaculated versus epididymal spermatozoa

Freshly ejaculated semen can be used for *in vitro* fertilization of porcine oocytes, which can be matured *in vivo* as well as *in vitro* (Mattioli *et al.* 1989; Yoshida *et al.* 1990; Rath and Niemann 1997). The successful penetration of oocytes after IVF with epididymal spermatozoa has also been reported (Nagai *et al.* 1984). However, when fresh spermatozoa are used for IVF, a high variability in fertilization results can be expected due to differences

between boars and day-to-day variations between ejaculates from the same boar (Rath and Niemann 1997).

On the other hand, when using frozen-thawed spermatozoa, subsequent IVF experiments can be performed with spermatozoa from the same origin (ejaculate or epididymis). It is generally accepted that the use of frozen-thawed spermatozoa minimizes variation between experiments. For that reason, most laboratories use frozen-thawed spermatozoa to standardize the male factor in IVF experiments. Nevertheless, a single IVF protocol may not result in similar fertilization rates for different batches of frozen-thawed spermatozoa (Gil *et al.* 2008).

The comparison of IVF results using fresh semen, frozen-thawed ejaculated and frozen-thawed epididymal spermatozoa of identical boars under uniform IVF conditions revealed that minimal variability was obtained using frozen-thawed epididymal spermatozoa (Rath and Niemann 1997). Spermatozoa flushed from the epididymis can be frozen with good results using a standard freezing protocol. Furthermore, epididymal spermatozoa have some advantages over ejaculated spermatozoa which are higher motility rates after thawing, higher fertilizing capacity *in vitro* and less generation of reactive oxygen species (Rath and Niemann 1997; Matás *et al.* 2010).

These characteristics are probably related with the fact that epididymal spermatozoa do not come into contact with seminal plasma (Nagai *et al.* 1984). Seminal plasma contains many proteins related to sperm function and motility. Exposure to seminal plasma results in binding of “decapacitation factors” to the sperm surface which protect spermatozoa from premature activation during transport in the female reproductive tract *in vivo*. The “decapacitated” spermatozoa have to be “recapacitated” before they are able to fertilize an oocyte. *In vitro*, spermatozoa are artificially placed in close contact with the oocytes, and consequently, there is no need to prevent capacitation. Moreover, various capacitation procedures have been described to facilitate IVF with fresh semen (Nagai *et al.* 1984; Yoshida *et al.* 1990; Rath 1992). In addition, spermatozoa from the seminal plasma-rich fraction of the ejaculate display a significantly lower fertilizing ability than spermatozoa from sperm-rich fractions, which has also been attributed to factors present in seminal plasma (Xu *et al.* 1996).

Seminal plasma contains a family of secretory proteins, spermadhesins, which bind to the surface of spermatozoa (Caballero *et al.* 2008). Seven spermadhesins, PSP-I, PSP-II, AQN-1, AQN-2, AQN-3, AWN and DQH, have been identified in boar seminal plasma (Strzezek *et al.* 2005). The biological effects of these proteins are complex and not fully elucidated. They are described to function in transport of spermatozoa in the female reproductive tract, suppression of the immune response against sperm antigens and gamete interaction (Strzezek

et al. 2005). The majority of spermadhesins are released from the sperm head during *in vitro* capacitation (Dostàlovà *et al.* 1994; Caballero *et al.* 2008). Spermadhesin AWN is detected on the membrane of boar spermatozoa bound to the zona pellucida *in vivo* (Rodríguez *et al.* 1998) and is also the only spermadhesin present on the surface of epididymal sperm (Dostàlovà *et al.* 1994). By lack of contact with seminal plasma, epididymal spermatozoa may not exhibit the full range of surface molecules compared to ejaculated spermatozoa. In fundamental research on gamete interaction, this difference between epididymal and ejaculated spermatozoa needs to be considered.

Sperm-oocyte co-incubation conditions

Fertilization results depend to a significant extent on the composition of the fertilization medium, the sperm concentration and the sperm-oocyte co-incubation time. Commonly used media for IVF include modified Tris-buffered medium (mTBM), Tissue Culture Medium 199 (TCM199) and modified Tyrode's medium (mTALP) (Mattioli *et al.* 1989; Yoshida *et al.* 1993b; Abeydeera and Day 1997; Rath *et al.* 1999). The presence of caffeine in the fertilization medium is able to stimulate capacitation, sperm motility and spontaneous acrosome reaction of spermatozoa from some boars, but not from others (Gil *et al.* 2008). Because of the variation in *in vitro* fertility between boars, the IVF protocol needs to be optimized for each individual boar and/or batch of frozen semen. There is a wide range of sperm concentrations reported in literature illustrating that fertilizing capacity depends on boar as well as the type of spermatozoa used (fresh vs. frozen thawed). In early porcine IVF protocols, sperm and oocytes were incubated during 12-16 h (Nagai *et al.* 1988). Mainly because of high penetration rates coinciding with high levels of polyspermy, there has been a tendency to decrease gamete co-incubation time. In current porcine IVF protocols, a 5 to 6 h period is commonly used (Abeydeera and Day 1997; Kikuchi *et al.* 1999; Coy *et al.* 2002; Gil *et al.* 2003).

1.2.3 *In vitro* culture of porcine embryos

Different developmental stages of embryos have different *in vitro* requirements. This is caused by the important biochemical and morphological changes which embryos undergo during the first week of life, such as the transition of maternal to embryonic genome control, compaction and development of a blastocoel (Swain *et al.* 2002). In pigs, the four-cell stage embryo is very sensitive to *in vitro* conditions. Early attempts to culture *in vivo* derived pig embryos were confronted with a developmental arrest at this stage, which coincided with the

activation of the embryonic genome and the onset of RNA synthesis (Telford *et al.* 1990). In the early 1990's, it was shown that a reduced NaCl concentration in the culture medium improved embryo development (Beckmann and Day 1993; Petters and Wells 1993).
The North Carolina State University medium NCSU23 described by Petters and Wells (1993) is the most widely used embryo culture medium. Other media for IVC include modified Whittens medium (Beckmann and Day 1993) and Beltsville embryo culture medium (BECM) (Dobrinsky *et al.* 1996). These media are able to support development of *in vitro* matured and fertilized oocytes to the blastocyst stage. However, there is still much room for improvement. As shown by Kikuchi *et al.* (1999), culture of *in vitro* produced zygotes in the oviduct of a synchronized recipient yields significantly higher blastocyst rates compared to culture *in vitro*. Furthermore, it was demonstrated that blastocysts collected at day 6 have a higher cell number when zygotes are transferred on the day of fertilization (day 0) compared to transfer after 2 days of IVC or after *in vitro* embryo culture without transfer (mean cell number 181.5, 58.2 and 38.4, respectively) (Kikuchi 2004). Thus, deficiencies in culture conditions seem to decrease viability of *in vitro* produced embryos. Furthermore, comparison of embryo metabolism showed an altered metabolic activity in pig embryos cultured *in vitro* compared to their *in vivo* counterparts (Swain *et al.* 2002).

1.3 Current limitations for large scale IVP of porcine embryos

From the start of porcine IVP, nuclear maturation of immature oocytes has been more easily achieved than cytoplasmic maturation. The current limitations of porcine IVP all relate in a variable degree to insufficient cytoplasmic development (Krisher *et al.* 2007).

The poor male pronuclear formation reported in early IVF studies has been improved by various modifications to the IVM system (Abeydeera 2002). The presence of follicle cells or cumulus cells during porcine IVM increases male pronuclear formation after subsequent IVF (Mattioli *et al.* 1988; Nagai *et al.* 1993; Ka *et al.* 1997). In general, cumulus enclosed oocytes are subjected to IVM in pigs, as in other domestic animals.

The major obstacle for efficient production of large numbers of IVP embryos remains the high incidence of polyspermic fertilization (Abeydeera 2002; Gil *et al.* 2010). Many researchers have identified small pieces of the puzzle, but there is still no complete understanding why polyspermy rate during porcine IVF often reaches more than 40% (Abeydeera and Day 1997; Suzuki *et al.* 2000; Coy *et al.* 2002; Gil *et al.* 2004a).

There is still a major gap between *in vitro* culture of embryos and physiological conditions, although several embryo culture media are available that support the development of the porcine zygote to the blastocyst stage. Timing of embryo development is an accurate and non-invasive way to evaluate the developmental competence of embryos (Mateusen *et al.* 2005). The four-cell stage, critical because of embryonic genome activation, lasts between 20 to 24 h for *in vivo* developing embryos (Hunter 1974), whereas *in vitro* cultured but *in vivo* derived embryos display a 4-cell lag phase of average 38 to 44 h (Anderson *et al.* 1999; Mateusen *et al.* 2005). This discrepancy can likely be attributed to imperfections of *in vitro* culture conditions. Further research focusing on metabolic activity and the changing needs of embryos according to their developmental stage will help to optimize culture conditions (Gardner 1998; Swain *et al.* 2002; Krisher and Prather 2012).

Adaptations to the IVF protocol to minimize polyspermic fertilization

The two major mechanisms *in vivo* that ensure an effective block to polyspermy are believed to be the cortical reaction followed by the "zona block" after the entry of the first sperm cell and a restriction in the number of sperm at the site of fertilization (Hunter *et al.* 1990). More recently, it has become evident that oviductal secretions contribute to the oocyte defense mechanisms against polyspermy (Coy and Avilés 2010). By mimicking the physiological conditions of fertilization *in vivo*, researchers have attempted to tackle the problem of polyspermy *in vitro*.

During fertilization, the oocyte is activated by sperm penetration and reacts with exocytosis of cortical granules, the "cortical reaction". Enzymes released from the cortical granules induce protein changes in the zona pellucida and remove sperm receptors, thereby blocking further binding and entry of spermatozoa, the so-called "zona block" (Yanagimachi 1994). The high polyspermy rate in porcine IVF has been associated with failures in the cortical reaction, such as a delayed or incomplete cortical reaction, or an uneven distribution of released cortical granule content into the perivitelline space (Cran and Cheng 1986). It has been found that the presence of beta-mercaptoethanol could significantly improve the exocytosis of cortical granules in *in vitro* matured oocytes (Funahashi 2005). However, there is inconsistency in the results obtained by studies evaluating the cortical reaction during porcine IVF. Wang *et al.* (1997) described that only 45% of IVM oocytes completed cortical granules exocytosis within 18 h after fertilization *in vitro*. Similarly, Coy *et al.* (2002) reported a slow cortical reaction in porcine oocytes after IVM/IVF, with a large proportion of cortical granules remaining in the oocyte at 5 h after IVF in different types of medium (mTBM, TCM-199 and mTALP). In

contrast, it was described that the ability to release the content of cortical granules does not differ between *in vitro* and *in vivo* matured porcine oocytes (Wang *et al.* 1998) and even that the cortical reaction in *in vitro* matured oocytes occurs faster than in oocytes matured *in vivo* (Romar *et al.* 2012). In the studies of Wang et al. (1998) and Romar et al. (2012), the cortical reaction was completed within 5-6 h after fertilization of *in vitro* matured oocytes. Interestingly, timing of cortical granule exocytosis was similar in polyspermic and monospermic oocytes (Wang *et al.* 1998) or faster in polyspermic oocytes (Romar *et al.* 2012). Differences in outcome might be explained by the assessment of cortical granule density at specific time points during the experiment, boar effects and the use of oocytes from gilts versus sows. Taken together, the results of studies investigating the cortical reaction indicate that a functional block to polyspermy includes events after exocytosis of the cortical granules. Multiple spermatozoa may have the chance to enter the oocyte when the zona reaction is delayed or incomplete.

Especially when high numbers of spermatozoa are near the oocyte, one can expect that multiple spermatozoa will enter, especially when the cortical reaction is delayed. The high sperm concentration used in IVF (>1000 spermatozoa per oocyte is common practice) compared to *in vivo* (close to 1 sperm cell per oocyte), is believed to be one of the main reasons for high polyspermy rates in porcine IVF (Hunter 1996). Several researchers developed a modified IVF method to reduce the concentration of spermatozoa near the oocytes by an increase in sperm selection. Examples are the climbing-over-a-wall (COW) method (Funahashi and Nagai 2000), the swim-up method (Park *et al.* 2009) and straw IVF (Li *et al.* 2003). These IVF methods are based on the selection of spermatozoa according to the degree of motility. A schematic cross-sectional view of the COW method, the swim-up method and straw IVF can be found in Figure 3. Although these methods decrease polyspermy rate, they are not able to completely avoid polyspermy. Recently, researchers have focused on modifying the gamete co-incubation time. It was shown that after 2-10 min of gamete incubation, the number of spermatozoa bound to the zona pellucida is sufficient for successful fertilization and oocytes can be transferred to fresh fertilization medium to avoid exposure to excessive number of spermatozoa (Grupen and Nottle 2000; Gil *et al.* 2004b; Almiñana *et al.* 2008). This modification of the IVF protocol is able to retain high penetration rates and improve blastocyst percentages, but efficiency is dependent on boar fertility (Gil *et al.* 2010).

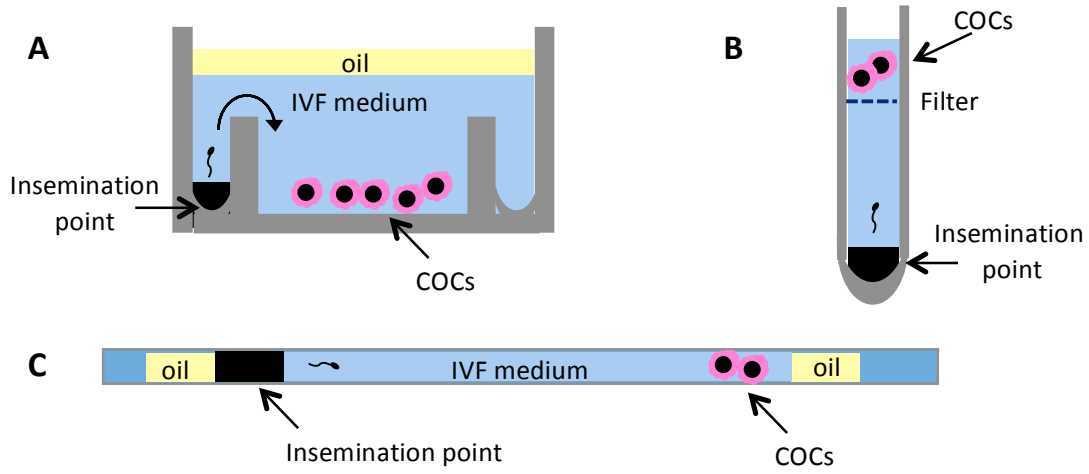


Figure 3: Schematic cross-sectional view of (A): Climbing-over-a-wall method (adapted from Funahashi and Nagai 2000), (B): Swim-up method (adapted from Park *et al.* 2009) and (C): Straw IVF (adapted from Li *et al.* 2003).

In vivo, fertilization takes place in the oviduct. The contribution of oviductal secretions to the regulation of polyspermy has been revealed in recent years. There is now convincing evidence that oviductal secretions, including oviduct-specific protein (OSP), modify the zona pellucida in preparation for normal fertilization in pigs (Coy and Avilés 2010). This process is called "pre-fertilization zona hardening" and is also described in other mammalian species. The term hardening refers to the increased resistance of the zona pellucida to proteolysis, which can be assessed *in vitro* by the time needed for ZP digestion in pronase solution, and to a decrease in sperm binding and sperm penetration (Coy and Avilés 2010). Pre-incubation of *in vitro* matured porcine oocytes with oviductal fluid increases their resistance to pronase digestion and decreases sperm penetration rate in subsequent IVF (Kim *et al.* 1996; Coy *et al.* 2008). Co-incubation of porcine gametes with oviductal epithelial cells in the fertilization medium was also shown to reduce polyspermy rate and improve embryo development (Bureau *et al.* 2000). Furthermore, exposure of *in vitro* matured oocytes to 10 µg/ml purified porcine OSP before and during IVF resulted in a significant lower penetration rate and polyspermy rate compared to the control group (0 µg/ml porcine OSP) (Kouba *et al.* 2000).

1.4 The role of proteases in fertilization

1.4.1 Fertilization

Mammalian fertilization requires the successful completion of a number of steps in a compulsory order (Yanagimachi 1994; Wassarman *et al.* 2001; Primakoff and Myles 2002). Fertilization begins with the movement of spermatozoa through the female reproductive tract towards the site of fertilization: the oviduct. In many species, including the pig, spermatozoa first adhere to oviductal epithelial cells at the caudal isthmus of the oviduct, which functions as a sperm reservoir (Töpfer‐Petersen *et al.* 2002). The adhesion between spermatozoa and oviductal epithelium is mediated by carbohydrate-binding proteins and oligosaccharides (Suarez 2000). At the sperm reservoir, spermatozoa are able to survive and maintain fertilizing capacity for a period of hours to days. Subsequently, around the time of ovulation, continuous release of low numbers of spermatozoa that underwent capacitation (a process including changes in metabolic activity, motility and organization of lipids and proteins in the membrane of spermatozoa) ensures that fertile spermatozoa will meet the ovulated oocytes in the oviduct (Rodriguez-Martinez 2007). Chemo-attractants direct the sperm cell to the oocyte, which is at that time surrounded by two extracellular layers: the zona pellucida (ZP) and the cumulus oophorus (Fig. 4).

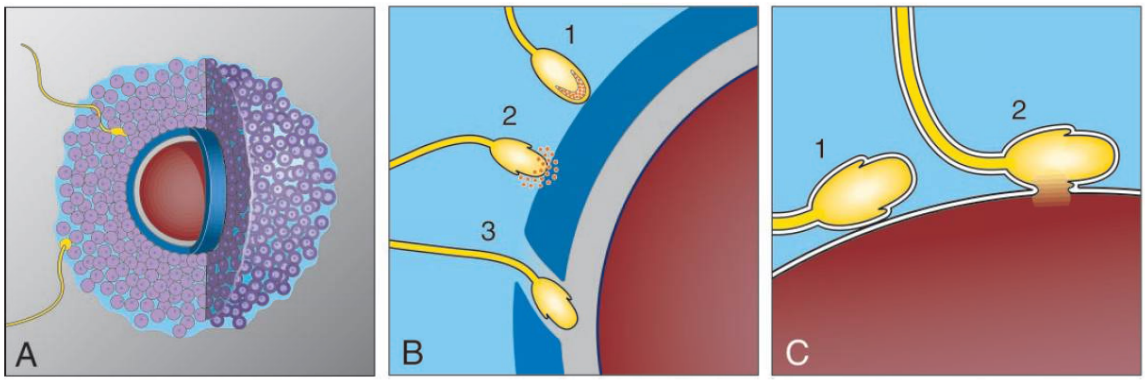


Figure 4: Mammalian fertilization. (**A**) Sperm penetration through cumulus cells (purple) to reach the zona pellucida (blue). (**B**) Oocyte, depicted with cumulus cells removed; sperm 1 binds to the zona pellucida (blue); sperm 2 undergoes exocytosis, releasing acrosomal contents (orange); sperm 3 penetrates the zona pellucida and begins entry into the perivitelline space (gray). (**C**) sperm 1 binds to the oocyte plasma membrane by the side of its head, in a central region (equatorial region); sperm 2 fuses with the oocyte plasma membrane (Adapted from Primakoff and Myles 2002).

Spermatozoa then find their way through the cumulus matrix using sperm motility and a sperm hyaluronidase. Only species-specific spermatozoa with an intact acrosome are able to bind to the ZP. Once bound to the ZP, spermatozoa are stimulated to undergo the acrosome reaction in order to penetrate the ZP. Based on recent findings, it appears that, at least in the mouse, not only the ZP but also the cumulus oophorus is a physiological site of the acrosome reaction (Yin *et al.* 2009; Jin *et al.* 2011; Sun *et al.* 2011). Nevertheless, sperm penetration of the ZP is generally accepted to involve proteolysis by proteases released from the acrosome together with sperm motility. After the sperm cell reaches the perivitelline space, binding and fusion with the oocyte membrane are the last steps to complete fertilization. From then, the fertilized oocyte is called a zygote.

1.4.2 Proteases: terminology and classification

The general term "protease" refers to a hydrolytic enzyme that acts on a protein or further degrades fragments of a protein (Beynon and Bond 2001). Proteases execute a diversity of functions in developmental, physiological and pathological processes. They are important for remodeling cellular and extracellular proteins and play a key role in regulatory pathways by activating or deactivating target proteins. Some proteolytic enzymes preferentially act on small peptides and are called "peptidases". Based on the site of cleavage, distinction is made between exoproteases/exopeptidases and endoproteases/endopeptidases. Exoproteases act on the terminal groups of proteins, whereas endoproteases cleave amide bonds within a protein.
Proteases with homology of amino acid sequence in the active site of the molecule are grouped into families. The five primary families of proteases are serine (S), cysteine (C), Threonine (T), aspartic (A) and metallo- (M) proteases (Deu *et al.* 2012). Their catalytic mechanism is illustrated in Figure 5. A detailed and up to date classification of proteases can be found in the MEROPS database (Rawlings *et al.* 2012).

Serine proteases

The S proteases are the largest and most thoroughly studied type of proteases and represent about one third of all known proteases (Di Cera 2009). Serine proteases are named for the nucleophilic Ser residue at the active site. Serine protease activity typically includes the formation of an ester bond between S and the substrate (Fig. 5). Most of the S proteases are endoproteases that cleave a polypeptide in the middle of the chain (Di Cera 2009). Next to S proteases that are secreted or sequestered in an organel, a group of membrane bound serine proteases has also been described. The "type II transmembrane serine proteases" are membrane proteins with an extracellular chymotrypsin-like S protease domain (Hooper *et al.*

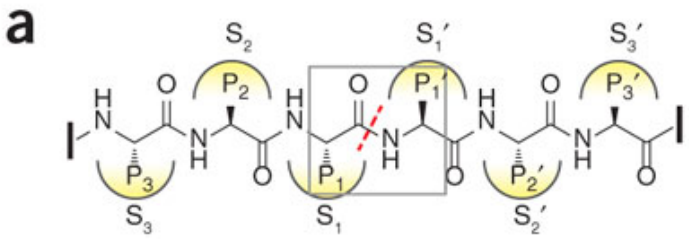
2001). These S proteases can interact with other proteins on the cell membrane, extracellular proteins or proteins on adjacent cells. It appears that type II transmembrane serine proteases are important for homeostasis and function either via hormone or growth factor activation or in the initiation of proteolytic cascades (Bugge *et al.* 2009).

Trypsin, chymotrypsin and elastase are pancreatic S proteases with a central role in digestion (Whitcomb and Lowe 2007). Based on the structure of the active site, most of the S proteases can be classified as member of the trypsin-like, chymotrypsin-like or elastase-like family of S proteases (Walker and Lynas 2001). Serine proteases are synthesized as inactive zymogens, which are mostly activated by proteolytic cleavage. The plasminogen/plasmin system, in which urokinase type and tissue type plasminogen activator (uPA and tPA) convert plasminogen into the active protease plasmin, is involved in various physiological and pathological processes, including fibrinolysis, wound healing, inflammation, oncogenesis and tumor metastasis (Miles and Parmer 2013). Blood coagulation and complement activation are two well-described examples of a cascade with several serine proteases and sequential zymogen activation (Spronk *et al.* 2003; Sim and Tsiftsoglou, 2004).

Cysteine proteases

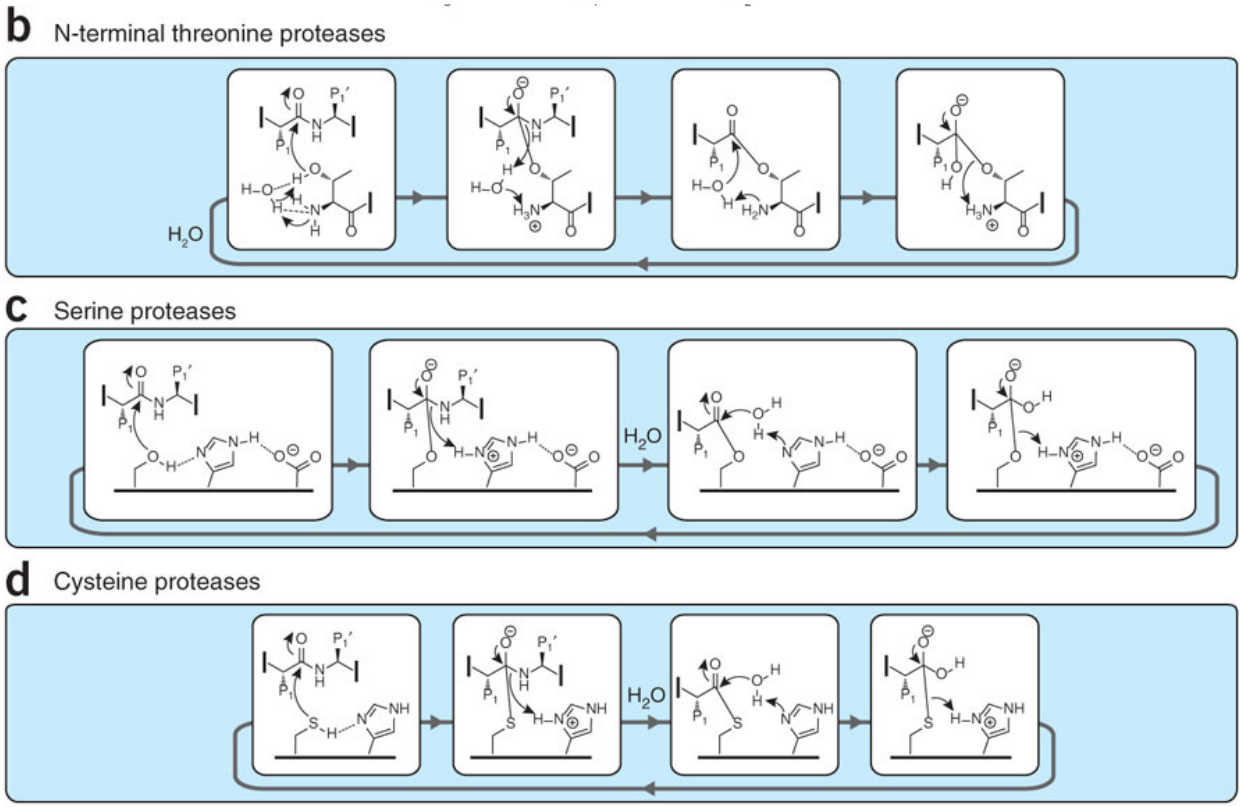
The common feature of all C proteases is that they use a nucleophilic Cys residue and a His residue as the general base for proton shuttling during substrate hydrolysis (Beynon and Bond 2001). Similar to the catalytic mechanism of S proteases, hydrolysis of a peptide bond by C proteases includes the formation of a covalent intermediate (Fig. 5). This explains why some S protease inhibitors, such as chymostatin and leupeptin, also inhibit several members of the C family (Otto and Schirmeister 1997). Cysteine proteases are implicated in a variety of biological processes. For example, lysosomal cathepsins were initially considered to be responsible for intralysosomal protein degradation but there is increasing evidence for their localization in other cellular compartments with specific roles in the immune system, bone remodelling and keratinocyte differentiation (Turk *et al.* 2012). Members of the calpain and caspase families of C proteases exert critical functions in the regulation of programmed cell death (Nakagawa and Yuan 2000; Ouyang *et al.* 2012).

Proteolysis



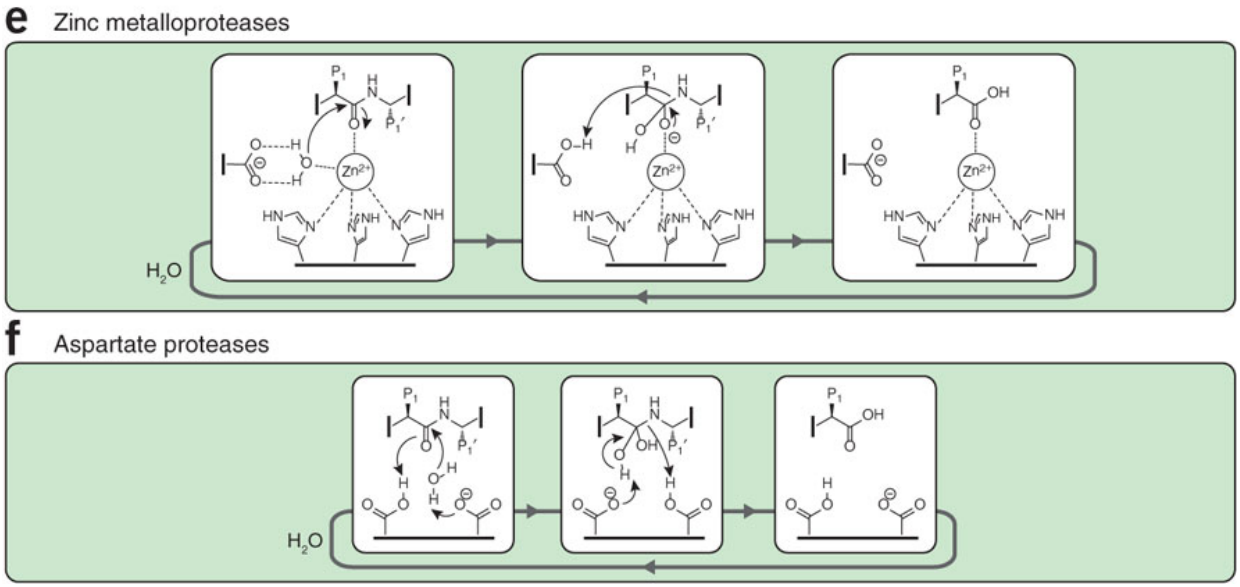
(a) Protease substrates bind through interactions of the side chain residues (P and P' residues) with the substrate pockets of the protease (S and S' pockets). The red dashed line indicates a scissile bond.

Covalent catalysis



(b-d) The architecture of the active site and mechanism of hydrolysis for N-terminal threonine (T), serine (S) and cysteine (C) proteases. Hydrolysis of a peptide bond starts with the nucleophilic attack by the catalytic side chain residue (respectively T, S or C) and the formation of an acyl-enzyme intermediate. Next, the intermediate is hydrolyzed by water with the release of the cleaved peptide and the regeneration of the active enzyme.

Non-covalent catalysis



(e,f) In the case of zinc-dependent metalloproteases (**e**) and aspartic proteases (**f**), a metal ion or carboxylic acid group activates a water molecule, leading to acid-base catalysis. There is no formation of a covalent intermediate between the enzyme and the substrate.

Figure 5. Mechanism of substrate hydrolysis by the primary families of proteases (Adapted from Deu *et al.* 2012).

Threonine proteases

Threonine proteases represent a third class of proteases in which a nucleophilic residue, in this case a Thr residue, attacks the substrate with the formation of an acyl-enzyme intermediate (Fig. 5). The proteasome, a multisubunit complex responsible for the degradation of ubiquitinated proteins, contains several protease domains with threonine protease activity (Glickman and Ciechanover 2002).

Metalloproteases

The enzymatic activity of metalloproteases depends on a metal ion, usually zinc. Three families of metalloproteases are acknowledged to modify and degrade extracellular matrix, the matrix metalloproteases (MMPs), the "A Disintegrin And Metalloprotease" family of proteases (ADAM) and the ADAM proteases with thrombospondin motifs (ADAMTS) (Shiomi *et al.* 2010). However, metalloproteases display a variety of functions, including matrix degradation, cell migration and shedding of various proteins, including cytokines and growth factors, from membrane anchored precursors (Black and White 1998).

Aspartic proteases

The activity of A proteases depends on the carboxyl group of two aspartic acid side chains and a water molecule bound to both of these carboxyl groups. There is no formation of a covalent intermediate between the enzyme and the substrate. Typically, this type of protease has a low pH optimum. This limits the function of A proteases to specific locations within organisms. Examples of A proteases are pepsin and chymosin in the stomach, cathepsin D and E in lysosomes and renin in the kidney (Tang and Wong 1987). However, their localization may not be that restricted as initially thought. The inactive form as well as enzymatically active Cathepsin D was found in the extracellular compartment and for both forms various biological functions were suggested (Benes *et al.* 2008).

More recently, the families of glutamate proteases (Fujinaga *et al.* 2004) and asparagine peptide lyases (Rawlings *et al.* 2011) have been described. Proteases of which the catalytic mechanism is not yet elucidated are classified as "unknown type of protease" (Rawlings *et al.* 2012).

1.4.3 Proteases and sperm maturation

Spermatozoa acquire motility and fertilizing capacity during their passage through the epididymis. During this period of sperm maturation, the sperm membrane undergoes extensive modifications, including adsorption of macromolecules from epididymal fluid, reorganization of molecules in the plasma membrane and modification of membrane-bound glycoproteins by enzymes such as glycosidases and proteases. Proteases present in the epididymis play a key role in the processing of sperm surface proteins that are functionally important in fertilization. For example, PH-30 is a protein located on the surface of guinea pig sperm and is implicated in the fusion of gametes (Primakoff *et al.* 1987). During sperm maturation in the epididymis, the precursor form of PH-30 is cleaved several times (Blobel *et al.* 1990). Final processing in the cauda epididymis results in the exposure of two new epitopes, of which at least one appears to be associated with sperm-oocyte fusion based on the recognition by a fusion-inhibiting monoclonal antibody (Blobel *et al.* 1990). Testase 1 (ADAM24) is a membrane-anchored protein on mouse spermatozoa of which the pro-domain is released by proteolytic processing during sperm transit of the epididymis (Zhu *et al.* 2001).

1.4.4 Proteases and sperm-cumulus interaction

Ovulated porcine oocytes are surrounded by two extracellular layers at the time of fertilization (Yanagimachi 1994). The first extracellular layer of the sperm cell comes into contact with is the cumulus oophorus, which consists of cumulus cells embedded in a viscous extracellular matrix. The cumulus matrix is composed of glycosaminoglycans, such as hyaluronan and chondroitin sulphate, which form a network by cross-linking with glycoproteins (Nakayama *et al.* 1996). Because hyaluronan is the structural backbone of the cumulus matrix, sperm hyaluronidase activity in combination with sperm motility have long been considered to be necessary for creating a path through the cumulus oophorus (Yanagimachi 1994). The detection of hyaluronidases on the sperm surface of numerous mammalian species supported this concept. However, the necessity of sperm hyaluronidase for fertilization has been questioned, especially since spermatozoa lacking hyaluronidase activity were found to pass through the cumulus matrix (Talbot *et al.* 1985; Baba *et al.* 2002; Kimura *et al.* 2009) and inhibitors of hyaluronidase effectively inhibited dispersal of cumulus cells but did not interfere with sperm penetration (Kang *et al.* 2010).

Proteases have not yet been implicated in sperm-cumulus interaction (Primakoff and Myles 2002). Nevertheless, there are several hypothetical ways by which hydrolytic enzymes could

be present during sperm-cumulus interaction and may aid in the dispersion of the cumulus matrix by hydrolyzing hyaluronan and/or polypeptides: (1) hydrolytic enzymes originating from epididymal fluid or accessory gland secretions may bind to the sperm surface during epididymal transit or ejaculation and remain bound until sperm interaction with the cumulus; (2) hydrolytic enzymes may diffuse from the acrosome to the sperm membrane during capacitation or (3) some spermatozoa may undergo acrosome reaction in the cumulus paving the way for other, acrosome-intact, spermatozoa (Tulsiani *et al.* 1998).

1.4.5 Proteases and sperm-zona pellucida interaction

After penetration of the cumulus oophorus, the sperm cell reaches the zona pellucida (ZP). In pigs, this extracellular coat of the oocyte is composed of three glycoproteins, ZPA, ZPB and ZPC, with species-specific post-translational modifications (Töpfer-Petersen 1999). The binding of a sperm cell to the ZP is a biphasic event starting with a non-specific loose attachment followed by a tight binding to carbohydrate moieties on ZP glycoproteins. This ligand-receptor binding is responsible for the species-specificity of fertilization. In the pig, sperm-binding activity has been linked with ZPB (Töpfer-Petersen 1999).

Mammalian spermatozoa have a specialized organelle, the acrosome, which is located in the anterior part of the sperm head and can be described as a bag-like structure filled with enzymes (proteases, glycohydrolases, sulfatases) (Tulsiani *et al.* 1998). Exocytosis of the acrosomal content is a prerequisite for successful fertilization. The sperm cell is triggered to undergo the acrosome reaction by contact with ZP glycoproteins. During the acrosome reaction, fusion and vesiculation of the outer acrosomal membrane and the overlying sperm membrane result in the release of acrosomal content (i.e. enzymes) at the ZP. The inner acrosomal membrane is then exposed and secondary binding ligands are able to interact with the ZP (Yanagimachi 1994).

The exploration of acrosomal proteases began with the observation that various trypsin-inhibitors markedly reduced sperm-ZP binding and sperm penetration of the ZP *in vitro* (Stambaugh and Buckley 1968; Zaneveld *et al.* 1969; Miyamoto and Chang 1973; Saling 1981; Fraser 1982). Since exocytosis of the acrosomal content was accepted to be a prerequisite for successful fertilization, putative zona proteases were believed to be localized in the acrosome. To date, various proteases have been detected in the acrosome of mammalian spermatozoa, among which the most extensively studied serine protease acrosin (described in most mammalian species), a collagenase-like protease (human, rat, cattle), a metalloprotease

(human, hamster, boar), a cathepsin D-like protease (mouse), a dipeptidyl peptidase II (guinea pig), a 75 kDa serine protease (mouse), a trypsin-like serine protease other than acrosin (pig) and plasminogen activator (human, cattle, pig) (Smokovitis *et al.* 1987; Tulsiani *et al.* 1998; Honda *et al.* 2002a). The family of serine proteases is most represented on male gametes with about fifteen sperm serine proteases identified by immunochemistry, western blot, in situ hybridization or proteomics, not only in the acrosomal content but also on acrosomal and sperm membranes (Cesari *et al.* 2010).

The serine protease acrosin (EC 3.4.2.10) and its precursor proacrosin are found specifically within the acrosome of mammalian spermatozoa (Tranter *et al.* 2000). In porcine spermatozoa, proacrosin is purified as a mixture of 55 and 53 kDa proteins with isoelectric points around 8 (Polakoski and McRorie 1973; Polakoski *et al.* 1973). These precursor proteins are converted into enzymatically active forms by auto-activation, which is mediated by sperm contact with ZP glycoproteins (Eberspaecher *et al.* 1991; Leggio *et al.* 1994). Most serine proteases are activated by a single cleavage of the precursor at either the N or C terminus of the protein. In contrast, conversion of proacrosin to acrosin involves processing at both ends of the molecule (Baba *et al.* 1989). The primary sequence of proacrosin has been characterized in several species, including boar, bull, rat, guinea pig, mouse and human (Tranter *et al.* 2000). Furthermore, the crystal structures of pig and ram acrosins have been solved in complex with a protease inhibitor, p-aminobenzamidine. From the three-dimensional structure of acrosin, two separate effector sites could be distinguished (Tranter *et al.* 2000). The first effector site of acrosin is a trypsin-like active site, which is associated with the protease activity of acrosin. Using homologous native ZP, boar acrosin was shown to cleave ZP glycoproteins (Dunbar *et al.* 1985; Urch and Patel 1991). The second effector site of acrosin is located adjacent to the trypsin-like site and displays lectin-like carbohydrate-binding properties (Tranter *et al.* 2000). It has been shown that acrosin and acrosin recombinant proteins are able to bind to solubilized ZP proteins as well as to the native ZP (Richardson and O'Rand 1996; Crosby and Barros 1999; Howes *et al.* 2001). Furthermore, protease inhibitors such as pancreatic trypsin inhibitor and T-α-Tosyl-L-Lysinyl-Chloro-methylketone (TLCK), which are able to inhibit acrosin, were shown to inhibit ZP binding and subsequent penetration (Saling 1981; Müller-Esterl *et al.* 1983).

Despite these lines of evidence for the involvement of acrosin in sperm-ZP interaction, studies with knock-out mice showed that a disruptive mutation in the gene encoding for acrosin does not alter sperm-ZP binding nor disables fertilization (Baba *et al.* 1994; Adham *et al.* 1997).

Nevertheless, mouse spermatozoa lacking acrosin show a delay in penetration of the ZP. Similar results were obtained in studies on deletion mutants for other sperm serine proteases: single knock-out mice are subfertile but do not completely loose their fertilizing capacity. Researchers have proposed several explanations for these findings. In general, most credence is given to the possible redundancy of serine proteases which would resemble a evolutionary mechanism to ensure fertilization via alternative pathways in case a certain protease is disabled (Honda *et al.* 2002a; Cesari *et al.* 2010). However, there is also the possibility that penetration of the ZP does not depend on acrosin or other acrosomal proteases (Bedford 1998). In the review paper of Bedford (1998), the author enumerates several reasons for questioning the zona lysis theory in mammalian fertilization, such as (1) there is no evident dissociation of the ZP after local release of acrosomal enzymes; (2) the inner acrosomal membrane (IAM) is exposed as acrosome-reacted spermatozoa penetrate the ZP but there are no indications for proteolytic action on the leading edge of the IAM; (3) the relative protease insensitivity and elastic character of the ZP and (4) the fact that embryonic development depends on the presence and integrity of the ZP in the first days after fertilization (Bedford 1998). According to Bedford (1998), eutharian spermatozoa have evolved towards penetration of the ZP by physical means (i.e. cutting thrust).
Nevertheless, Ferrer *et al.* (2012) very recently demonstrated that the IAM of bull spermatozoa exhibits proteolytic activity. Moreover, these authors revealed the presence of proacrosin/acrosin and matrix metalloprotease 2 (MMP2) on the IAM. These findings may reinvigorate the discussion on whether or not ZP penetration depends on proteolysis, compared to a rather modest, supportive, role for proteases proposed in previous years.

The proteaseome, a multi-subunit protease with specificity for ubiquitinated protein substrates, has been implicated in sperm penetration of the ZP in several mammalian species, including human, mouse and pig (Morales *et al.* 2003; Sun *et al.* 2004; Sutovsky *et al.* 2004). The ubiquitin-proteasome pathway is generally known as a pathway for intracellular protein degradation in eukaryotic cells (Glickman and Ciechanover 2002). Protein modification by ubiquitin occurs by attachment of an ubiquitin residue to a lysine residue within the target protein. This ubiquitination is regulated by ubiquitin activating and conjugating enzymes. The attachment of one ubiquitin molecule to a protein can be followed by binding of additional ubiquitin molecules, because ubiquitin itself carries several lysine residues. “Poly-ubiquitination” marks proteins for degradation and facilitates docking of these proteins to the 26S proteasome complex. The 26S proteasome is a hollow cylindrical structure, which can be

imagined as a sort of paper (protein) shredder. On one side, the ubiquitinated proteins enter the proteasome, in the inner part proteins are dissected and on the opposite side small peptides are released into the cytoplasm. Before dissection of the proteins, ubiquitin molecules are removed for reuse. The 20S complex of the proteasome has several protease subunits with threonine protease activity (Glickman and Ciechanover 2002). Surprisingly, ubiquitin-associated protein degradation not only occurs within eukaryotic cells, but cell surface proteins can be ubiquitinated and processed by secreted proteasomes as well (Sawada *et al.* 2002a). The link between the ubiquitin-proteasome pathway and fertilization was first established in ascidians. It was shown that proteasomes, ubiquitin and conjugating enzymes were released during acrosomal exocytosis of sperm from the ascidian *Halocynthia roretzi*. Subsequent ubiquitination of an ascidian homologue of mammalian ZP proteins, Vesp70, preceded sperm penetration of the vitelline envelope (Sawada *et al.* 2002b). More recently, it was shown that proteasomal inhibitors and anti-proteasome antibodies were able to prevent sperm penetration of the ZP during porcine fertilization (Sutovsky *et al.* 2003; Sutovsky *et al.* 2004). Removal of the ZP abolished the effect of proteasomal inhibitors or antibodies on fertilization. Furthermore, proteasomal subunits were found in the boar sperm acrosome. These findings led to a model of mammalian ZP penetration similar to the ubiquitin-proteasome dependent penetration of the vitelline envelope in ascidians (Sutovsky *et al.* 2004). However, in contrast to ascidians, ZP proteins of mammalian oocytes appear to be ubiquitinated before fertilization (Sutovsky *et al.* 2004).

1.4.6 Proteases and sperm-oocyte interaction

In contrast to the well-characterized ligand-receptor binding of spermatozoa to the ZP, the molecular basis by which acrosome-reacted spermatozoa bind to the oocyte and initiate gamete fusion is still unclear. There are various molecules assumed to play a role in sperm-oocyte interaction, but their contribution to gamete fusion has not yet been clearly defined.

Several researchers have proposed a role for sperm metalloproteases. Díaz-Pérez and Meizel (1992) reported that an ‘early’ metalloprotease activity during the acrosome reaction is important for gamete fusion of human spermatozoa with zona-free hamster oocytes (Díaz‐Pérez and Meizel 1992). In sea urchins, inhibition of a ‘late’ metalloprotease activity, acting after gamete binding, completely blocked gamete fusion (Roe *et al.* 1988). Similarly, Correa et al.(2000) describe the involvement of a metalloprotease in membrane fusion after sperm-oocyte binding in mice.

Much attention has been paid to the A Disintegrin and Metalloprotease (ADAM) family of metalloproteases because they contain a disintegrin domain, which is a potential adhesion partner for integrins on the oocyte membrane (Bigler *et al.* 1997; Evans 2001; Primakoff and Myles 2002). Several members of the ADAM family are present on spermatozoa of at least one mammalian species, such as ADAM1 (fertilin α), ADAM2, (fertilin β), ADAM3 (cyritestin), ADAM5, ADAM16 and ADAM19 (Evans 2001). From these ADAMs, sperm proteins fertilin, a hetero-dimeric complex of ADAM 1 and ADAM 2, and cyritestin (ADAM 3) have been studied most extensively. Initially, fertilin was isolated at the sperm surface of guinea-pig spermatozoa (Primakoff *et al.* 1987). Expression of fertilin in porcine spermatozoa has recently been described (Fabrega *et al.* 2011). Both fertilin and cyritestin are associated with sperm adhesion to the oocyte membrane. Fertilization rate is strongly decreased by the presence of monoclonal antibodies directed to the binding site of these sperm proteins or by peptides mimicking the disintegrin domain (Yuan *et al.* 1997). Both fertilin β and cyritestin are described to interact with integrin $\alpha_6\beta_1$ (Almeida *et al.* 1995; Chen and Sampson 1999), although this was not confirmed in other studies (Takahashi *et al.* 2001; Zhu and Evans 2002). Spermatozoa lacking fertilin β or cyritestin are deficient in adhesion to the oocyte membrane, nevertheless, gamete fusion is still possible (Nishimura *et al.* 2001). Surprisingly, deletion of these sperm proteins also interferes with sperm binding to the zona pellucida. The interpretation of these results is complicated because deletion of either fertilin β or cyritestin resulted in a reduction of both sperm proteins and even others. The finding that fertilization is not blocked in oocytes lacking integrin α6β1 (Miller *et al.* 2000) further illustrates that gamete fusion involves much more than the interaction of fertilin or cyritestin on spermatozoa and $\alpha_6\beta_1$ on oocytes (Evans 2001; Rubinstein *et al.* 2006). There are several other candidate ADAMs and integrins which may play a role in gamete adhesion and fusion and may be the subject of further research in this area (Evans 2001; Tomczuk *et al.* 2003).

Gamete fusion occurs first at the level of the sperm equatorial segment. The posterior region of the sperm head and the sperm tail fuse with the oocyte membrane, whereas the anterior region of the head is incorporated by the oocyte in a phagocytotic manner (Yanagimachi 1994). In the past decades, two compatible models have been described to explain the sperm-oocyte signaling prior to oscillatory changes in the intracellular [Ca^{2+}] in the oocyte, the resumption of meiosis and the exocytosis of cortical granules (Evans and Kopf 1998; Whitaker 2006; Wakai *et al.* 2011). The first model includes membrane receptor-mediated signal transduction via kinases and phospholipase C -γ (PLC-γ), activation of the

phosphoinositide signaling pathway and production of the second messenger 1,4,5-inositol-triphosphate (IP_3) (Lee *et al.* 2006; Whitaker 2006). The second model attributes oocyte activation to sperm components that are introduced in the oocyte during or after gamete fusion. The 'sperm-borne oocyte-activating factor' (SOAF) represents a multifactorial entity which comprises at least two components, a protein-nature component SOAF-I and a sub-membranous component SOAF-II (Perry *et al.* 2000). The combined effect of both components is important for the induction of [Ca^{2+}] oscillations in the oocyte. The protein part of SOAF (SOAF-I) is well conserved across mammalian species and is also referred to as the 'sperm factor' (SF) (Wu *et al.* 1997). This factor has been identified as a member of the PLC family, a sperm-specific isoform named PLCζ (Saunders *et al.* 2002; Swann *et al.* 2004). Interestingly, Perry *et al.* (2000) found that release of SOAF-I (SF, PLCζ) is sensitive to protease inhibitors. This suggests that at least one of the components involved in oocyte activation needs to be processed by a protease (Perry *et al.* 2000). Recently, Kurokawa *et al.* (2007) showed that after cleavage of PLCζ, the fragments remain associated and form functional complexes during fertilization. More specifically, these researchers were able to induce intracellular [Ca^{2+}] oscillations in mouse oocytes when mimicking the cleavage products by expression of cRNA encoding residues 1-361 and 362-647 of mouse PLCζ.

The intracellular [Ca^{2+}] oscillations at fertilization initiate a cascade of events designed to protect the oocyte from polyspermic fertilization. Exocytosis of cortical granules into the perivitelline space leads to modifications of the zona, cleavage of ZP glycoproteins and reorganization of disulfide bonds.

In mouse, cattle and pig, it has been shown that ZPB (ZP2) is cleaved by a protease released from the cortical granules (Iwamoto *et al.* 1999). In mouse, the protease involved has been identified as the oocyte-specific metalloprotease ovastacin (Burkart *et al.* 2012). By use of specific antiserum, Burkart *et al.* (2012) showed the presence of ovastacin in cortical granules before but not after fertilization. They also demonstrated that ZP2 was a direct substrate of this metalloprotease. In sea urchins, a protease is released by the female gamete within seconds after sperm penetration. This protease, which is inhibited by soybean trypsin inhibitor, alters the vitelline layer and prevents further sperm entry, similar to the process of zona hardening described in mammalian oocytes (Vacquier *et al.* 1973; Carroll Jr and Epel 1975). Furthermore, fertilization of sea urchin oocytes in the presence of soybean trypsin inhibitor results in higher polyspermy rates (Vacquier *et al.* 1972; Vacquier *et al.* 1973). Serine protease activity derived from the cortical granules has been shown to contribute to the oocyte's defense mechanism against polyspermy in the hamster and in the mouse (Cherr *et al.*

1988; Hoodbhoy and Talbot 1994). Similarly, a role has been proposed for serine protease activity in the regulation of sperm penetration in bovine oocytes, mediated by the release of tissue-type plasminogen activator (tPA) from the oocyte and activation of plasminogen into the serine protease plasmin (Rekkas *et al.* 2002).

Recent studies have provided new insights on how the plasminogen-plasmin system may contribute to the regulation of sperm penetration during porcine fertilization (Coy *et al.* 2012; Mondéjar *et al.* 2012). As demonstrated by Coy *et al.* (2012), the porcine oocyte releases tPA and urokinase-type plasminogen activator (uPA) upon contact with spermatozoa. These plasminogen activators convert plasminogen into plasmin, which protease activity can break the tight binding of spermatozoa to the ZP and lead to detachment of spermatozoa previously attached to the ZP. In the study of Coy *et al.* (2012), plasminogen activators were dectected in the peri-oolemma content whereas cortical granules were un-reactive to specific antibodies against tPA and uPA. The proposed mechanism by which the plasminogen-plasmin system removes supernumerary spermatozoa is supported by the detection of plasminogen in porcine oviductal fluid (the precursor of plasmin is thus present at the site of gamete interaction *in vivo*) and results from *in vitro* experiments (Mondéjar *et al.* 2012). *In vitro*, a decrease in sperm-ZP binding and sperm penetration was observed when plasminogen was supplemented to the IVF medium in concentrations similar to those detected in oviductal fluid (Mondéjar *et al.* 2012).

An overview of the proteases associated with fertilization is given in table 1.

Table 1. Proteases involved in fertilization

Fertilization step	Protease	Type of protease	Species	Reference
Sperm motility	A serine protease with lys and arg ester bond specificity (compatible with plasmin)	Serine	Human, Cattle, Rabbit, Rat	de Lamirande and Gagnon (1986)
	Plasmin	Serine	Cattle	Smokovitis *et al.* (1987)
Sperm capacitation	Urokinase- Plasminogen activator (u-PA)/Plasmin	Serine	Rhesus monkey	Liu (2007)
Sperm-ZP binding	110 kDa collagenase-like peptidase	Metallo	Human, Rat, Cattle	Koren and Milkovic (1973)
and zona lysis	Trypsin-like activity	Serine	Mouse	Saling (1981)
	Acrosin	Serine	Mammals	Fraser (1982); Tranter *et al.* (2000)
	P-aminobenzamidine-sensitive protease other than acrosin	Serine	Mouse	Yamagata *et al.* (1998a)
	TESP5/testisin	Serine	Mouse	Honda *et al.* (2002b)
	Urokinase-plasminogen activatior (u-PA)/Plasmin	Serine	Mouse	Huarte *et al.* (1993)
	Testase 1 (ADAM24)	Metallo	Mouse	Zhu *et al.* (2001)
	BSP66	Serine	Cattle	Cesari *et al.* (2005)
Acrosome reaction	Trypsin and chymotrypsin activity	Serine	Cattle	Deppe *et al.* (2008)
	Trypsin-like activity	Serine	Human	Llanos *et al.* (1993)
	Chymotrypsin-like activity	Serine	Human	Morales *et al.* (1994)
	Acrosin	Serine	Human	Tesarik *et al.* (1990)

Fertilization step	Protease	Type of protease	Species	Reference
	Acrosin	Serine	Mouse	Yamagata *et al.* (1998b)
	Proprotein convertase subtilisin/kexin type 4 (PCSK4)	Serine	Mouse	Gyamera-Acheampong and Mbikay (2009)
Sperm-oocyte	Fertilin (ADAM 1+ADAM 2)	Metallo	Guinea pig	Primakoff *et al.* (1987)
binding and fusion	Cyritestin (ADAM3)	Metallo	Mouse	Yuan *et al.* (1997)
	TIMP3 sensitive, Zn-dependent metalloprotease	Metallo	Mouse	Correa *et al.* (2000)
	Metalloprotease activity	Metallo	Human Hamster	Díaz‐Pérez and Meizel (1992)
	Metalloprotease activity	Metallo	Sea urchin	Roe *et al.* (1988)
Zona reaction	Protease	Serine	Sea urchin	Vacquier *et al.* (1972); Vacquier *et al.* (1973)
	Protease activity	-	Human, Cattle	Iwamoto *et al.* (1999)
	Protease	-	Mouse	Bleil *et al.* (1981); Moller and Wassarman (1989)
	Tissue-type Plasminogen Activator	Serine	Rat	Zhang *et al.* (1992)
	Tissue-type Plasminogen Activator and Urokinase-type Plasminogen Activator	Serine	Porcine	Coy *et al.* (2012)
	Ovastacin	Metallo	Mouse	Burkart *et al.* (2012)
			Pig	Hatanaka *et al.* (1992)

1.5 Inhibition of proteases

In vivo, protease activity is strictly regulated at several levels because uncontrolled proteolysis could lead to severe cell and tissue damage. Many proteases are formed as inactive precursors that are activated under specific conditions. Examples in reproduction are the plasminogen-plasminogen activator system in spermatogenesis and the activation of proacrosin to acrosin during fertilization (Eberspaecher *et al.* 1991; Le Magueresse-Battistoni 2007). Once activated, proteases are further regulated by the non-specific inhibitor alpha$_2$-macroglobulin in the plasma or by more specific protease inhibitors in the cellular and extracellular environment. Alpha$_2$-macroglobulin is a plasma glycoprotein of 725 kDa. It inhibits proteases of all classes by entrapping the protease and masking the catalytic site. Bound proteases may retain activity towards small substrates but activity against large substrates is greatly reduced. After reaction with a protease, the alpha$_2$-macroglobulin-protease complex is rapidly cleared from the circulation (Barrett and Starkey 1973; Feldman *et al.* 1985; Borth 1992). Most endogenous protease inhibitors exhibit specificity towards a single class of proteases; cystatins act on cysteine proteases, serpins are directed to serine proteases and tissue inhibitors of metalloproteases (TIMPs) are physiological inhibitors of metalloproteases (Beynon and Bond 2001; Mason and Joyce 2011).

Disturbance of the tight regulation of protease activity can lead to pathological processes, such as arthritis, fibrosis, cardiovascular disease and cancer (Walker and Lynas 2001; Shiomi *et al.* 2010). In this respect, much effort has been undertaken to study the mechanisms of *in vivo* protease inhibition and the translation of this knowledge into the development of synthetic protease inhibitors as potential therapeutic agent (Baker *et al.* 2002; Zhong and Groutas 2004). In general, inhibitors are designed to fit the protease active site and to interfere with the catalytic mechanism of the protease. There is a wide range of synthetic protease inhibitors commercially available. These inhibitors differ in their specificity (action towards proteases from different classes versus action towards proteases from one class or a single protease), mode of action (reversible, irreversible), molecular weight (high molecular weight versus low molecular weight), stability in aqueous solution, cell permeability and cell toxicity. Table 2 gives an overview of commercially available protease inhibitors that have been used in previous *in vivo* and *in vitro* fertilization studies.

Table 2. Commercially available protease inhibitors used in *in vivo* or *in vitro* fertilization studies* (list not exhaustive)

Name	Class	Specificity**	Mode of Action**	Molecular Weight (g/mol)**
AEBSF (Pefabloc)	Serine	All serine proteases	Irreversible	Low (239.5)
p-aminonenzamidine	Serine	Trypsin-like serine proteases	Reversible	Low (208.9)
Antithrombin III	Serine/ Cysteine	Trypsin-like serine proteases	Reversible	High (67000)
Leupeptin	Serine / Cysteine	Trypsin-like serine proteases and some cysteine proteases	Reversible	Low (426.6)
PMSF	Serine	All serine proteases	Irreversible	Low (174.2)
STI	Serine	Trypsin-like serine proteases and to a lesser extent chymotrypsin and plasmin	Reversible	High (20100)
TLCK	Serine	Trypsin-like serine proteases	Irreversible	Low (332.5)
TPCK	Serine	Chymotrypsin-like serine proteases	Irreversible	Low (351.5)
E-64	Cysteine	All cysteine proteases	Irreversible	Low (357.4)
Pepstatin A	Aspartic	Some aspartic proteases, including cathepsin D, pepsin and renin	Reversible	Low (685.9)
Bestatin	Metallo	Aminopeptidases	Reversible	Low (308.4)
EDTA	Metallo	All metalloproteases	Reversible	Low (372.2)
1,10-phenanthroline	Metallo	All metalloproteases	Reversible	Low (198.2)
Phosporamidon	Metallo	Bacterial metallo-endopeptidases and few of mammalian origin	Reversible	Low (543.6)
TIMP 1-4	Metallo	Matrix metalloproteases	Reversible	High (20000-30000)

* Zaneveld *et al.* 1970; Miyamoto and Chang 1973; Saling *et al.* 1981; De Lamirande and Gagnon 1986; Flaherty and Swann 1993; Yamagata *et al.* 1998; Correa *et al.* 2000; Tranter *et al.* 2000; Deppe *et al.* 2008.

**Beynon and Bond 2001; Sigma-Aldrich Co.

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CHAPTER 2

AIMS OF THE STUDY

Up to now, large-scale production of porcine embryos is compromised by a high incidence of polyspermic fertilization. The high polyspermy rate during porcine IVF has been associated with failures in the cortical reaction leading to an incomplete “zona block” and with the high number of spermatozoa near the oocyte when compared to in vivo conditions. Proteases have been recognized to play a role in different steps of mammalian fertilization. Previous research has identified about fifteen sperm serine proteases in the acrosomal content and on acrosomal and sperm membranes, mainly in human and mouse. The serine protease activity of spermatozoa was first associated with zona lysis, and more recently with other fertilization events such as sperm capacitation, dispersion of acrosomal content and activation of oocyte transmembrane receptors. Furthermore, there is evidence for the involvement of metalloprotease activity in gamete fusion and the subsequent cortical reaction. Accordingly, these proteases represent interesting candidates to explore which step(s) during porcine IVF depend on the action of proteases and how the fertilization process is affected by protease inhibition. Specific protease inhibitors might be useful as regulators of sperm penetration in porcine IVF, lessening the problem of polyspermic fertilization and increasing the efficiency of embryo production.

Among the different approaches that can be used to investigate the involvement of proteases during fertilization, the use of inhibitors has the advantage to distinguish active proteases from inactive proteins, in contrast to methods such as immunochemistry or proteomics which allow to demonstrate the localization of a protease but not its functionality. There are various biological protease inhibitors known to be of utmost importance in the strict regulation of protease activity in vivo. Moreover, we have recently identified that alpha$_2$-macroglobulin, a plasma protein and non-specific protease inhibitor, hampers cumulus expansion during IVM of porcine oocytes in medium with 10% serum. Cumulus cells are known to secrete several serine proteases and metalloproteases, which have been proposed to function in oocyte maturation and ovulation. Further investigation of the mechanism underlying the inhibitory effect of alpha$_2$-macroglobulin on cumulus expansion might improve our understanding of how the action of proteases is required to prepare the oocyte for successful fertilization.

The general aim of the present thesis was to gain more insight in the role of proteases during *in vitro* maturation and *in vitro* fertilization of porcine cumulus-oocyte-complexes.

The specific scientific aims were:

1. To assess the effect of serine protease inhibitors on sperm viability, motility and the capability to undergo the acrosome reaction (**Chapter 3**)
2. To assess the effect of serine protease inhibitors on total fertilization, polyspermic fertilization and sperm penetration rate during porcine IVF and to evaluate whether serine protease inhibitors can be used as a tool to reduce polyspermic fertilization (**Chapter 3**)
3. To explore the effect of serine protease inhibitors during sequential steps of porcine fertilization (**Chapter 4**)
4. To assess the effect of metalloprotease inhibitors on sperm viability, motility and the capability to undergo the acrosome reaction (**Chapter 5**)
5. To evaluate the effect of metalloprotease inhibitors on total fertilization, polyspermic fertilization and sperm penetration rate during porcine IVF and to evaluate whether metalloprotease inhibitors can be used as a tool to reduce polyspermic fertilization **(Chapter 5)**
6. To explore the effect of metalloprotease inhibitors during sequential steps of porcine fertilization (**Chapter 5**)
7. To investigate two hypotheses by which excess of $alpha_2$-macroglobulin might hamper cumulus expansion during porcine IVM: 1) the binding of EGF to A2M followed by decreased availability of EGF, and 2) the inhibition of matrix metalloproteases (**Chapter 6**).

CHAPTER 3

A CRITICAL ASSESSMENT OF THE EFFECT OF SERINE PROTEASE INHIBITORS ON PORCINE FERTILIZATION AND QUALITY PARAMETERS OF PORCINE SPERMATOZOA *IN VITRO*

Modified from

A CRITICAL ASSESSMENT OF THE EFFECT OF SERINE PROTEASE INHIBITORS ON PORCINE FERTILIZATION AND QUALITY PARAMETERS OF PORCINE SPERMATOZOA *IN VITRO*

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Abstract

Proteases play an important role during mammalian fertilization. Their function is frequently investigated using specific inhibitors. We analyzed four serine protease inhibitors [4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF), soybean trypsin inhibitor from glycine max (STI), N_{α}-tosyl-L-lysine-chloromethyl ketone hydrochloride (TLCK) and N_{p}-tosyl-L phenylalanine-chloromethyl ketone (TPCK)] for their *in vitro* effect on fertilization and sperm quality in pigs. Inhibitor concentrations were chosen based on the reduction of fertilization rate during preliminary dose-response experiments with cryopreserved epididymal spermatozoa. The inhibitor effects on *in vitro* fertilization (IVF) and sperm parameters (membrane and acrosomal integrity, motility and mitochondrial membrane potential - mMP) were evaluated using diluted fresh semen. AEBSF (100 μM), TLCK (100 μM) and TPCK (100 μM) decreased total fertilization and polyspermy rates by at least 50%. STI (5 μM) lowered total fertilization rates but not the level of polyspermy. AEBSF and TPCK reduced fertilization parameters to a similar degree using cryopreserved epididymal spermatozoa (dose-response experiment) or diluted fresh semen. Inhibition by STI was more pronounced using cryopreserved epididymal spermatozoa, whereas TLCK inhibited IVF only with diluted fresh semen. AEBSF and STI had no effect on sperm parameters, and TLCK significantly reduced motility. TPCK diminished mMP and motility and affected membrane and acrosomal integrity in a negative way**.** In summary, serine protease inhibitors differed in the way they reduce the fertilization rate. These results emphasize the necessity of inhibitor testing before they can be applied in fertilization studies. AEBSF and STI can be used in the future IVF studies without compromising sperm quality.

Introduction

Serine proteases play a key role in many biological pathways as well as in the modulation of cellular and extracellular proteins (Beynon and Bond 2001). Their involvement in fertilization has been acknowledged in mammalian as well as in non-mammalian species (Sawada *et al.* 1984; Yanagimachi 1994). Based on early inhibitor studies reporting a decrease in fertilization rate by serine protease inhibition (Stambaugh and Buckley 1968; Zaneveld *et al.* 1969; Miyamoto and Chang 1973; Saling 1981), sperm serine protease activity has been mainly associated with sperm binding to and penetration of the zona pellucida (ZP) (Saling 1981; Brown and Cheng 1985; Jones 1987; Urch and Patel 1991). Since then, the sperm specific serine protease acrosin has been described in several mammalian species and serine protease activity has also been associated with other fertilization events such as sperm capacitation, dispersion of acrosomal content and activation of oocyte transmembrane receptors (Yamagata *et al.* 1998; Smith *et al.* 2000; Liu 2007). Various serine proteases have been detected in the acrosome and on the sperm and acrosomal membranes by using techniques such as immunochemistry, western blot, in situ hybridization and proteomics (for review see Cesari *et al.* (2010)). Still, researchers have not been able to establish a direct link between a single serine protease and a specific physiological role in fertilization.

The research on the role of serine proteases during fertilization has been carried out mainly in the mouse (Baba *et al.* 1994; Adham *et al.* 1997; Yamagata *et al.* 1998; Jin *et al.* 2011). However, the various characteristics of the sperm proteases described so far point out that protease systems substantially differ between mouse and other animals (Honda *et al.* 2002). It is therefore not appropriate to extrapolate results from mouse to other mammalian species. In the pig, the sperm-specific serine protease acrosin and an acrosin-like protease have been described (Polakoski and McRorie 1973; Polakoski *et al.* 1973; Polakoski and Parrish 1977; Jones and Brown 1987; Baba *et al.* 1989; Akama *et al.* 1994; Tranter *et al.* 2000; Puigmule *et al.* 2011). The proteasome, a multi-subunit protease with including trypsin- and chymotrypsin-like serine protease activity, has been implicated in sperm penetration of the ZP (Sutovsky *et al.* 2004; Yi *et al.* 2007). Nevertheless, a general view on how trypsin- and chymotrypsin-like serine proteases contribute to the different steps of porcine fertilization is still lacking.

Among the different approaches that can be used in fertilization studies, protease inhibitors have the advantage that they can be used to distinguish active proteases from inactive zymogens, in contrast to methods such as immunochemistry or proteomics which allow only

to demonstrate the localization of a protease but not its functionality. In general, inhibitors are designed to bind to the active site of the protease and to interfere with the catalytic mechanism of the protease. In this study, we investigated the effect of inhibitors of trypsin-like and chymotrypsin-like serine proteases on total fertilization and polyspermy rate during porcine IVF in relation to their effect on four important sperm quality parameters: sperm membrane integrity, motility, mitochondrial membrane potential and acrosomal integrity. Four inhibitors were used: 4-(2-Aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF), Soybean Trypsin Inhibitor from Glycine Max (STI), N_{α}-Tosyl-L-lysine-chloromethyl ketone hydrochloride (TLCK) and N_{p}-Tosyl-L phenylalanine-chloromethyl ketone (TPCK). AEBSF is a broad spectrum serine protease inhibitor and irreversibly inhibits proteases belonging to the trypsin-, chymotrypsin- and elastin-like serine protease family. STI and TLCK inhibit trypsin-like serine proteases, respectively in a reversible and irreversible manner (Kunitz 1947). TPCK is used to inhibit proteases of the chymotrypsin-like family of serine proteases and inhibits these proteases in an irreversible manner (Beynon and Bond 2001). AEBSF, TLCK and TPCK are of low molecular weight, whereas STI is a high molecular weight inhibitor (20.1 kDa).

Results show that serine protease inhibitors differ in the way they reduce fertilization rates. AEBSF and STI inhibited fertilization rate without compromising sperm quality parameters and were found suitable for further research on the role of serine proteases during porcine fertilization.

Materials and methods

Media

All chemicals used in this study were purchased from Sigma-Aldrich (Bornem, Belgium), unless otherwise stated. The basic medium used for the collection and washing of cumulus oocyte complexes (COCs) was a modified HEPES-buffered Tyrode balanced salt solution (HEPES-TM) with 10 µg/ml gentamycin sulfate, 10 mM HEPES and 3 mg/ml BSA. Oocyte maturation medium consisted of BSA-free 'North Carolina State University' 23 (NCSU23) (Petters and Wells 1993) supplemented with 0.57 mM cysteine, 10 ng/ml epidermal growth factor, 10 IU/ml equine Chorionic Gonadotropin (eCG) (Folligon®, Intervet, The Netherlands), 10 IU/ml human Chorionic Gonadotropin (hCG) (Chorulon®, Intervet, The Netherlands) and 10% porcine follicular fluid. The basic medium for IVF was Tyrode's albumin lactate pyruvate medium (TALP medium)(Rath *et al.* 1999) supplemented with 0.3%

BSA (FERT-TALP). Presumed zygotes were washed in HEPES-buffered TALP medium (HEPES-TALP), i.e. TALP medium with 25 mM HEPES. The embryo culture medium was NCSU23 with 0.4% BSA. The pH of oocyte maturation medium, FERT-TALP, HEPES-TALP and embryo culture medium were respectively 7.4, 7.5, 7.4 and 7.4. Prior to IVF and fluorescent staining, boar spermatozoa were washed (390 X g, 3 min) in Androhep extender (pH: 6.7) (Minitüb®, Tiefenbach, Germany).

Protease inhibitors

All inhibitors were used at different concentrations within the range of effective concentrations reported by the manufacturer (Sigma-Aldrich, Bornem, Belgium), and AEBSF and STI were also used in lower concentrations. More specifically, the tested concentrations were 40, 100 and 250 µM AEBSF; 1, 5 and 25 µM STI; 40 and 100 µM TLCK; 40 and 100 µM TPCK. Inhibitors STI (T6522) and TLCK (90182) were stored desiccated. AEBSF (A8456) was dissolved in deionized water (stock solution of 10 mM) and TPCK (T4376) in dimethyl sulfoxide (DMSO) (stock solution of 20 mM). Protease inhibitor dilutions were prepared in HEPES-TM with 10 µg/ml gentamycin sulfate, 10 mM HEPES and 3 mg/ml BSA. For the sperm experiments, all dilution steps were performed with HEPES-TM medium.For IVF experiments, the preparation of inhibitor working solution included a final 1:9 dilution step in fertilization medium. From this inhibitor dilution in fertilization medium, 10 µl was added to the fertilization droplets (90 µl) approximately 15 min before matured COCS were assigned to the different treatments. Inhibitor AEBSF was stored in deionized water. To adjust the concentration of medium compounds, a 1:1 dilution was made of stock solution in deionized water and medium with twofold concentration of medium components. The possible negative effect of 0.5% DMSO in the fertilization medium, as solvent of inhibitor TPCK, was excluded in preliminary experiments.

Effects of serine protease inhibitors on fertilization rates after IVF

Oocytes were derived from porcine ovaries (Piétrain (boar line) x commercial hybrid (sow line)) collected at a local slaughterhouse and prepared following the protocol of Bijttebier *et al.* (2008). *In vitro* maturation and *in vitro* fertilization were performed as described by Beek *et al.* (2012) with few modifications in sperm preparation for IVF. Briefly, groups of 100 immature cumulus-oocyte-complexes (COCs) were cultured for 22 h in 500 µl maturation medium, followed by 22 h in hormone-free maturation medium (39°C, 5% CO_2). Prior to IVF, protease inhibitor dilutions were prepared in concentrated stocks and added to the

droplets of fertilization medium approximately 15 min before matured COCS were assigned to the different treatments.

In preliminary dose-response IVF experiments, different concentrations of protease inhibitors were tested: 0, 40, 100 or 250 µM AEBSF; 0, 1, 5 or 25 µM STI; 0, 40 or 100 µM TLCK; 0, 40 or 100 µM TPCK (total n=1657 oocytes). For dose-response experiments, cryopreserved epididymal spermatozoa were used from one landrace boar with known fertility in IVF and cryopreserved according to the protocol of Rath and Niemann (1997). Using cryopreserved epididymal spermatozoa, variability in IVF outcome under control conditions is minimal compared to the use of cryopreserved ejaculated or fresh diluted spermatozoa (Rath and Niemann 1997). After thawing in a water bath at 38°C for 60 sec, epididymal spermatozoa were washed by centrifugation (3 min at 390 X g) in 9.5 ml of Androhep. The sperm pellet was resuspended in fertilization medium and diluted to a concentration of $1.25x10^6$ spermatozoa/ml. Post-thaw sperm motility was evaluated by computer-assisted sperm analysis (CASA) and only samples with satisfactory motility (>70% of total motility) were used for IVF experiments. The final concentration during gamete co-incubation was $1.25x10^5$ spermatozoa/ml. After a co-incubation period of 6 h, the presumed zygotes were vortexed for 3 min in 2.5 ml HEPES-TALP to remove loosely bound spermatozoa, washed three times in culture medium and cultured for 18 h in a modular incubator chamber (39°C, 5% CO_2).

Next, to evaluate the effect of inhibitors on porcine IVF and sperm quality parameters using spermatozoa from the same boar, protease inhibitors (100 µM AEBSF, 5 µM STI, 100 µM TLCK or 100 µM TPCK) were tested using diluted fresh semen in our IVF system (total n=840 oocytes, 3 boars). For IVF with diluted fresh semen, 5 ml of a commercial semen dose (Piétrain boar line) was centrifuged (3 min at 390 X g). Afterwards, the sperm pellet was resuspended in fertilization medium. The final concentration during gamete co-incubation was $2.5x10^6$ spermatozoa/ml.

Assessment of fertilization parameters

Fertilization parameters were assessed after staining of presumed zygotes with fluorescent stain Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). More specifically, 22 - 24 h after *in vitro* fertilization, presumed zygotes were fixed overnight with 4% paraformaldehyde in PBS. Subsequently, presumed zygotes were incubated in 10 µg/ml Hoechst 33342 for 10 min, then washed in 0.1% (w/v) poly-vinyl pyrrolidone (PVP) in phosphate buffered saline (PBS) and mounted in a droplet of glycerol with (25 mg/ml) 1,4-diazabicyclo (2.2.2) octane (DABCO; Acros, Ghent, Belgium). In the zygotes stained with

Hoechst 33342 (excitation wavelength: 350 nm, emission wavelength: 461 nm), nuclear DNA was visualized using a Leica DMR fluorescence microscope (Leica Microsystems, Belgium). Oocytes with a metaphase plate and a polar body were classified as MII stage. The presence of two or more pronuclei and two polar bodies was indicative of fertilization. Polyspermic fertilization was defined as zygotes with more than two pronuclei or more than one decondensed sperm head. In cleaved embryos, fertilization was evaluated based on the number of nuclei and polar bodies. Total fertilization and percentage of polyspermy was calculated on total number of inseminated oocytes. Accordingly, total fertilization (%) = [n zygotes with > 1 pronucleus / n inseminated oocytes] x 100, and polyspermy (%) = [n zygotes with >2 pronuclei or >1 decondensed sperm head / n inseminated oocytes] x 100.

Effects of serine protease inhibitors on sperm quality parameters

Commercial semen doses from Piétrain boars (100 mL per blister, containing ± $3x10^9$ freshly ejaculated spermatozoa diluted in a commercial extender) were bought from a certified AI center and transported at 17°C in isotherm boxes to the laboratory (n=3 boars, 1 semen dose per boar).. Upon arrival at the laboratory, semen was divided into aliquots and pre-heated at 37°C for 30 min. Subsequently, each of the aliquots was submitted to one of the following treatments: (1) incubation with 100 µM AEBSF, (2) incubation with 5 µM STI, (3) incubation with 100 µM TLCK, (4) incubation with 100 µM TPCK or (5) incubation with 20 µl HEPES-TM per ml, equal to the volume of protease inhibitor solution (in HEPES-TM medium) added in group 1 to 4. Sperm samples were not pooled. All incubations were carried out at 37°C (incubator IN, Memmert GmbH + Co.KG, Germany) for a period of 6 h to assess membrane integrity, motility and acrosome integrity, and for a period of 1 h for evaluation of mitochondrial function.

Membrane integrity

Sperm membrane integrity was assessed by evaluation of the membrane integrity using an one-step eosin-nigrosin staining (Björndahl *et al.* 2003). Aliquots of each treatment group were stained by eosin-nigrosin at different time points during incubation (0 h, 1 h, 4 h and 6 h) and visualized with a light microscope. For each time point and sample, we calculated the average of two counts of 100 spermatozoa per boar (n=3 boars)(3 replicates).

Motility

Aliquots of diluted semen were incubated with protease inhibitors at 37°C. The control group consisted of spermatozoa incubated with 20 µl HEPES-TM per ml of semen. Motility parameters were evaluated by CASA (Hamilton Thorne, HTR Ceros 12.3 semen analyzer, Hamilton-Thorne Research, Beverly, MA, USA) at different time points during incubation (0 h, 1 h, 4 h and 6 h). Briefly, 10 µl of each sperm suspension was mounted on a glass slide and maintained at 37°C using a minitherm stage warmer. Five randomly selected microscopic fields were scanned 5 times each and 4 motility parameters were assessed: total motility (MOT), progressive motility (PROGR), average velocity (VAP) and straight-linear velocity (VSL). Additionally, these motility parameters were evaluated each ten minutes during the first hour of incubation in the control, TLCK and TPCK group. Software settings for the analysis of boar sperm were used as described by Vyt *et al.* (2004). Statistical analysis was performed using the mean of the 5 scans of each microscopic field (3 replicates).

Mitochondrial function

Mitochondrial function was evaluated by flow cytometric analysis of spermatozoa stained by the fluorochrome 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (T3168, Molecular Probes, Invitrogen, Eugene, Oregon, USA). JC-1 stain accumulates in the mitochondria as a green-fluorescent monomer (~529 nm). In the presence of a high mitochondrial membrane potential (mMP), fluorescence emission shifts from green to red (~590 nm). This potential-dependent color shift was used to study the mitochondrial membrane potential of spermatozoa and to determine the percentage of spermatozoa with a high membrane potential within each sample. After 1 h incubation of spermatozoa with and without protease inhibitor, sperm samples were diluted in Androhep to a concentration of $2x10^6$ spermatozoa/ml. A sperm sample treated with 50 µM carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP) (C2759, Sigma Aldrich, Bornem, Belgium) was used as a control to confirm that the JC-1 response was sensitive to changes in membrane potential. Five µl JC-1 (200 µM) were added to 1 mL of each sperm sample and incubated for 15 min in the dark (37ºC). Subsequently, spermatozoa were washed by centrifugation (3 min at 390xg) and resuspended in 1 mL of Androhep. Analyses were performed using a double-laser flow cytometer (BD FACSCanto II, California, USA) with an excitation wavelength of 488 nm. The green fluorescent signal of spermatozoa with a low mitochondrial membrane potential was measured through a 530/30 nm band pass filter (FL-1-A), the red fluorescent signal of spermatozoa with a high mitochondrial membrane potential was measured through a 585/42

nm band pass filter (FL-2-A). In each run, a negative unstained control sample (tube 1), an untreated positive control sample stained with JC-1 (tube 2) and a positive control sample stained with JC-1 and treated with 50 µM CCCP (tube 3) were included. CCCP causes depolarization of the mitochondrial membrane, greatly reducing the FL-2-A red fluorescence signal, providing a green-signal only (FL-1-A). Spermatozoa were first evaluated based on their forward scatter (FSC) and side scatter (SSC) properties. From all samples, 10,000 events were collected and analyzed. All data were acquired and processed using FACSdiva Software (Becton Dickinson, Belgium)(3 replicates with 3 different boars, 1 ejaculate per boar).

Acrosome integrity

Acrosome integrity was evaluated by fluorescence microscopy using fluorescein isothyiocyanate-conjugated Pisum sativum agglutinin (PSA-FITC; L0770, Sigma-Aldrich, Bornem, Belgium) in combination with Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). Boar semen samples were centrifuged and the sperm pellet was resuspended in HEPES-TM to obtain a concentration of $2x10^6$ spermatozoa/ml. Subsequently, aliquots of 2 ml were warmed in an incubator (Memmert GmbH + Co.KG, Germany) at 37ºC. Sperm suspensions without protease inhibitor were used as a control. Other sperm suspensions were treated with 100 µM AEBSF, 5 µM STI, 100 µM TLCK, and 100 µM TPCK, respectively. At different time points (0 h, 1 h, 4 h, 6 h), one aliquot of each sperm suspension was stained for evaluation of acrosomal integrity. First, 1 µl of Hoechst was added to the sperm aliquot and incubated for 3 min (37°C). After centrifugation at 720 x g for 10 min, the supernatant was removed and the sperm pellet was resuspended in 50 µl of 96% ethyl alcohol and incubated for 30 min at 4°C. Then, 15 µl of each sperm aliquot was smeared on a glass slide and air-dried. Afterwards, 15 µl of PSA-FITC (2 mg PSA-FITC diluted in 2 ml PBS) was added. The glass slides were kept for 15 min at 4°C, washed 5 times with deionized water and air-dried. Two counts of 100 spermatozoa of each sperm suspension were evaluated using a Leica DMR fluorescence microscope (Leica Microsystems, Belgium)(3 replicates). All spermatozoa were stained with Hoechst 33342. The acrosomal region of acrosome-intact spermatozoa was PSA-FITC positive and labeled green, while acrosome-reacted spermatozoa retained only an equatorial labeled band with little or no labeling of the anterior head region.

Statistical analysis

Differences in fertilization parameters between control and treatment groups were analyzed by means of binary logistic regression. The data of the sperm quality parameters at different

time points during incubation were analyzed using repeated measures analysis of variance (ANOVA). In this model, the percentage of membrane intact spermatozoa, the different CASA parameters and the percentage of acrosome intact spermatozoa were considered as dependent variables. Group was included as independent factor, replicate as covariate, and time was the within subjects variable. When significant differences were found for a parameter in the repeated measures analysis, one-way ANOVA was performed at the different time points during the incubation period. The percentage of spermatozoa with a high mitochondrial membrane potential was analyzed by one-way ANOVA. No direct comparisons between treatment groups were analyzed. Hypothesis testing was performed using a significance level of 5% (two-sided tests)(SPSS 20.0).

Results

Effects of serine protease inhibitors on fertilization parameters after porcine IVF

The presence of AEBSF (40-250 μM), STI (1-5 μM) or TPCK (10-100 μM) during porcine IVF with cryopreserved epididymal spermatozoa induced a clear dose-response effect on total fertilization and polyspermy rate (Fig. 6). In contrast, TLCK had no effect on IVF with cryopreserved epididymal spermatozoa when tested within the range of recommended working concentrations (Fig. 6C). In higher concentrations (≥ 250 μM), TLCK completely blocked fertilization but this coincided with signs of oocyte degeneration, more specifically degenerative changes of the ooplasm and DNA fragmentation.

Using diluted fresh semen for IVF, the presence of 100 μM AEBSF resulted in 31.0% total fertilization compared to 57.5% in the control group. Polyspermy rates were 3.5% and 23.8% for AEBSF and control oocytes, respectively ($P<0.05$). In the presence of 5 μM STI, 42.8% of oocytes were fertilized and 14.0% of oocytes were penetrated by more than one sperm cell during IVF with diluted fresh semen. Inhibitor TPCK significantly decreased total fertilization rate from 57.5% to 7.5% and polyspermy rate from 23.8% to 1.5%. The inhibitory effect of 100 μM AEBSF and 100 μM TPCK on total fertilization and polyspermy rate was similar between IVF experiments with crypreserved epididymal spermatozoa and diluted fresh semen. The inhibitory effect of STI was more pronounced during IVF with cryopreserved epididymal spermatozoa. In contrast, TLCK did not interfere with fertilization by cryopreserved epididymal spermatozoa, yet strongly inhibited total fertilization and polyspermy rate using diluted fresh semen for IVF (Fig. 7).

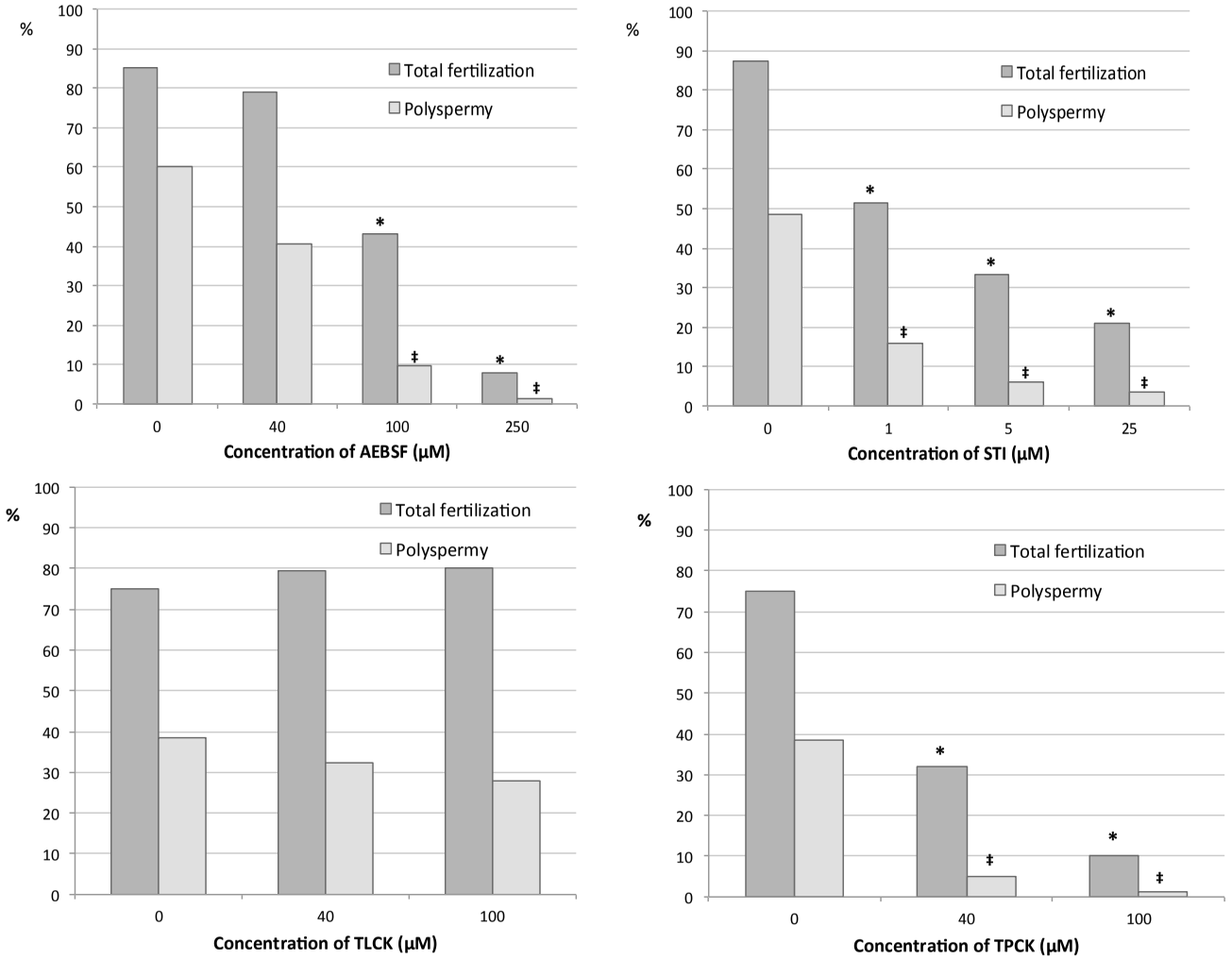

Figure 6: Effect of different concentrations of serine protease inhibitors on total fertilization and polyspermy rate of cumulus-intact porcine oocytes after IVF with epididymal spermatozoa (mean of 3 replicates). Values significantly different from control ($P<0.05$) are marked with * (total fertilization rate) or ‡ (polyspermy rate).

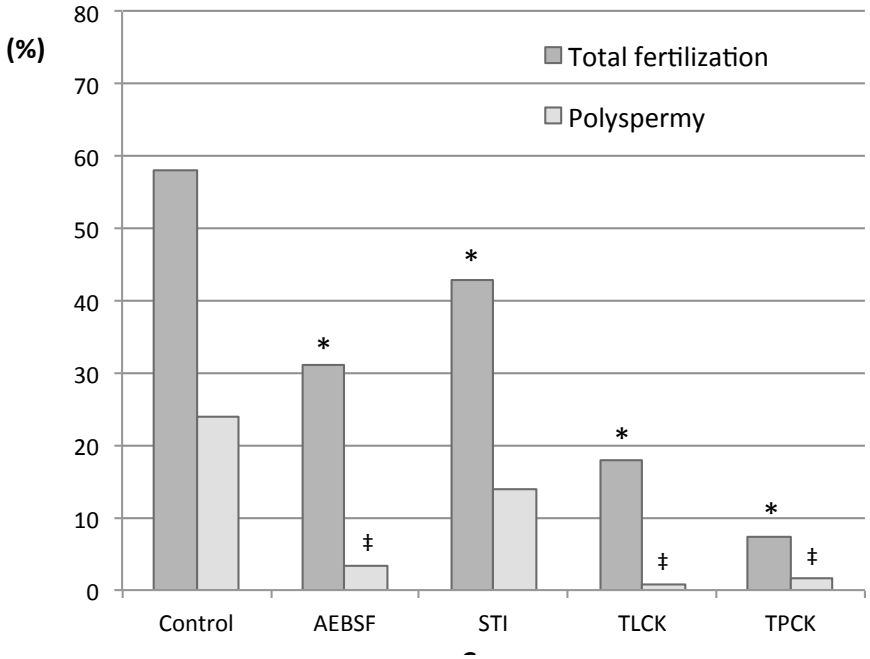

Figure 7: Effect of serine protease inhibitors AEBSF (100 µM), STI (5 µM), TLCK (100 µM) and TPCK (100 µM) on total fertilization and polyspermy rate of cumulus-intact porcine oocytes after IVF with diluted fresh semen (mean of 4 replicates). Values significantly different from control ($P<0.05$) are marked with * (total fertilization) or ‡ (polyspermy).

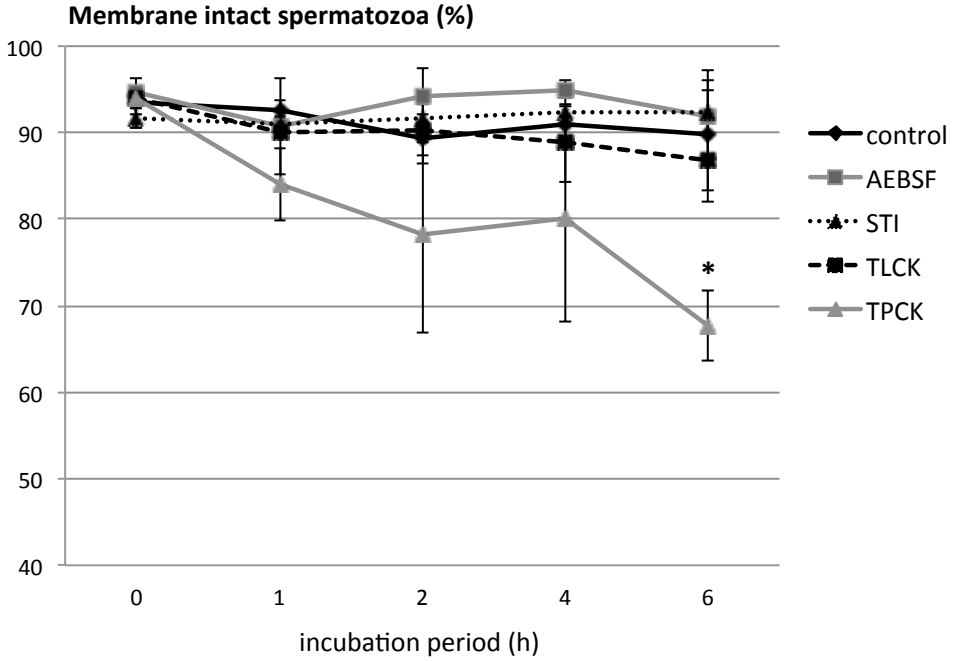

Figure 8: Sperm membrane integrity at different time points during a 6 h incubation period in medium without protease inhibitor (control) or with either AEBSF (100 µM), STI (5 µM), TLCK (100 µM) or TPCK (100 µM) (37°C). Data represent mean ± SD of 3 replicates. *Value significantly different from control ($P<0.05$).

Effects of serine protease inhibitors on sperm membrane integrity and motility

There was a tendency towards a loss of membrane integrity when spermatozoa were incubated in medium with 100 μM TPCK. The percentage of membrane intact spermatozoa in TPCK was significantly different from the control only after 6 h of incubation ($P<0.05$)(Fig. 8). Other inhibitors did not affect sperm membrane integrity.

Total motility, progressive motility, VAP and VSL of spermatozoa are shown in Figure 9 and 10. Spermatozoa incubated in medium with 100 μM AEBSF showed a decrease in motile spermatozoa after 6 h of incubation, yet progressive motility, VAP and VSL remained unaltered (Fig. 9). STI (5 μM) exerted no effect upon sperm motility, progressive motility and velocity. In contrast, total and progressive motility were almost completely blocked by 100 μM TPCK and 100 μM TLCK within 1 and 2 h, respectively. Therefore, the effect of TPCK and TLCK on motility was studied more in detail during the first hour of incubation. Noticeably, the negative effect of 100 μM TPCK on sperm motility already reached its maximum within 20 minutes. The effect of TLCK on motility was shown to occur more gradually over a time period of 2 hours (Fig. 10). Both inhibitors were shown to decrease sperm velocity of motile spermatozoa, indicated by a significant lower average path velocity (VAP) and straight line velocity (VSL) compared to the control group.

Effect of serine protease inhibitors on sperm mitochondrial potential

In control samples, 72.0 ± 15.6% of the spermatozoa displayed a high mMP. Incubation in 100 μM AEBSF or 5 μM STI had no significant effect on the mMP, with 58.1 ± 14.7% and 77.4 ± 1.9% of spermatozoa showing a high mMP respectively. After 1 h of incubation in 100 μM TLCK, 56.4 ± 9.7% of spermatozoa exhibited a high mMP, which was numerically but not significantly lower than in the control group (72.0 ± 15.6%). In contrast, more than 99% of spermatozoa incubated in 100 μM TPCK had a low mMP (99.4 ± 0.3%)($P<0.05$).

Effect of serine protease inhibitors on the acrosome reaction

The acrosomal status of spermatozoa was not influenced by incubation with 100 μM AEBSF or 100 μM TLCK (Fig. 11). In the presence of 5 μM STI, the percentage of spermatozoa with intact acrosome was significantly higher than in control medium at 4 h of incubation. At 6 h of incubation, acrosome integrity did not differ between the control group (94.7 ± 0.6%) and STI group (98.0 ± 1.0%; $P>0.05$). TPCK reduced acrosome integrity over time. At 6 h of incubation, 89.3 ± 1.5% of spermatozoa incubated in TPCK had intact acrosome compared to 96.0 ± 1.4% in the control group ($P<0.01$).

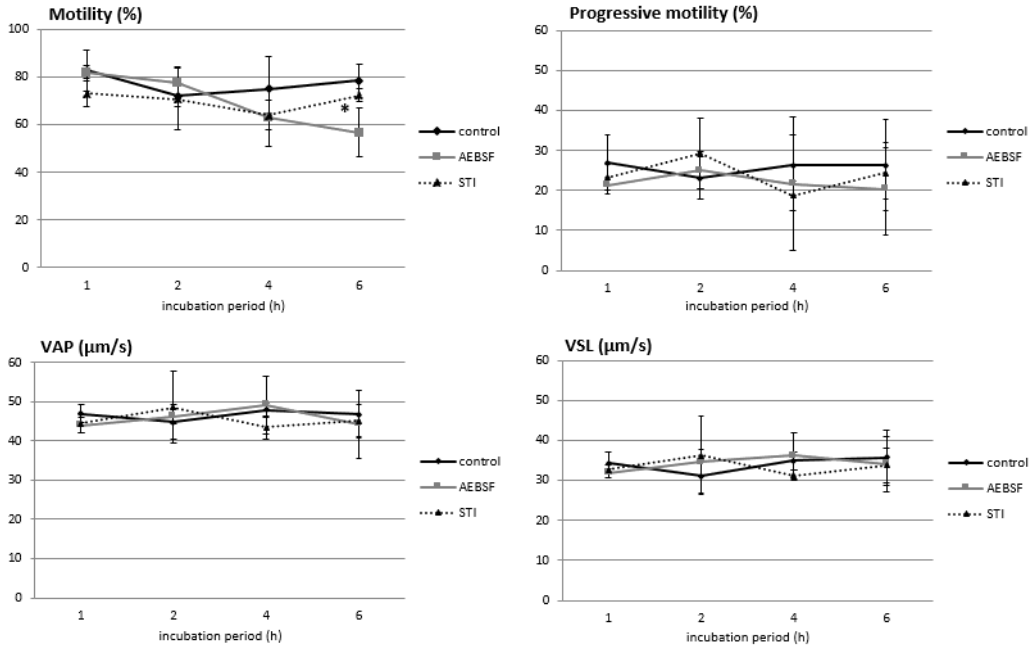

Figure 9: CASA parameters of spermatozoa at different time points during a 6 hour incubation period in standard medium (control; no inhibitor) or medium with either 100 µM AEBSF or 5 µM STI. Data represent mean and standard deviation (SD) of Motility (%): percentage of motile spermatozoa; Progressive motility (%): percentage of spermatozoa with progressive movement; VAP: average path velocity (µm/s); VSL: straight line velocity (µm/s); (3 replicates). *Value significantly different from control ($P<0.05$).

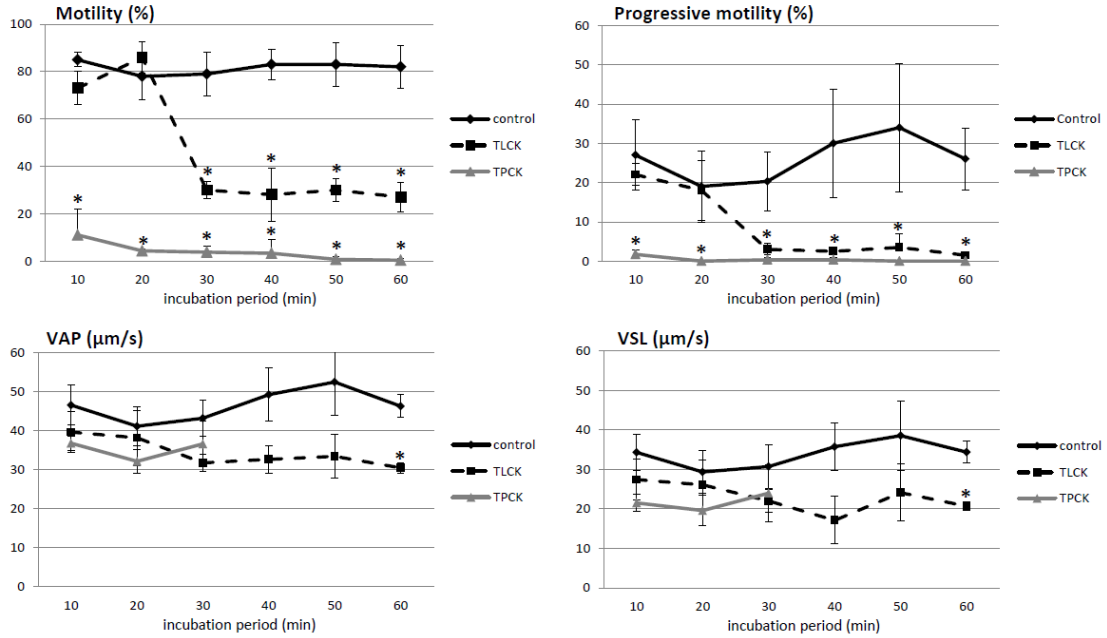

Figure 10: CASA parameters of spermatozoa at different time points during one hour incubation period in standard medium (control; no inhibitor) or medium with either 100 µM TLCK or 100 µM TPCK. Data represent mean and standard deviation (SD) of Motility (%): percentage of motile spermatozoa; Progressive motility (%): percentage of spermatozoa with progressive movement; VAP: average path velocity (µm/s); VSL: straight line velocity (µm/s); (3 replicates). *Value significantly different from control ($P<0.05$)

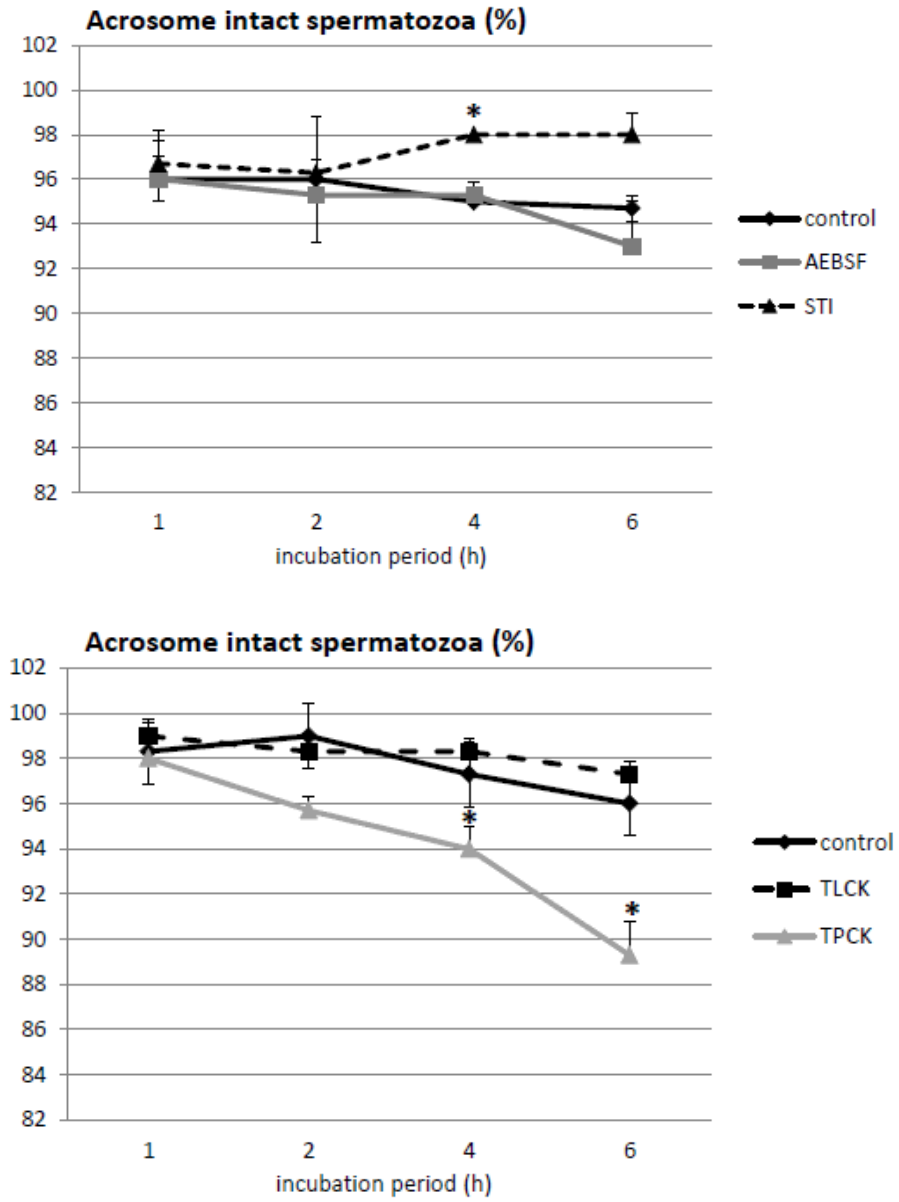

Figure 11: Acrosome integrity at different time points during a 6 h incubation period in medium without protease inhibitor (control) or with either AEBSF (100 µM), STI (5 µM), TLCK (100 µM) or TPCK (100 µM) (37°C). Data represent mean ± SD of 3 replicates. *Value significantly different from control ($P<0.05$).

Discussion

The four tested serine protease inhibitors showed a significant inhibitory effect on porcine fertilization *in vitro*. Two types of spermatozoa, cryopreserved epididymal and diluted freshly ejaculated spermatozoa, were used in these IVF experiments. Epididymal spermatozoa differ from ejaculated spermatozoa in that they have not been in contact with seminal plasma. They have a higher *in vitro* fertilizing potential than ejaculated spermatozoa, which was attributed to their superior motility and the lack of contact with capacitation-inhibiting factors present in seminal plasma (Rath and Niemann 1997). Compared to the control group, the inhibitory effects of AEBSF and TPCK were similar for both types of spermatozoa. In contrast, TLCK inhibited fertilization only during IVF with ejaculated spermatozoa. In the presence of TLCK, motility of ejaculated spermatozoa was seriously hampered from 20 min of incubation onwards. This negative effect on motility likely contributed to a decrease in sperm penetration during IVF with diluted fresh semen. It has been shown that high penetration rates in porcine IVF can be achieved with a gamete co-incubation period as short as 10 minutes, yet this largely depends on sperm fertilizing capacity (Gil *et al.* 2007; Almiñana *et al.* 2008). In line with this, the superior motility of epididymal spermatozoa might enabled similar fertilization rates in 100 μM TLCK as in the control group during the dose-response experiment. Inhibitor STI significantly decreased total fertilization and polyspermy rate during IVF using both epididymal or ejaculated spermatozoa but when using ejaculated spermatozoa, only fifty percent of the inhibitory effect was obtained compared to epididymal spermatozoa. A possible explanation for the reduced effect of 5 μM STI during IVF with ejaculated spermatozoa could be saturation of this inhibitor because of the much higher (20 times higher) concentration of spermatozoa during IVF with diluted fresh semen compared to the IVF dose-response experiment with epididymal spermatozoa. Apart from the type of spermatozoa, the effect of inhibitors might be influenced by the process of cryopreservation, since freezing and thawing are known to alter lipid composition and lipid-protein organization in the sperm plasma membrane (Buhr *et al.* 1994; Maldjian *et al.* 2005). Further research is needed to elucidate whether type of spermatozoa and/or cryopreservation changed the effect of STI and TLCK on fertilization rate.

Importantly, serine protease inhibitors AEBSF and STI did not induce premature acrosome reaction nor did they affect membrane integrity, mitochondrial membrane potential or motility parameters, which makes them suitable for further research on the specific roles of serine proteases during porcine fertilization *in vitro*. For that reason, the decrease in fertilization

rates in the presence of these inhibitors further indicates the involvement of serine proteases during porcine IVF.

The irreversible inhibitor of trypsin-like serine proteases TLCK did not influence on sperm membrane integrity, acrosomal integrity or mitochondrial function but did affect the motility of boar spermatozoa already at a relatively low concentration. During the evaluation of sperm motility in the presence of 100 μM TLCK, a strong decrease in total and progressive motility of ejaculated spermatozoa was recorded within a 1-h incubation period. De Lamirande and Gagnon (1986) evaluated the effect of several serine protease inhibitors (including STI, TLCK and TPCK) as well as protease substrates on motility of human, mouse, rat and bovine spermatozoa. They used concentrations of 2000 to 2250 μM TLCK but did not observe any inhibitory effect on sperm motility. In other studies, rabbit spermatozoa was reported to maintain motility for 8 h in medium with 200 μM TLCK (Zaneveld *et al.* 1970), whereas motility of hamster spermatozoa decreased after 15 min incubation in concentrations starting from 135 μM (0.005%) TLCK (Miyamoto and Chang 1973).

Although De Lamirande and Gagnon (1986) used demembranated spermatozoa and reactivation by Mg-ATP as *in vitro* model to test the effect of inhibitors on sperm motility (whereas we evaluated the effect on membrane-intact spermatozoa), the comparison of their study with ours led to two conclusions of general interest. First, the range of effective concentrations within the same experimental set up strongly differs between serine protease inhibitors, irrespective of equivalence in inhibitor spectrum (for example STI versus TLCK). And secondly, there are explicit differences in susceptibility of spermatozoa towards these inhibitors between different mammalian species, with the pig and the hamster being particularly susceptible. Surprisingly, previous fertilization studies with serine protease inhibitors (Stambaugh *et al.* 1969; Miyamoto and Chang 1973; Hoshi *et al.* 1981; Saling 1981; Deppe *et al.* 2008) did not elaborate on the negative effect of inhibitors on sperm motility, nor provided any quantitative data of possible toxic effects.

From the four inhibitors tested in this study, TPCK exerted the most pronounced effect on sperm quality parameters of diluted fresh semen, which explains the strong inhibition of fertilization in 100 μM TPCK. Almost no spermatozoa retained motility after 20 min of incubation. Furthermore, visualization of the mitochondrial membrane potential (mMP) by means of JC-1 staining showed that more than 99% of spermatozoa had a low mMP after 1 h incubation in 100 μM TPCK. Changes in the permeability of the mitochondrial membrane are a common feature of different pathways leading to apoptosis or programmed cell death (Ly *et*

al. 2003). All apoptosis-signaling pathways described so far involve a collapse of the mMP. The fluorescent probe JC-1 has a high sensitivity and specificity in the detection of changes in the mMP that occur during apoptosis (Mathur *et al.* 2000; Ly *et al.* 2003). TPCK has been shown to inactivate chymotrypsin and chymotrypsin-like serine proteases but not trypsin. TPCK is also able to inhibit cysteine proteases. Even though TPCK is mostly used for protease inhibition, other biochemical effects have been reported including dual effects on cell death (Frydrych and Mlejnek 2008). More specifically, TPCK has been shown to block several features of apoptosis via inhibition of caspases, but also to induce cell death itself or to enhance cell toxicity of other agents. Ambiguous effects on cell death have also been described for TLCK, although with lower cytotoxicity (Murn *et al.* 2004; Frydrych and Mlejnek 2008). Frydrych and Mlejnek (2008) demonstrated that TPCK enhanced the collapse of mMP, whereas TLCK partially prevented the reduction in mMP when apoptosis was induced by cytotoxic agents. These possible effects of TLCK and TPCK on cell death are important since they may induce death of sperm cells too. Loss of mMP and sperm motility possibly reflects early stages of cell death, whereas the loss of membrane and acrosomal integrity may represent later features of cell death. Next to that, a low mMP coincided with a low fertilization rate *in vitro* in our study. This finding supports the usefulness of JC-1 staining in sperm quality assessments, which has been described previously for several species including the human, the pig and the horse (Garner *et al.* 1997; Gravance *et al.* 2000; Guthrie and Welch 2006; Espinoza *et al.* 2009).

Taken together; our findings underline the importance of toxicity testing of inhibitors for a correct application of these inhibitors during experiments. The negative effect of TLCK and TPCK on sperm quality parameters makes them unacceptable for use in fertilization studies. Inhibitors AEBSF and STI decreased sperm penetration during porcine IVF but had no effect on viability or motility of porcine spermatozoa. These two serine protease inhibitors can thus be used in future experiments to elucidate the specific roles of serine proteases during porcine fertilization *in vitro*.

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CHAPTER 4

INHIBITORS OF SERINE PROTEASES DECREASE SPERM PENETRATION DURING PORCINE FERTILIZATION *IN VITRO* BY INHIBITION OF SPERM BINDING TO THE ZONA PELLUCIDA AND THE ACROSOME REACTION

Modified from

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Abstract

Serine proteases are involved in mammalian fertilization. Inhibitors of serine proteases can be applied to investigate at which point these enzymes exert their action. We selected two serine protease inhibitors, 4-(2-Aminoethyl)benzene sulfonyl fluoride hydrochloride (AEBSF, 100 μM) and Soybean Trypsin Inhibitor from Glycine Max (STI, 5 μM) via previous dose-response and sperm toxicity tests. In the present study, we evaluated how these inhibitors affect porcine fertilization *in vitro* as calculated on total fertilization rate, polyspermy rate and the sperm penetration index (SPI) of cumulus-intact (CI), cumulus-free (CF) and zona-free oocytes. In the control group (no inhibitor), total fertilization rate, polyspermy rate and SPI were respectively 86%, 49% and 2.2 for CI and 77%, 43% and 2.2 for CF oocytes. AEBSF and STI significantly reduced total fertilization rate in CI and CF oocytes. Inhibition rates were higher in CF than CI oocytes, indicating that inhibitors exerted their action after sperm passage through the cumulus. AEBSF significantly reduced sperm binding to the zona pellucida. The acrosome reaction was inhibited by both inhibitors ($P<0.05$). Only 40.4% (AEBSF) and 11.4% (STI) of spermatozoa completed a calcium-induced acrosome reaction compared to 86.7% in the control. There was no effect on sperm binding or fertilization parameters in zona-free oocytes. In conclusion, sperm-zona binding and acrosome reaction were inhibited by serine protease inhibitors during porcine IVF.

Introduction

Mammalian fertilization requires the successful completion of a number of steps in a compulsory order (Yanagimachi 1994; Primakoff and Myles 2002). Previous studies have demonstrated that the important players involved in fertilization, i.e. the sperm cell, the oocyte and its surrounding cumulus cells, all contain and/or secrete several proteases. The family of serine proteases, named after the serine residue in the active site of the enzyme, is the largest family of proteases and also the most represented on gametes (Cesari *et al.* 2010). The serine protease acrosin and its precursor proacrosin are found specifically within the acrosome of mammalian spermatozoa (Tranter *et al.* 2000). Activation of proacrosin to acrosin is mediated by contact with glycoproteins of the zona pellucida (ZP). The sperm protease acrosin is associated with several steps of fertilization, including sperm binding to the ZP, dispersal of acrosomal content, subsequent zona lysis and activation of oocyte transmembrane receptors (Yamagata *et al.* 1998b; Smith *et al.* 2000; Tranter *et al.* 2000). Up to now, about fifteen sperm serine proteases are described, not only in the acrosomal content but also on acrosomal and sperm membranes (Cesari *et al.* 2010).

Serine protease activity derived from the cortical granules has been shown to contribute to the oocyte's defense mechanism against polyspermy in the hamster and in the mouse (Cherr *et al.* 1988; Hoodbhoy and Talbot 1994). Similarly, a role has been proposed for serine protease activity in the regulation of sperm penetration in bovine oocytes, mediated by the release of tissue-type plasminogen activator (tPA) from the oocyte and activation of plasminogen into the serine protease plasmin (Rekkas *et al.* 2002). Furthermore, recent studies have provided new insights on how the plasminogen-plasmin system may contribute to the regulation of sperm penetration during porcine fertilization (Coy *et al.* 2012; Mondéjar *et al.* 2012). As proposed by Coy et al.(2012), the oocyte releases tPA and urokinase-type plasminogen activator (uPA) upon contact with spermatozoa. These plasminogen activators convert plasminogen into plasmin, which protease activity will lead to detachment of spermatozoa previously attached to the ZP. This model is supported by the detection of plasminogen in porcine oviductal fluid (Mondéjar *et al.* 2012). The precursor of plasmin is thus present at the site of gamete interaction in vivo. In addition, a decrease in sperm-ZP binding and sperm penetration was observed when plasminogen was supplemented to the IVF medium in concentrations similar to those detected in oviductal fluid (Mondéjar *et al.* 2012).

In several mammalian species, such as the pig, oocytes are still surrounded by the cumulus oophorus at the time of fertilization in vivo (Van Soom *et al.* 2002). The presence of cumulus

cells during porcine IVF has beneficial effects on fertilization outcome (Romar *et al.* 2003). In the mouse, cumulus cells have been shown to synthesize and secrete several proteases, including tPA and uPA (D'Alessandris *et al.* 2001). The secretion of plasminogen activators by cumulus cells rapidly increases when cumulus expansion is completed and matrix disassembly begins. Activation of the plasminogen-plasmin system is thus proposed to play a regulatory role in cumulus matrix disaggregation (D'Alessandris *et al.* 2001).

Serine proteases are thus involved in several aspects of gamete interaction and in mechanisms that work either conceptive or contraceptive. These features make them interesting candidates for further research on how serine protease inhibition may affect porcine fertilization *in vitro*. Up to now, the high degree of polyspermic fertilization during porcine IVF hampers large scale *in vitro* production of porcine embryos. Specific protease inhibitors could be useful as regulators of sperm penetration during porcine IVF, lessening the problem of polyspermy. The present study investigated which fertilization step(s) during porcine IVF are affected by serine protease inhibitors 4-(2-Aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF) and Soybean Trypsin Inhibitor from Glycine Max (STI). These two inhibitors were previously tested in our laboratory and were shown not to compromise membrane integrity and motility of porcine spermatozoa.

Materials and methods

Media

All chemicals used in this study were purchased from Sigma-Aldrich (Bornem, Belgium), unless otherwise stated. The basic medium used for the collection and washing of cumulus-oocyte- complexes (COCs) was a modified HEPES-buffered Tyrode balanced salt solution (HEPES-TM) with 10 µg/ml gentamycin sulfate, 10 mM HEPES and 3 mg/ml BSA. Immature oocytes were matured in BSA-free 'North Carolina State University' 23 (NCSU23) (Petters and Wells 1993) supplemented with 0.57 mM cysteine, 10 ng/ml epidermal growth factor, 10 IU/ml equine Chorionic Gonadotropin (eCG) (Folligon®, Intervet, The Netherlands), 10 IU/ml human Chorionic Gonadotropin (hCG) (Chorulon®, Intervet, The Netherlands) and 10% (v/v) porcine follicular fluid. Follicular fluid was collected from 5-6 mm follicles of prepubertal gilts. After centrifugation of follicular fluid (100xg, 10 min), the supernatant was aspirated, filtered (0.22 µm) and stored at -80ºC until use. The basic medium for IVF was Tyrode's albumin lactate pyruvate medium (TALP medium) (Rath *et al.* 1999) supplemented with 0.3% BSA (FERT-TALP). Frozen-thawed epididymal boar spermatozoa were centrifuged (390xg, 3 min) in Androhep extender consisting of 27 mM Tri-

natriumcitrate, 2.7 mM Titriplex III EDTA, 2.5 mg/ml BSA (fraction V), 14 mM $NaHCO_3$, 38 mM Hepes, 144 mM D(+)-glucose.H_2O and 50 µg/ml gentamycine sulfate. Presumed zygotes were washed in HEPES-buffered TALP medium (HEPES-TALP), i.e. TALP medium with 25 mM HEPES. The embryo culture medium was NCSU23 with 0.4% BSA.

Protease inhibitors

Protease inhibitors were purchased from Sigma-Aldrich (Bornem, Belgium). Inhibitor STI (T6522) was stored desiccated and dissolved in FERT-TALP medium prior to use. Inhibitor AEBSF (A8456) was dissolved in deionized water to a stock solution of 10 mM. The supplementation of AEBSF stock solution with deionized water to the fertilization medium was corrected by the addition of equal volume of medium with twofold concentration of medium components. Inhibitor AEBSF was added to the fertilization medium resulting in a final concentration of 100 µM. The final concentration of STI in the fertilization medium was 5 µM. These concentrations were chosen based on previous dose-response IVF experiments and were shown to have no adverse effects on sperm membrane integrity and sperm motility during a 6 h incubation period (Beek et al., *accepted for publication*).

In vitro *maturation (IVM) and* in vitro *fertilization (IVF) of porcine oocytes*

Ovaries of prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory in physiological saline at 37°C. Immature cumulus-oocyte-complexes (COCs) were aspirated from follicles with a diameter ranging from 3 to 6 mm. Then COCs were selected based on a homogeneous ooplasm and a multilayered cumulus. Groups of 100 immature COCs were placed into 500 µl maturation medium and cultured for the first 22 h (39°C, 5% CO_2). Subsequently, COCs were cultured in hormone-free maturation medium for another 22 h. After 44 h of IVM, the matured COCs of each well were transferred to 500 µl of FERT-TALP under mineral oil for 15 min. Protease inhibitor dilutions were prepared in concentrated stocks and added to droplets of FERT-TALP to obtain the final concentration required. Approximately 10 min after the addition of protease inhibitors, the matured COCs were randomly assigned to the fertilization droplets (45-50 COCs/droplet unless stated otherwise). Frozen epididymal semen was thawed for 60 sec in a water bath at 38°C and spermatozoa were washed by centrifugation (3 min at 390xg) in 9.5 ml of Androhep. The sperm pellet was resuspended in 1 ml FERT-TALP and sperm concentration was assessed using a Bürker counting chamber. Spermatozoa were added to the droplets containing the COCs resulting in a final concentration of $0.25x10^5$ or $1.25x10^5$ spermatozoa/ml, as indicated

in the experimental design. The control COCs were fertilized in fertilization medium without addition of a protease inhibitor. After a co-incubation period of 6 h, the presumed zygotes were vortexed during 3 min in 2.5 ml HEPES-TALP to remove loosely bound spermatozoa, washed three times in culture medium and cultured for 18 h in a modular incubator chamber (39°C, 5% CO_2).

Assessment of fertilization parameters

Approximately 24 h after insemination, presumed zygotes were washed in 0.1% (w/v) poly-vinyl pyrrolidone (PVP) in phosphate buffered saline (PBS). Subsequently, the zygotes were fixed overnight with 4% paraformaldehyde in PBS and stained with 10 µg/ml *bis*-benzimide (Hoechst 33342; Molecular Probes, Leiden, The Netherlands) for 10 min. Zygotes were mounted in a droplet of glycerol with (25 mg/ml) 1,4-diazabicyclo (2.2.2) octane (DABCO; Acros, Ghent, Belgium) and nuclear DNA was visualized using a Leica DMR fluorescence microscope (Leica Microsystems, Belgium). Oocytes with a metaphase plate and a polar body were classified as being in the MII stage. The presence of two pronuclei or cleaved embryos with two equally sized blastomeres was indicative for normal fertilization, whereas zygotes with more than two pronuclei or more than one decondensed sperm head were classified as polyspermic. In case of polyspermic fertilization, the number of penetrated spermatozoa was counted. The following fertilization parameters were assessed: total fertilization rate (%), polyspermy rate (%) and the sperm penetration index (SPI), that is the mean number of penetrated spermatozoa per fertilized oocyte.

Assessment of sperm acrosome integrity

Acrosome integrity was evaluated by fluorescence microscopy using fluorescein isothyiocyanate-conjugated Pisum sativum agglutinin (PSA-FITC; L0770, Sigma-Aldrich, Bornem, Belgium) in combination with Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). First, 1 µl of Hoechst was added to the sperm aliquot and incubated for 3 min (37°C, incubator IN, Memmert GmbH + Co.KG, Germany). After centrifugation at 720 x g for 10 min, the supernatant was removed and the sperm pellet was resuspended in 50 µl of 96% ethyl alcohol and incubated for 30 min at 4°C. Then, 15 µl of each sperm aliquot was smeared on a glass slide and air-dried. Afterwards, 15 µl of PSA-FITC (2 mg PSA-FITC diluted in 2 ml PBS) was added. The glass slides were kept for 15 min at 4°C, washed 5 times with deionized water and air-dried. Then, each sperm suspension was evaluated using a Leica DMR fluorescence microscope (Leica Microsystems, Belgium). All spermatozoa were stained

with Hoechst 33342. The acrosomal region of acrosome-intact spermatozoa was PSA-FITC positive and labeled green, while acrosome-reacted spermatozoa retained only an equatorial labeled band with little or no labeling of the anterior head region (Fig. 15a).

Experimental design

Effect of serine protease inhibitors on sperm penetration through the cumulus oophorus

In vitro matured COCs were randomly assigned to 6 groups of approximately 50 COCs (3 replicates). Three groups of COCs were denuded by vortexing with 0.1% (w/v) hyaluronidase in HEPES-TM (cumulus-free or CF), the other groups were kept cumulus-intact (CI). Both CF and CI oocytes were fertilized in standard fertilization medium and in fertilization medium supplemented with either 100 μM AEBSF or 5 μM STI. The CF and CI oocytes were co-incubated with $1.25x10^5$ spermatozoa/ml for 6 h (39°C, 5% CO_2). Prior to fixation, all groups were vortexed to remove loosely bound spermatozoa and cultured during 18 h. After Hoechst staining, the oocytes were evaluated for fertilization parameters.

Effect of serine protease inhibitors on sperm-zona binding

In vitro matured COCs were denuded by vortexing with 0.1% (w/v) hyaluronidase in HEPES-TM and randomly assigned to 2 groups of 20 oocytes (3 replicates for each inhibitor). The first group was co-incubated with spermatozoa in standard fertilization medium (control), the second group in fertilization medium supplemented with protease inhibitor, either 100 μM AEBSF or 5 μM STI. For unambiguous counting of the number of spermatozoa bound to the ZP, oocytes were co-incubated with $0.25x10^5$ spermatozoa/ml (39°C, 5% CO_2) in each group. After 4 h of co-incubation, oocytes were washed five times to remove loosely bound spermatozoa, and subsequently fixed and stained with Hoechst 33342. Of each oocyte, the average of two counts was taken into account as the number of sperm bound to the ZP.

Effect of serine protease inhibitors on sperm-oolemma binding and IVF of zona-free oocytes

After *in vitro* maturation, COCs were denuded by vortexing with 0.1% (w/v) hyaluronidase in HEPES-TM during 3 min. Subsequently, the CF oocytes were washed in PBS and shortly incubated in a 0.1% (w/v) pronase solution in PBS (Kim *et al.* 1996) to dissolve their ZP. Oocytes were continuously observed under a stereomicroscope for dissolution of ZP. After a recovery period of 30 min in FERT-TALP, the ZP-free oocytes (45 to 50 oocytes per group) were randomly assigned to 3 different media: standard fertilization medium and fertilization medium supplemented with either 100 μM AEBSF or 5 μM STI. The ZP-free oocytes were

co-incubated with spermatozoa at a final concentration of 0.25 x10^5 spermatozoa/ml in 100 µl droplets of medium covered with mineral oil. Prior to insemination, the sperm dilution was incubated for 30 min in FERT-TALP (39°C, 5% CO_2) to allow capacitation.
One hour after insemination, 10 oocytes from each group were washed five times to remove loosely bound spermatozoa, fixed and stained with Hoechst 33342. Per presumed zygote, the number of spermatozoa bound to the oolemma was counted. All other oocytes were co-incubated with spermatozoa for 6 h. These presumed zygotes were then vortexed to remove loosely bound spermatozoa and cultured during 18 h, as described before. After fixation and staining, fertilization parameters were evaluated as described before.

Effect of pre-incubation of gametes with serine protease inhibitors

For the pre-incubation experiment, *in vitro* matured COCs were divided into four groups of approximately 50 COCs. For pre-incubation of female gametes, COCs of group 1 and group 2 were incubated in standard fertilization medium and in fertilization medium with protease inhibitor (either 100 µM AEBSF or 5 µM STI), respectively, for 30 min prior to fertilization. Subsequently, COCs were washed, transferred to standard fertilization medium and co-incubated with 1.25x10^5 spermatozoa/ml (39°C, 5% CO_2) during 6 h. The two other groups of COCs, group 3 and group 4, were used to evaluate pre-incubation of male gametes. Both groups were co-incubated with 1.25x10^5 spermatozoa/ml in standard fertilization medium, but with differently prepared spermatozoa. Group 3 was inseminated with spermatozoa previously incubated in standard fertilization medium for 30 min, whereas group 4 was inseminated with spermatozoa previously incubated for 30 min in fertilization medium supplemented with protease inhibitor.
After a gamete co-incubation period of 6 h, all presumed zygotes were vortexed during 3 min in 2.5 ml HEPES-TALP to remove loosely bound spermatozoa, washed three times in culture medium and cultured for 18 h in a modular incubator chamber (39°C, 5% CO_2). At 24 hours after insemination, all presumed zygotes were fixed, stained with Hoechst and fertilization parameters were assessed (4 replicates with inhibitor AEBSF, 3 replicates with inhibitor STI).

Effect of protease inhibitors on the acrosome reaction

Spermatozoa were washed by centrifugation (3 min at 390xg) and the sperm pellet was resuspended in HEPES-TM to obtain a sperm concentration of 2x10^6 sp/ml and divided into aliquots of 1 ml. Aliquot 1 without inhibitor was used as negative control. A second aliquot was supplemented with 1 µM A23187 calcium ionophore as full response control. Sperm

aliquots 3 and 4 were treated with 100 µM AEBSF, aliquots 5 and 6 were treated with 5 µM STI. Sperm aliquots 4 and 6 were then supplemented with 1 µM A23187 calcium ionophore to evaluate the response in the presence of a protease inhibitor. All incubations were carried out for 1 h at 37°C (incubator IN, Memmert GmbH + Co.KG, Germany). Subsequently, acrosome integrity was evaluated using PSA-FITC staining as described in 2.6. The acrosomal region of acrosome-intact spermatozoa was PSA-FITC positive and labeled green, while acrosome-reacted spermatozoa retained only an equatorial labeled band with little or no labeling of the anterior head region (Fig 15a). For each replicate, the average of two counts of 100 spermatozoa was calculated per sperm aliquot. Three replicates were performed.

Statistical analysis

Differences in total fertilization and polyspermy rate between control and treatment group or between cumulus-intact and cumulus-free oocytes within a group were analyzed by means of binary logistic regression. ANOVA was used to evaluate the sperm penetration index (SPI), the differences in mean number of spermatozoa bound to the ZP, in mean number of spermatozoa bound to the oolemma, and the percentage of acrosome intact spermatozoa. In all experiments, the control group was compared with each treatment group and no direct comparisons between treatment groups were analyzed. Hypothesis testing was performed using a significance level of 5% (two-sided tests)(SPSS 21.0).

Results

Effect of serine protease inhibitors AEBSF and STI on sperm penetration through the cumulus oophorus during porcine IVF

To evaluate the effect of protease inhibition on sperm penetration through the cumulus, fertilization parameters of cumulus-intact (CI) and cumulus-free (CF) oocytes were compared (Fig. 12). In comparison with the control group, total fertilization and polyspermy rate decreased significantly in both CI and CF oocytes when either inhibitor AEBSF (100 μM) or STI (5 μM) was added to the fertilization medium. Total fertilization rate was reduced more in CF than in CI oocytes by both inhibitors. Polyspermy rate decreased in medium with AEBSF independent of the presence of the cumulus cells. Compared to the control group, polyspermy was inhibited with 67% and 70% for CI and CF oocytes, respectively ($P>0.05$). The presence of 5 μM STI in the fertilization medium resulted in a significant reduction of polyspermic fertilization in CI oocytes compared to the control group (-93%). Its inhibitory effect on polyspermy was even higher in CF oocytes (-99%)($P<0.05$). Within each group, the sperm penetration index was similar in cumulus-intact and cumulus-free oocytes.

Effect of serine protease inhibitors AEBSF and STI on sperm binding to the zona pellucida

The number of spermatozoa bound to the ZP decreased significantly when AEBSF was added to the fertilization medium ($P<0.05$) (Fig. 13). After 4 h of gamete co-incubation, the average number of spermatozoa bound to the ZP was 92 ± 8.7 (Mean ± SD) in the control group and 75 ± 6.5 in 100 μM AEBSF (n = 120). The supplementation of 5 μM STI to the fertilization medium did not decrease the number of sperm bound to the ZP when compared to the respective control group (n = 120).

Effect of serine protease inhibitors AEBSF and STI on sperm-oolemma binding and fertilization of zona-free oocytes

Supplementation of either AEBSF (100 μM) or STI (5 μM) did not significantly influence sperm-oolemma binding (n = 90). However, small numerical differences were observed between groups ($P>0.05$). Compared to the control group (8.6 ± 1.0), the number of sperm bound per oocyte slightly increased to 8.9 ± 1.0 (STI) and 11 ± 1.2 (AEBSF). Serine protease nhibitors AEBSF and STI did not inhibit total fertilization, polyspermy rate nor sperm penetration index in zona-free oocytes ($P>0.05$) (n = 341).

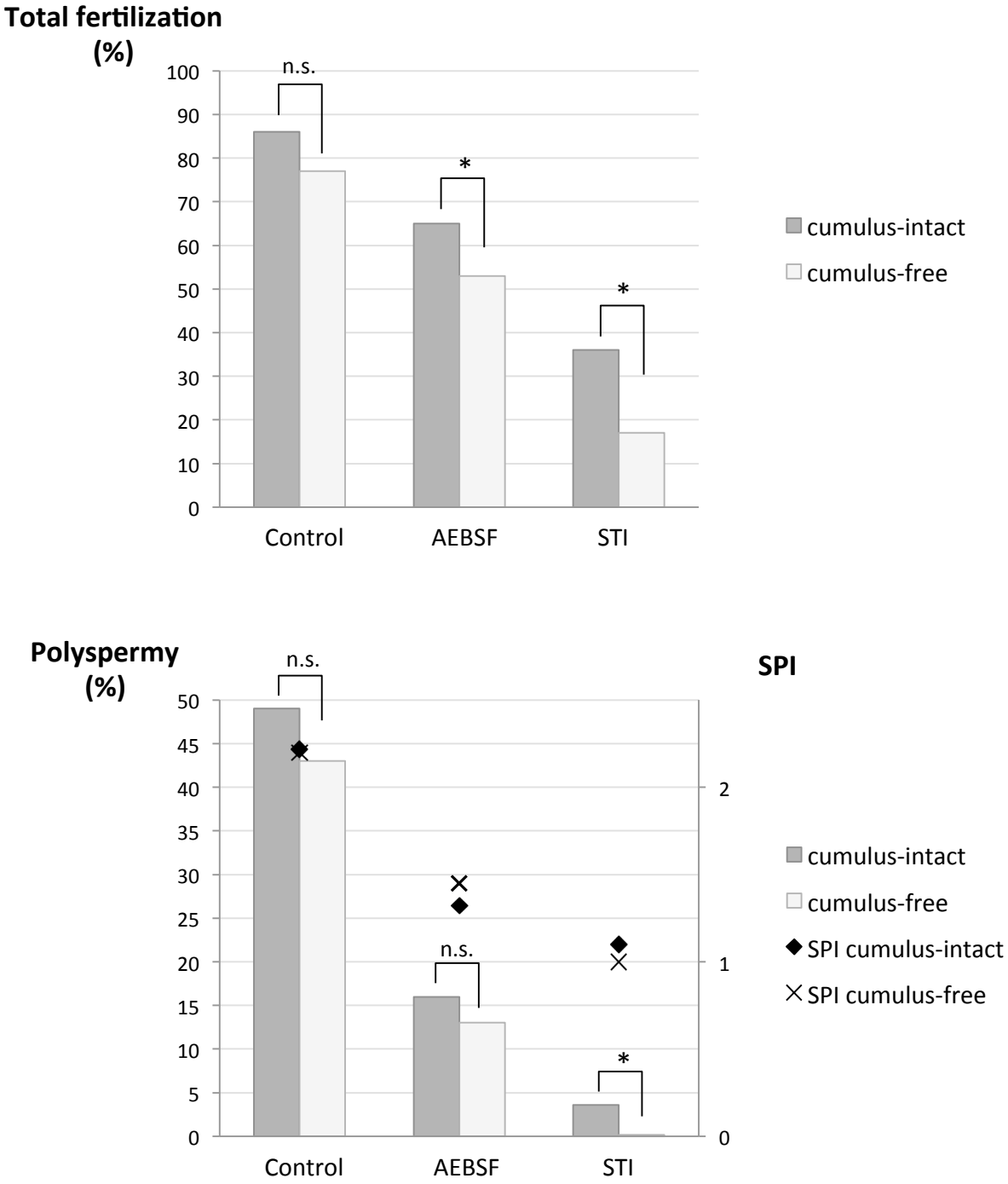

Figure 12: Effect of AEBSF (100 µM) and STI (5 µM) on total fertilization rate, polyspermy rate and the sperm penetration index (SPI) in cumulus-intact (CI) and cumulus-free (CF) porcine oocytes. Data represent mean of 3 replicates (total n = 820 oocytes). Values significantly different from control ($P<0.05$) are marked with different capitals (CI oocytes) or different small letters (CF oocytes). * ($P<0.05$). n.s.: not significant.

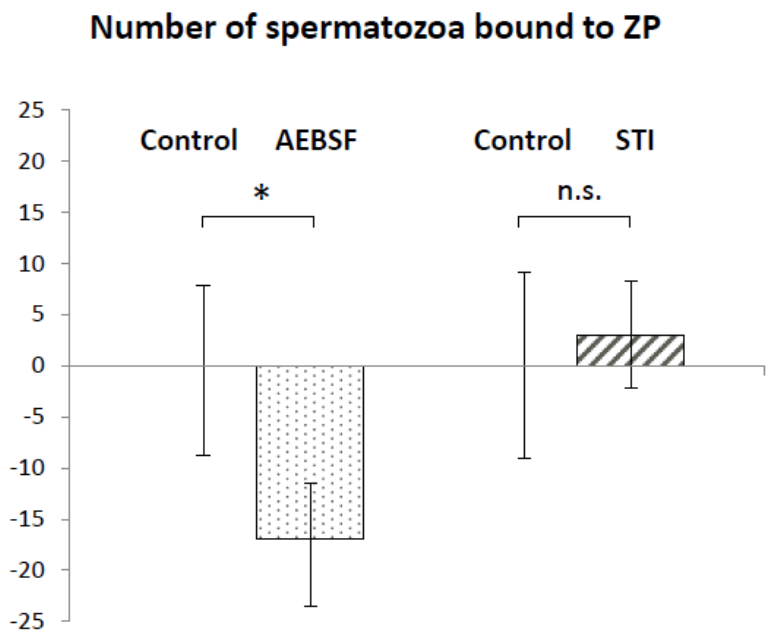

Figure 13: Differences in mean number of spermatozoa bound to the zona pellucida (ZP) compared to the control group after 4 h of gamete co-incubation in medium with either AEBSF (100 µM) or STI (5 µM). Data represent mean difference ± SD of 3 replicates (total n = 240 oocytes). *($P<0.05$). n.s.: not significant.

Effect of pre-incubation of gametes with serine protease inhibitors AEBSF and STI on fertilization parameters after porcine IVF

The results of the pre-incubation experiments are presented in Fig. 14. Pre-incubation of COCs with 100 µM AEBSF for 30 min prior to fertilization significantly decreased total fertilization rate compared to the control group: 83.0% (AEBSF) versus 93.5% (control). Polyspermy rate was also significantly lower when COCs were pre-incubated in fertilization medium with AEBSF (100 µM). Pre-incubation of spermatozoa with AEBSF prior to IVF did not affect fertilization parameters.

When COCs were pre-incubated with STI (5 µM), only 46% of the COCs became fertilized during subsequent IVF, compared to 75% of the COCs in the respective control group. Similarly, polyspermy rate and the number of penetrated sperm per fertilized oocyte were lower in COCs pre-incubated in 5 µM STI compared to COCs pre-incubated in medium without protease inhibitor ($P<0.05$). Pre-incubation of spermatozoa with STI (5 µM) resulted in a significant reduction of total fertilization rate, polyspermy rate and sperm penetration index compared to the control group.

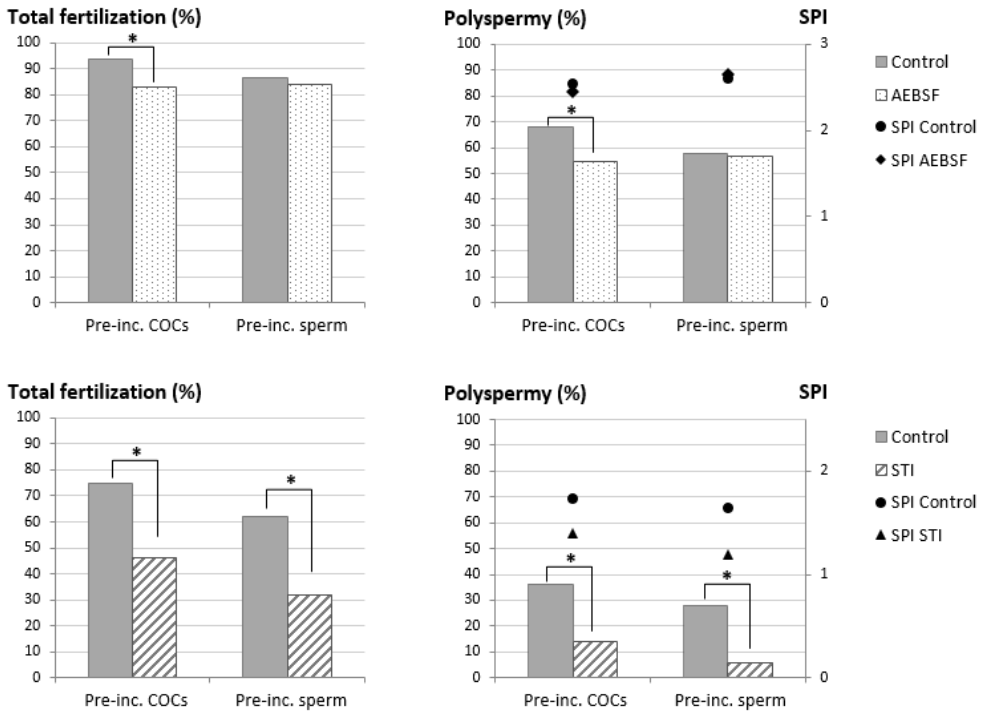

Figure 14: Effect of gamete pre-incubation with AEBSF (100 μM) and STI (5 μM) on total fertilization rate, polyspermy rate and the sperm penetration index (SPI). Data represent mean of 3 (STI, n = 523) and 4 (AEBSF, n = 756) replicates. *(P<0.05).

The effect of serine protease inhibitors on the calcium-induced acrosome reaction

The acrosomal status of spermatozoa in medium without calcium-ionophore was not influenced by incubation with 100 µM AEBSF or 5 µM when compared to the control group (Fig. 15b). Treatment with calcium ionophore induced the acrosome reaction in the majority of spermatozoa in the full response control, with only 13.3 ± 5.7% of spermatozoa with intact acrosome after 1 h of incubation. Inhibitors AEBSF and STI significantly inhibited the calcium-induced acrosome reaction ($P<0.05$). In the presence of 100 µM AEBSF, 59.6 ± 8.5% of the spermatozoa remained acrosome intact. In 5 µM STI, even 88.6 ± 5.6% of the spermatozoa remained acrosome intact (Fig. 15b).

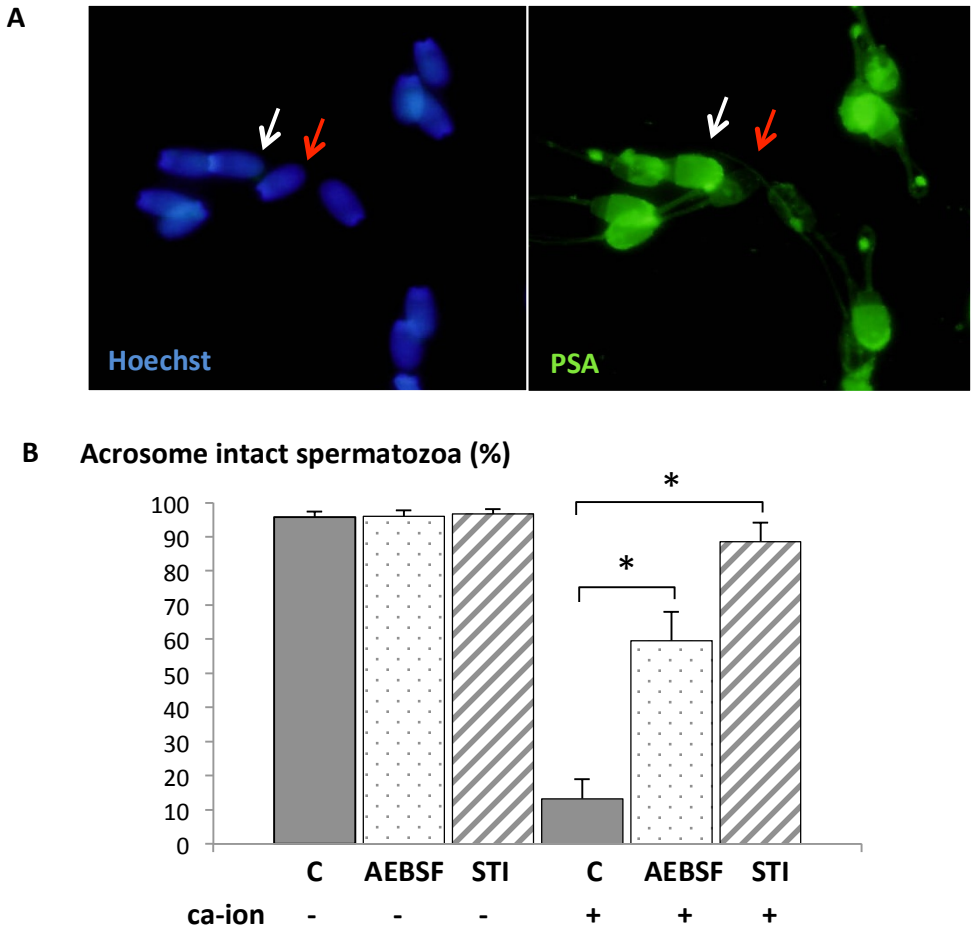

Figure 15. A: Fluorescent images of spermatozoa stained with FITC-labeled Pisum Sativum Agglutinin (PSA) and Hoechst (original magnification X 600). White arrow indicates an acrosome-intact sperm cell, red arrow an acrosome-reacted sperm cell. **B:** Percentage of acrosome intact spermatozoa after 1 h incubation in control medium, in medium with AEBSF (100 µM) or in medium with STI (5 µM), either without (-) or with (+) supplementation of calcium ionophore (A23187). Data represent mean ± SD of 3 replicates. *($P<0.05$).

Discussion

Serine proteases are generally classified according to their proteolytic activity. Three main families can be distinguished: trypsin-like, chymotrypsin-like and elastase-like serine proteases (Beynon and Bond 2001). The present study investigated the effect of two serine protease inhibitors, AEBSF and STI, during different steps of porcine IVF. Inhibitor AEBSF is a broad spectrum serine protease inhibitor and inhibits serine proteases of all three families irreversibly. The trypsin inhibitor from Glycine max (Soybean) Type I-S (STI) inhibits proteases with mechanisms similar to trypsin, so-called trypsin-like serine proteases in a reversible manner (Kunitz 1947).

The functional significance of proteases in sperm passage through the cumulus matrix is largely unknown, whereas sperm hyaluronidase and sperm motility are generally accepted to be imperative (Yanagimachi 1994; Primakoff and Myles 2002). During porcine IVF, metalloprotease activity was shown to be involved in sperm passage through the cumulus matrix (Beek *et al.* 2012). Inhibitors of zinc-dependent metalloproteases induced a strong inhibitory effect on sperm penetration in CI oocytes, but not in CF oocytes. In the present study, sperm passage through the cumulus was not hindered by serine protease inhibitors. When AEBSF or STI were added during porcine IVF of CI and CF oocytes, the inhibitory effect on total fertilization rate was less pronounced in CI oocytes than in CF oocytes. Nevertheless, in both CI and CF oocytes, sperm penetration and polyspermy rate decreased significantly in the presence of a serine protease inhibitor.

Sperm-oolemma binding and IVF of zona-free oocytes were unaltered in the presence of AEBSF or STI, which indicated that these serine protease inhibitors had an effect prior to sperm-oolemma interaction. As sperm passage through the cumulus was not hindered by AEBSF or STI, our results strongly indicate that inhibitors influenced fertilization parameters via an effect on sperm-ZP binding and/or zona penetration. Previous studies have described similar effects of trypsin-like serine protease inhibitors during fertilization in rabbit (Zaneveld *et al.* 1971; Stambaugh and Buckley 1972), hamster (Miyamoto and Chang 1973) and mouse (Saling 1981). Moreover, these early reports of decreased zona penetration in the presence of serine protease inhibitors, the lytic effect of acrosomal extracts on the ZP and the necessity of the acrosome reaction for successful fertilization, are the foundation of the paradigm in which zona lysis by acrosomal proteases enables sperm to penetrate the ZP (Yanagimachi 1994).
The present study demonstrated a small but significant inhibitory effect on sperm-ZP binding by AEBSF. Based on previous research, hampered sperm motility can be excluded with a

high degree of certainty as reason for the decrease in sperm-ZP binding. More specifically, total and progressive motility were not affected until 6 h incubation in fertilization medium supplemented with 100 μM AEBSF or without inhibitor (data not shown). The effect of AEBSF in the sperm-ZP binding experiment is in line with previous studies reporting interference of sperm-ZP binding by other serine protease inhibitors in the pig (Jones and Brown 1987; Jones 1991; Urch and Patel 1991). Furthermore, analysis of the crystal structure of porcine acrosin showed the presence of an effector side that could act as receptor for ZP glycoproteins in secondary sperm binding to the ZP (Tranter *et al.* 2000). Surprisingly, inhibitor STI showed no significant effect on sperm-ZP binding, whereas inhibitors AEBSF and STI are both able to inhibit members of the family of trypsin-like serine proteases (Beynon and Bond 2001). Differences in the properties of AEBSF and STI may have caused this discrepancy. Similar to our findings, Müller-Esterl *et al.* (1983) described that low molecular weight inhibitors inhibited porcine acrosin more effectively than inhibitors with a high molecular weight and furthermore, that irreversible inhibitors appeared to perform better than reversible inhibitors.

Sperm penetration of the ZP can be divided in 1) binding of the sperm cell to the ZP (starting with a loose attachment followed by species-specific tight binding), 2) the acrosome reaction with release of acrosomal enzymes at the surface of the zona pellucida and subsequently 3) zona lysis and sperm penetration of the zona pellucida. The observed inhibition of zona penetration in the presence of serine protease inhibitors can be due to inhibition of one or more of these steps. As in mouse (Honda *et al.* 2002), human (De Jonge *et al.* 1989) and bovine (Deppe *et al.* 2008), serine protease inhibitors may interfere with the acrosome reaction and delay zona penetration. The effect of AEBSF and STI on the acrosome reaction of porcine spermatozoa was therefore investigated. In the presence of AEBSF or STI in the medium, less than 50% of spermatozoa completed the acrosome reaction in response to treatment with calcium ionophore, compared to more than 85% in medium without inhibitor. Furthermore, the inhibitory effect of STI on the acrosome reaction induced by calcium ionophore was about 1.5 times stronger than the inhibitory effect of AEBSF. Exocytosis of the acrosomal content is a prerequisite for successful fertilization as only acrosome-reacted spermatozoa are able to penetrate the ZP (Yanagimachi 1994). Therefore, we consider inhibition of the acrosome reaction by serine protease inhibitors a major reason for the decrease in sperm penetration during porcine IVF.

It remains to be clarified to what extent inhibition of acrosomal proteases involved in zona lysis has contributed to the decrease in sperm penetration, as by our approach we could not distinguish between interference with the release of acrosomal content and zona lysis. This requires a more complex experimental design. Real time imaging of sperm-oocyte interaction could be a first approach to evaluate the time necessary for zona penetration in medium with and without serine protease inhibitors. However, the starting point of zona penetration is the release of acrosomal content, real time imaging should thus be accompanied by fluorescent live staining of spermatozoa allowing to evaluate the onset and completion of the acrosome reaction. For now, there are only few studies in mouse in which the release of acrosomal content and the subsequent zona lysis was studied separately (Yamagata *et al.* 1998a; Yamagata *et al.* 1998b).

Pre-incubation of oocytes with AEBSF and STI reduced sperm penetration rate in subsequent IVF, which was not in line with the results of the fertilization experiments with zona-free oocytes and the predominant effect of inhibitors on sperm penetration of the ZP. The ambiguity between the results from the oocyte pre-incubation experiment versus the other experiments cannot readily be explained and we can only speculate about possible reasons. To us, the most probable explanation is that the viscous matrix of the cumulus oophorus might have captured protease inhibitor after which, by carry over to the fertilization droplet, the inhibitor could have been present during sperm interaction with the ZP. The incorporation of proteins from follicular fluid or medium into the cumulus matrix of COCs has been observed before (Bijttebier *et al.* 2009).

The high incidence of polyspermic fertilization during porcine IVF is still a major problem to be solved. Several modifications of the IVF protocol have been proposed to minimize polyspermy rate (Funahashi and Nagai 2000; Li *et al.* 2003; Almiñana *et al.* 2008; Park *et al.* 2009). The finding that serine protease inhibitors were able to decrease sperm penetration raised the question whether this characteristic could be used to minimize polyspermic fertilization, preferentially without affecting total fertilization rate. Unfortunately, the decrease in polyspermy rate by inhibitors AEBSF and STI coincided with a decrease in total fertilization. Possibly, the use of protease specific inhibitors would allow to regulate sperm penetration more precisely and to increase efficiency of porcine IVF.

In conclusion, the results of the present *in vitro* study confirm the involvement of a serine protease in sperm binding to the ZP and demonstrate a role for trypsin-like serine protease activity in the acrosome reaction. Further research is warranted to unravel the identity of the involved serine proteases in combination with their exact mode of action.

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CHAPTER 5

INHIBITORS OF ZINC-DEPENDENT METALLO-PROTEASES HINDER SPERM PASSAGE THROUGH THE CUMULUS OOPHORUS DURING PORCINE FERTILIZATION *IN VITRO*

Modified from

INHIBITORS OF ZINC-DEPENDENT METALLOPROTEASES HINDER SPERM PASSAGE TRHOUGH THE CUMULUS OOPHORUS DURING PORCINE FERTILIZATION *IN VITRO*

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Abstract

In the present study we report for the first time on a possible contribution of metalloproteases in sperm passage through the cumulus matrix in pigs. The presence of 20 μM 1,10-Phenanthroline (1,10-PHEN), inhibitor of zinc-dependent metalloproteases, strongly inhibited the degree of sperm penetration in cumulus-intact (CI), but not in cumulus-free (CF) porcine oocytes during IVF. The inhibitory effect of 1,10-PHEN was due to the chelation of metal ions since a non-chelating analogue (1,7-PHEN) did not affect *in vitro* fertilization rates. Furthermore, incubation with 1,10-PHEN did not affect sperm binding to the zona pellucida nor sperm motility, membrane integrity or acrosomal status. These findings led to the assumption that 1,10-PHEN interacts with a sperm- or cumulus-derived metalloprotease. Metalloproteases are key players in physiological processes involving degradation or remodeling of extracellular matrix. *In vivo*, their proteolytic activity is regulated by tissue inhibitors of metalloproteases (TIMP-1 - TIMP-4). We tested the effect of TIMP-3 on fertilization parameters after porcine IVF. Similarly to 1,10-PHEN, TIMP-3 inhibited total fertilization rate of CI but not CF oocytes, and did not influence sperm quality parameters. Although the inhibitory effect was stronger in CI oocytes, TIMP-3 also reduced the degree of sperm penetration in CF oocytes suggesting the involvement of a metalloprotease in a subsequent step during fertilization. In conclusion, our results indicate the involvement of TIMP-3-sensitive, zinc-dependent metalloprotease in sperm passage through the cumulus oophorus in pigs. The results should provide the basis for further biochemical research towards the localization and identification of the metalloprotease involved.

Introduction

During mammalian fertilization, the sperm cell interacts with the extracellular layers of the oocyte, the cumulus oophorus and the zona pellucida (ZP), to finally reach the oocyte plasma membrane. The cumulus oophorus consists of cumulus cells embedded in a matrix rich in hyaluronic acid. Traditionally, sperm hyaluronidases in combination with sperm motility are thought to enable spermatozoa to pass through the cumulus matrix and reach the ZP (Kim *et al.* 2008). The sperm binding to the ZP is then believed to induce the acrosome reaction with release of the acrosomal content, *e.g.* hydrolytic enzymes which serve in sperm penetration of the glycoprotein coat surrounding the oocyte, the ZP. However, due to recent findings, this accepted model of the acrosome reaction needs to be re-evaluated. At least in the mouse, not only the ZP but also the cumulus oophorus appears to be a physiological site of the acrosome reaction (Yin *et al.* 2009; Jin *et al.* 2011; Sun *et al.* 2011).

Several proteases have been found on sperm membranes and in the acrosome of mammalian spermatozoa, including a collagenase-like peptidase, a cathepsin D-like protease, dipeptidyl peptidase II, (pro)acrosin, trypsin-like proteases other than acrosin and testicular serine protease 5 (TESP5) (Honda *et al.* 2002). In pigs, the sperm-specific serine protease acrosin (Polakoski and McRorie 1973; Polakoski *et al.* 1973; Brown and Cheng 1985) as well as the proteasome, a multi-subunit protease with specificity for ubiquitinated protein substrates, have been implicated in fertilization of cumulus-free oocytes (Sutovsky *et al.* 2004; Yi *et al.* 2007). Rather surprisingly, most of the studies on the role of proteases in mammalian fertilization did not evaluate sperm passage through the cumulus, although in most mammals the cumulus oophorus is still present at the time of fertilization and can thus be considered as the first site of sperm-oocyte interaction (Van Soom *et al.* 2002). As a consequence, little is known about the functional significance of proteases in sperm passage through the cumulus oophorus. However, proteolytic activity may facilitate sperm passage through the viscous cumulus matrix by hydrolysis of bonds between hyaluronic acid and hyaluronic acid-binding proteins.

Three families of metalloproteases are acknowledged to modify and degrade extracellular matrix, the matrix metalloproteases (MMPs), the “A Disintegrin And Metalloprotease” family of proteases (ADAM) and the ADAM proteases with thrombospondin motifs (ADAMTS) (Shiomi *et al.* 2010). Their catalytic site typically contains a zinc binding motif and cleavage of substrates requires a zinc ion. The MMP family is mainly subdivided according to their domain structures and substrate specificity, *e.g.* collagenases (MMP1, MMP8, MMP13),

gelatinases (MMP2 and MMP9) and stromelysins (MMP3, MMP10). Most of the MMPs are secreted, although there are at least six membrane-type MMPs which contain a transmembrane domain (Nagase *et al.* 2006). Some secreted MMPs can be associated with cells by interaction with cellular proteins. For example, MMP9 interacts with the hyaluronic acid receptor CD44 (Yu and Stamenkovic 1999) and MMP2 can bind to integrin αvβ3 (Brooks *et al.* 1996). The main function of MMPs has been considered to be the hydrolysis of matrix components. Next to that, MMPs may have a more complex role and function to disrupt cell-cell and cell-matrix interactions and mediate release of growth factors and cytokines (Stetler-Stevenson and Yu 2001; Shiomi *et al.* 2010).

The diverse functions addressed to ADAMs can be explained by their typical structure containing a disintegrin domain and a metalloprotease domain (Reiss and Saftig 2009). They are capable of mediating cell adhesion and migration via their disintegrin domain (*e.g.* integrin binding) as well as via proteolysis of cell adhesion molecules. ADAMs also have a prominent role in signaling pathways by cleavage of membrane-bound proteins and release of biologically active factors, such as growth factors. Most of the ADAMs are membrane-anchored proteins but some members are secreted and are shown to cleave extracellular matrix components, such as ADAMTS1 and ADAMTS4 (Russell *et al.* 2003). ADAMTS1 and ADAMTS4 are both produced and secreted by porcine cumulus cells (Shimada *et al.* 2004). It has been hypothesized that ADAMTS1-mediated cleavage of versican, present in the cumulus matrix, may serve to stabilize the expanding matrix of the cumulus-oocyte-complex (COC) by release of 70 kDa N-terminal fragment that binds to hyaluronic acid (Shimada *et al.* 2004). Studies with ADAMTS1$^{-/-}$ mice have demonstrated a lower cleavage of versican in expanded COCs, an impaired structural organization of the extracellular matrix, and also a delay in the degradation of the COC matrix after ovulation (Brown *et al.* 2010). Thus, ADAMTS1 may also function in the gradual shedding of cumulus cells and disassembly of the matrix after ovulation, which could enhance sperm penetration at the time of fertilization.

The objective of the present study was to determine the possible involvement of metalloproteases during porcine fertilization *in vitro*. Since metalloproteases are associated with breakdown and remodeling of extracellular matrix, we focused on IVF experiments with cumulus-intact oocytes to evaluate the effect of metalloprotease inhibitors on sperm passage through the cumulus oophorus.

Materials and methods

Media

All chemicals used in this study were purchased from Sigma-Aldrich (Bornem, Belgium), unless otherwise stated. The basic medium used for the collection and washing of cumulus oocyte complexes (COCs) was a modified HEPES-buffered Tyrode balanced salt solution (HEPES-TM) with 10 µg/ml gentamycin sulfate, 10 mM HEPES and 3 mg/ml BSA. Immature oocytes were matured in BSA-free 'North Carolina State University' 23 (NCSU23) (Petters and Wells 1993) supplemented with 0.57 mM cysteine, 10 ng/ml epidermal growth factor, 10 IU/ml equine Chorionic Gonadotropin (eCG) (Folligon®, Intervet, The Netherlands), 10 IU/ml human Chorionic Gonadotropin (hCG) (Chorulon®, Intervet, The Netherlands) and 10% porcine follicular fluid. Follicular fluid was collected from 5-6 mm follicles of prepubertal gilts. After centrifugation of follicular fluid (100x*g*, 10 min), the supernatant was aspirated, filtered (0.22 µm) and stored at -80ºC until use. The basic medium for IVF was Tyrode's albumin lactate pyruvate medium (TALP medium) (Rath *et al.* 1999) supplemented with 0.3% BSA (FERT-TALP). Frozen-thawed epididymal boar spermatozoa were centrifuged (390x*g*, 3 min) in Androhep extender consisting of 27 mM Tri-natriumcitraat, 2.7 mM Titriplex III EDTA, 2.5 mg/ml BSA (fraction V), 14 mM $NaHCO_3$, 38 mM Hepes, 144 mM D(+)-glucose.H_2O and 50 µg/ml gentamycine sulfate. Presumed zygotes were washed in HEPES-buffered TALP medium (HEPES-TALP), i.e. TALP medium with 25 mM HEPES. The embryo culture medium was NCSU23 with 0.4% BSA.

Protease inhibitors

Protease inhibitors were purchased from Sigma-Aldrich (Bornem, Belgium), and used at different concentrations. Both 1,10-PHEN (P9375) and 1,7-PHEN (301841) were dissolved in methanol (stock solution: 200 mM). Phosphoramidon was dissolved in FERT-TALP medium (500 µM) and recombinant human TIMP-3 (TIMP-3) was dissolved in deionized water (1000 nM).

Oocyte collection

Ovaries of prepubertal gilts were collected at a local slaughterhouse and prepared following the protocol of Bijttebier *et al.*(2008). Briefly, immature cumulus-oocyte-complexes (COCs) were aspirated from follicles with a diameter ranging from 3 to 6 mm. Only COCs with a homogeneous ooplasm and a multilayered cumulus were selected.

In vitro *maturation (IVM) and* in vitro *fertilization (IVF) of porcine cumulus-oocyte-complexes*

Groups of 100 immature COCs were placed into 500 µl maturation medium and cultured for the first 22 h (39°C, 5% CO_2). Subsequently, COCs were cultured in hormone-free maturation medium for 22 h. After *in vitro* maturation of COCs, protease inhibitor dilutions were prepared in concentrated stocks and added to droplets of FERT-TALP to obtain the final concentration required. Since TIMP-3 was dissolved in deionized water, the concentration of components in the medium was adjusted by adding FERT-TALP-2x in the same volume as the stock solution of TIMP-3 to each fertilization droplet. Subsequently, the COCs were randomly assigned to the fertilization droplets (50 COCs/droplet). Frozen epididymal semen was thawed for 60 sec in a water bath at 38°C and spermatozoa were washed by centrifugation (3 min at 390xg) in 9.5 ml of Androhep. The sperm pellet was resuspended in 1 ml FERT-TALP and sperm concentration was assessed using a Bürker counting chamber. Spermatozoa were added to the droplets containing the COCs resulting in a final concentration of $1.25x10^5$ spermatozoa/ml. The control group consisted of COCs fertilized under standard conditions. After a co-incubation period of 6 h, the presumed zygotes were vortexed during 3 min in 2.5 ml HEPES-TALP to remove loosely bound spermatozoa, washed three times in culture medium and cultured for 18 h in a modular incubator chamber (39°C, 5% CO_2).

Assessment of fertilization parameters

Approximately 24 h after insemination, presumed zygotes were washed in 0.1% (w/v) poly-vinyl pyrrolidone (PVP) in phosphate buffered saline (PBS). Subsequently, the zygotes were fixed overnight with 4% paraformaldehyde in PBS and stained with 10 µg/ml *bis*-benzimide (Hoechst 33342; Molecular Probes, Leiden, The Netherlands) for 10 min. Zygotes were mounted in a droplet of glycerol with (25 mg/ml) 1,4-diazabicyclo (2.2.2) octane (DABCO; Acros, Ghent, Belgium) and nuclear DNA was visualized using a Leica DMR fluorescence microscope (Leica Microsystems, Belgium). Oocytes with a metaphase plate and a polar body were classified as being in the MII stage. The presence of two pronuclei or cleaved embryos with two equally sized blastomeres was indicative for fertilization, whereas oocytes without penetrated sperm heads were considered as not fertilized. Zygotes with more than two pronuclei or more than one decondensed sperm head were classified as polyspermic. In case of polyspermic fertilization, the number of penetrated spermatozoa was counted. The following fertilization parameters were assessed: total fertilization rate (%), monospermy (%),

polyspermy (%) and the sperm penetration index (mean number of penetrated spermatozoa per fertilized oocyte).

Effect of metalloprotease inhibitors on fertilization parameters after porcine IVF

Cumulus-oocyte-complexes were fertilized in the presence of 1,10-PHEN (5 μM, 20 μM) (n=483) or Phosphoramidon (10 μM, 100 μM) (n=453). Control COCs were fertilized under standard conditions (n=251 and n=245 for the experiments with 1,10-PHEN and Phosphoramidon, respectively). Each experimental unit consisted of 2 droplets of 40-50 oocytes. This experiment was repeated three times for each protease inhibitor.

Effect of metalloprotease inhibitors on fertilization parameters after IVF of cumulus- intact versus cumulus- free porcine oocytes

In vitro matured COCs were randomly assigned to 8 groups of approximately 40 oocytes each (3 replicates). Half of the COCs (4 groups) were denuded by vortexing with 0.1% (w/v) hyaluronidase in HEPES-TM (cumulus-free or CF), the other half were kept cumulus-intact (CI). Both CF and CI oocytes were fertilized under standard conditions (n=291) and in the presence of either 1,10-PHEN (20 μM) (n=273), 1,7-PHEN (20 μM) (n=279) or TIMP-3 (400nM) (n=302). Final inhibitor concentrations were based on preliminary dose-response experiments. The CF and CI oocytes were co-incubated with $1.25x10^5$ spermatozoa/ml for 6 h (39°C, 5% CO_2). Prior to fixation, all groups were vortexed to remove excess spermatozoa and cultured during 18 h. After Hoechst staining, the oocytes were evaluated for fertilization parameters as described above.

Effect of 1,10-PHEN on sperm-zona binding

In vitro matured COCs were denuded by vortexing with 0.1% (w/v) hyaluronidase in HEPES-TM and randomly assigned to 2 groups of 20 oocytes (3 replicates). The first group was fertilized in standard fertilization medium (control), the second group in the presence of 20 μM 1,10-PHEN. For unambiguous counting of the number of spermatozoa bound to the zona pellucida, oocytes were co-incubated with $0.25x10^5$ spermatozoa/ml (39°C, 5% CO_2). After 4 h of co-incubation (Fazeli *et al.* 1995), the oocytes were washed five times in PBS to remove loosely bound spermatozoa, and subsequently fixed and stained with Hoechst 33342. Of each oocyte, the average of two counts was taken into account as the number of sperm bound to the zona pellucida.

Evaluation of sperm membrane integrity, sperm motility and acrosomal status

To exclude a possible negative influence on spermatozoa, parameters related to sperm quality were evaluated over time in the presence of protease inhibitor concentrations which were used in the experiments. Sperm membrane integrity, motility and the acrosomal status were evaluated at different time points during incubation.

Boar semen samples were diluted in HEPES-TM to obtain a concentration of $20x10^6$ spermatozoa/ml and warmed in an incubator at 37ºC. After 30 min of warming, aliquots of diluted semen were incubated with and without the protease inhibitor concerned. Then at different time points (0 h, 1 h, 4 h and 6 h), one aliquot per test group was evaluated for membrane integrity. Membrane integrity was assessed using eosin-nigrosin staining which is considered a reliable and feasible technique (Maes *et al.* 2011). The average of three counts of 100 spermatozoa per aliquot was taken into account. Total and progressive motility were assessed by means of computer-assisted sperm analysis (Hamilton Thorne). After 0 h, 1 h, 2 h and 6 h of incubation, 5 µl of each sperm fraction was mounted on a Makler Counting Chamber and maintained at 37°C using a minitherm stage warmer. Five randomly selected microscopic fields were scanned for 5 times each. Statistical analysis was performed using the mean of the 5 scans of each microscopic field.

Acrosomal status was evaluated using fluorescein isothyiocyanate-conjugated Pisum sativum agglutinin (PSA-FITC; L0770, Sigma-Aldrich, Bornem, Belgium). Frozen epididymal semen was thawed for 60 sec in a water bath at 38°C and spermatozoa were washed by centrifugation (3 min at 390xg) in 9.5 ml of Androhep. The sperm pellet was resuspended in FERT-TALP to obtain a sperm concentration of $2x10^6$ sp/ml and divided into 6 aliquots of 1 ml. Aliquot 1 without inhibitor was used as negative control. A second aliquot was supplemented with 1 µM A23187 calcium ionophore as full response control. Sperm aliquots 3 and 4 were treated with 20 µM 1,10-PHEN, aliquots 5 and 6 were treated with 400 nM TIMP-3. Sperm aliquots 4 and 6 were then supplemented with 1 µM A23187 calcium ionophore to evaluate the response in the presence of a protease inhibitor. All incubations were carried out for 1 h at 39°C in a 5% CO_2 incubator. Subsequently, 1 µl of Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) was added to each aliquot and sperm aliquots were incubated for 3 min at 37°C. After centrifugation at 720 x g for 10 min, the supernatant was removed and the sperm pellets were resuspended in 50 µl of 96% ethyl alcohol and incubated for 30 min at 4°C. Afterwards, 15 µl of each sperm aliquot was smeared on a glass slide and air-dried. Then 15 µl of PSA-FITC (2 mg PSA-FITC diluted in 2 ml PBS) was

added. The glass slides were kept for 15 min at 4°C, washed 5 times with deionized water and air-dried. At least 100 spermatozoa in each sperm aliquot were evaluated using a Leica DMR fluorescence microscope (Leica Microsystems, Belgium). All spermatozoa were stained with Hoechst. The acrosomal region of acrosome-intact spermatozoa was PSA-FITC positive and labeled green, while acrosome-reacted spermatozoa retained only an equatorial labeled band with little or no labeling of the anterior head region.

Visualization of spermatozoa within the cumulus oophorus

Frozen-thawed spermatozoa were washed by centrifugation (3 min at 390xg) in 9.5 ml of Androhep. The sperm pellet was diluted in HEPES-TM to a concentration of $20x10^6$ spermatozoa/ml and spermatozoa were incubated with 250 nM Mitotracker (M7511, Molecular probes, Leiden, The Netherlands) during 20 min at 38°C. Subsequently, spermatozoa were washed twice by centrifugation in HEPES-TM (3 min at 390xg) and diluted in FERT-TALP. The mitotracker-labeled spermatozoa were added to 25 µl fertilization droplets containing *in vitro* matured COCs (1 COC per droplet) resulting in a final concentration of $1.25x10^5$ spermatozoa/ml. Gamete co-incubation was performed in standard FERT-TALP medium and in FERT-TALP with 20 µM 1,10-PHEN. After 2, 4 and 6 h of incubation, COCs were washed individually in PBS and fixed with 2% paraformaldehyde for 20 min at room temperature. To stain the nuclei, COCs were incubated with 10 µg/ml Hoechst 33342 for 3 min at room temperature, followed by two washes in PBS. Each COC was mounted in glycerol with DABCO and evaluated for the presence of mitotracker-labeled spermatozoa in the cumulus oophorus using a Leica DMR fluorescence microscope (Leica Microsystems, Belgium).

Statistical analysis

Differences in percentages of total fertilization and polyspermy rate were analyzed by means of binary logistic regression. ANOVA was used to evaluate the sperm penetration index and the differences in mean number of spermatozoa bound to the ZP. Differences in membrane integrity, sperm motility and acrosomal status were evaluated using repeated measures analysis of variance. Hypothesis testing was performed using a significance level of 5% (two-sided tests)(SPSS 16.0).

Results

Fertilization parameters after porcine IVF are affected by 1,10-Phenanthroline due to its capacity to chelate metal ions

The possible involvement of metalloproteases in porcine fertilization was evaluated by assessing the inhibitory effect of two metalloprotease inhibitors, Phosphoramidon and 1,10-Phenanthroline (1,10-PHEN). Phosphoramidon is often used in the classification of newly identified metalloproteases (Beynon and Bond 2001) although it has the limitation of targeting mainly bacterial metalloproteases and only few of mammalian origin (Bond and Butler 1987). The metal ion chelator 1,10-PHEN can be used to inhibit zinc-dependent metalloproteases, without affecting the Ca^{2+} in the fertilization medium, as it has a much higher stability constant for Zn^{2+} than for Ca^{2+} (Correa *et al.* 2000). Both inhibitors were used at different concentrations within the range of effective concentrations reported by the manufacturer, and 1,10-PHEN was also used in lower concentrations. The presence of Phosphoramidon (1-100 μM) during porcine fertilization *in vitro* did not affect total fertilization (control: 84.4%; Phosphoramidon 100 μM: 85.5%) nor polyspermy rate (control: 45.4%; Phosphoramidon 100 μM: 52.4%). Furthermore, the sperm penetration index (mean number of penetrated spermatozoa per fertilized oocyte) was very similar for COCs fertilized in control medium and medium with 100 μM Phosphoramidon, 2.29 and 2.31, respectively. In contrast, 1,10-PHEN (20 μM) was found to strongly decrease both total and polyspermic fertilization rate compared to the control group ($P<0.05$)(Fig. 16). The lowest concentration of 1,10-PHEN with a significant effect on fertilization rate was 20 μM. Therefore, this concentration was used in the subsequent experiments with 1,10-PHEN.

To evaluate the effect of 1,10-PHEN on sperm penetration of the cumulus oophorus, we compared fertilization parameters of cumulus-intact (CI) and cumulus-free (CF) oocytes The results showed a striking difference in inhibition of total fertilization rate between CI and CF oocytes (Fig. 17). Total fertilization rate of CI oocytes was inhibited with 90% compared to the respective control group, whereas a very small and no significant inhibition was recorded after denudation of oocytes. In CI oocytes, monospermic as well as polyspermic fertilization were markedly reduced by 1,10-PHEN ($P<0.01$), whereas in CF oocytes both parameters were not different from the control group ($P=0.469$ and $P=0.427$, respectively). Furthermore, the sperm penetration index (SPI) was significantly reduced by 1,10-PHEN in CI oocytes, 1.2 (1,10-PHEN) vs. 2.2 (control), but not in CF oocytes (SPI 2.2 and 2.1).

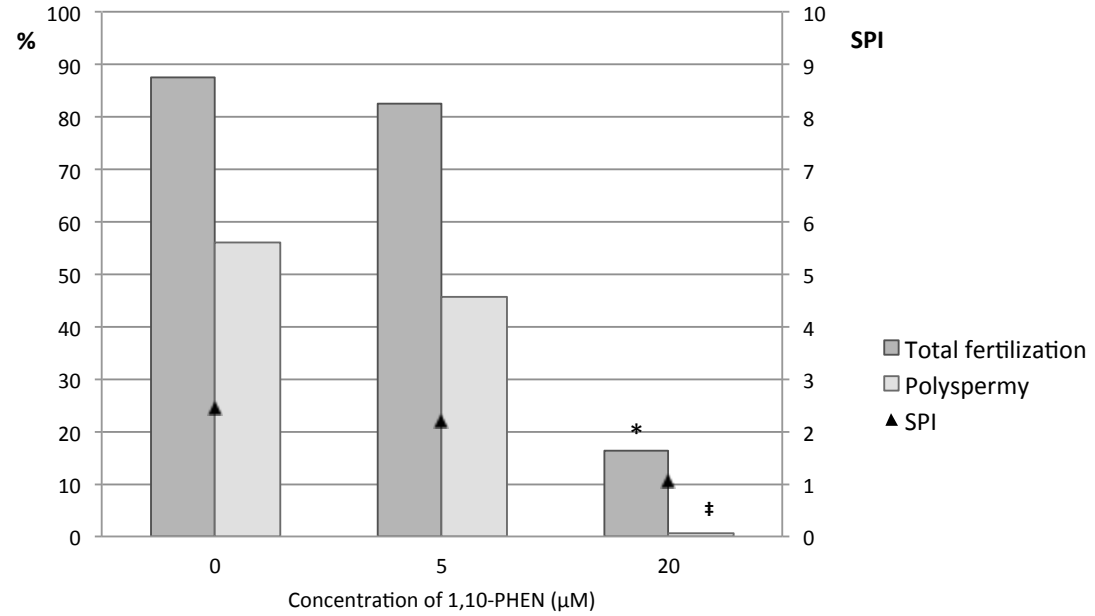

Figure 16: Effect of different concentrations of 1,10-PHEN (5 µM, 20 µM) on total fertilization, polyspermy rate and the sperm penetration index (SPI) of cumulus-intact porcine oocytes. Data represent mean of 3 replicates. Values significantly different from control ($P<0.05$) are marked with * (total fertilization) or ‡ (polyspermy).

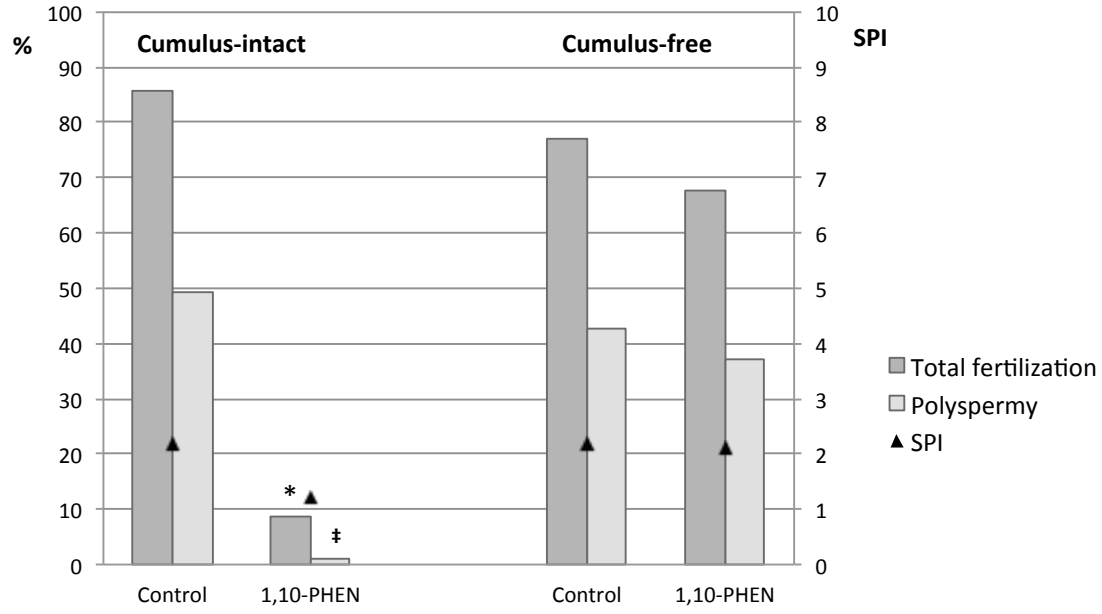

Figure 17: Effect of 20 µM 1,10-PHEN on total fertilization, polyspermy rate and the sperm penetration index (SPI) of cumulus-intact and cumulus-free porcine oocytes. Data represent mean of 3 replicates. Within each type of oocytes, values significantly different from control ($P<0.05$) are marked with * (total fertilization) or ‡ (polyspermy)

In order to confirm that the inhibitory effect of 1,10-PHEN was mediated by its capacity to bind metal ions, CI and CF oocytes were fertilized in medium with and without 20 μM of a non-chelating analogue, 1,7-PHEN. There were no significant differences in fertilization parameters between either CI or CF oocytes fertilized in standard medium or medium with 20 μM 1,7-PHEN (Fig. 18). Since 1,10-PHEN mainly targets zinc, we hypothesized the involvement of a zinc-dependent metalloprotease in sperm passage through the cumulus oophorus during porcine IVF.

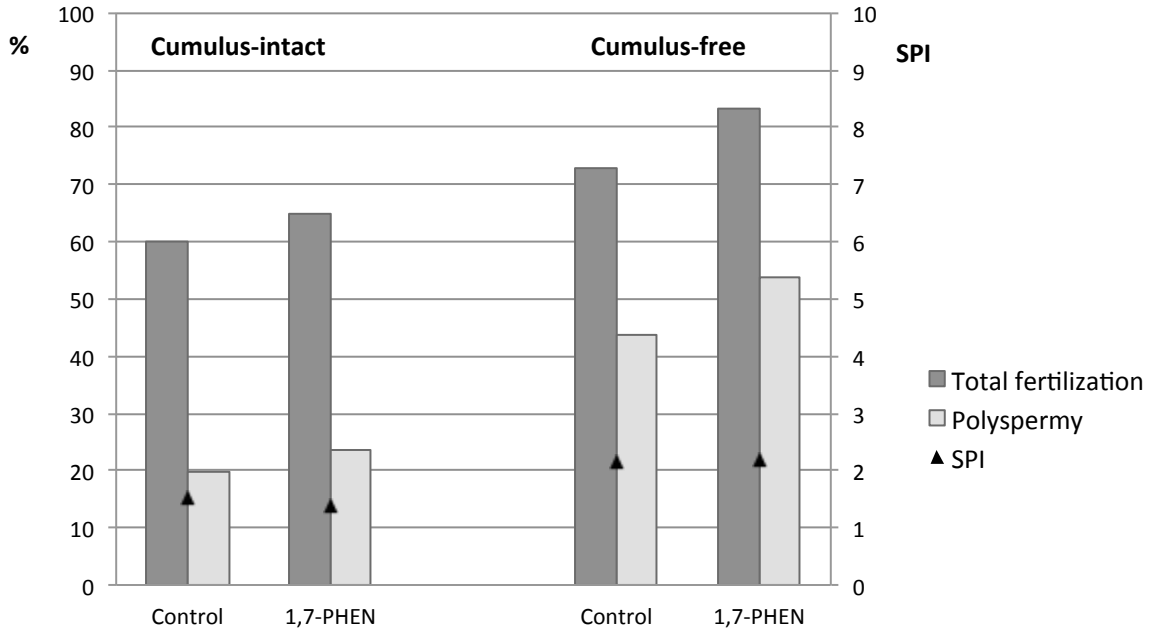

Figure 18: Effect of 20 μM 1,7-PHEN on total fertilization, polyspermy rate and the sperm penetration index (SPI) of cumulus-intact and cumulus-free porcine oocytes. Data represent mean of 3 replicates. Within each type of oocytes, none of the parameters were significantly different between control and treatment group ($P>0.05$).

When COCs were co-incubated with mitotracker-labeled spermatozoa in fertilization medium with and without 20 μM 1,10-PHEN, less spermatozoa were observed in the inner layers of the cumulus oophorus of COCs fertilized in medium with 1,10-PHEN (Fig. 19).
1,10-PHEN did not substantially affect sperm binding to the zona pellucida (ZP) of CF oocytes. After 4 h of gamete co-incubation, the average number of spermatozoa bound to the ZP (± SD) was 92 ± 26 in the control group and 73 ± 21 in the group of 1,10-PHEN. The high number of spermatozoa that bound to the zona pellucida in this experiment is dependent on the sperm concentration in the fertilization droplet (Fazeli *et al.* 1995). The major fraction of these spermatozoa would not succeed in penetration of the zona pellucida. Together these findings indicate that the inhibitory effect of 1,10-PHEN is predominantly situated at the level of sperm interaction with the cumulus.

1,10-PHEN has no significant effect on membrane integrity, motility and acrosomal status of porcine spermatozoa

To exclude a possible negative influence of 1,10-PHEN on the spermatozoa, parameters related to the fertilizing capacity of spermatozoa were evaluated during a 6 h incubation period in medium supplemented with and without 20 μM 1,10-PHEN. Sperm membrane integrity and motility were not affected by 1,10-PHEN as shown in Fig. 20A and 20B, respectively. Next to that, the acrosomal status of frozen-thawed epididymal spermatozoa incubated in 20 μM 1,10-PHEN was not significantly different ($P>0.05$) from that of spermatozoa in control medium (Fig. 20C). After 1 h of incubation with 1 μM A23187 calcium ionophore, the percentage of spermatozoa that underwent the acrosome reaction was similar in both groups. In the control group, 9.3 ± 3.1% of the spermatozoa remained acrosome intact versus 9.5 ± 3.1% of acrosome intact spermatozoa in medium with 20 μM 1,10-PHEN.

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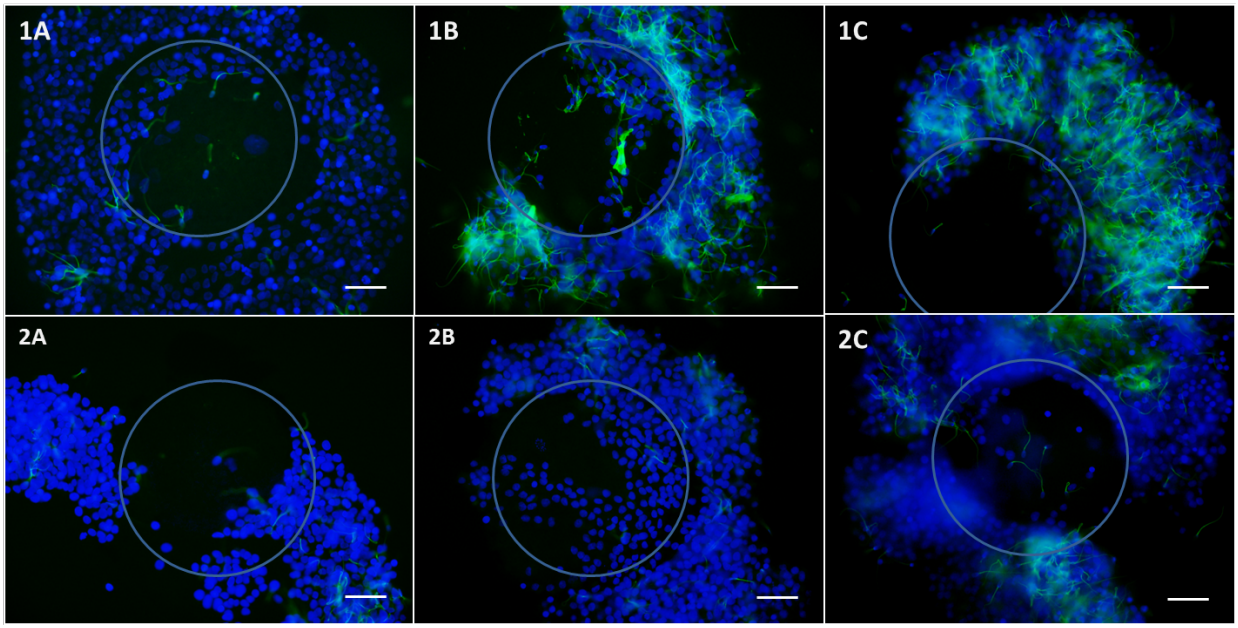
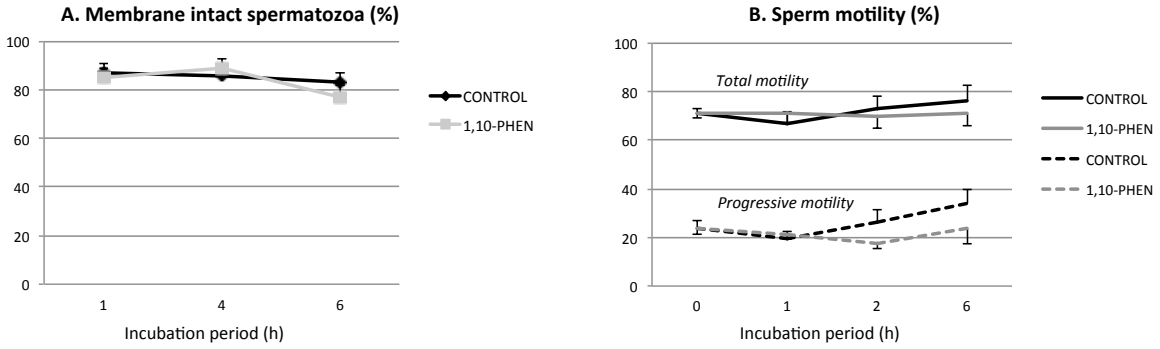


Figure 19: Visualization of spermatozoa within the cumulus oophorus after 2 h (A), 4 h (B), and 6 h (C) of gamete co-incubation in (1) standard medium and (2) medium with 20 µM 1,10-PHEN. The outline of the oocytes is represented by a blue circle. Spermatozoa were labeled by Mitotracker Green, nuclei were stained with Hoechst. (Original Magnification 400x; Bar = 25 µm).



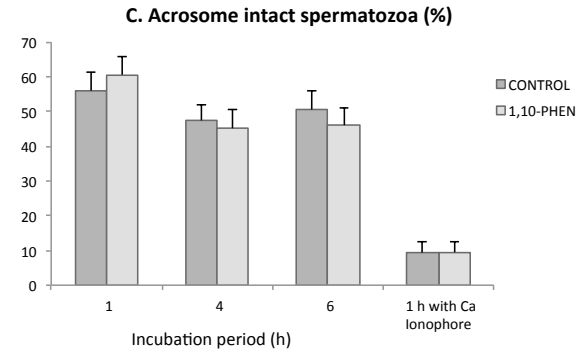


Figure 20: Effect of 20 µM 1,10-PHEN on membrane integrity (A), motility (B) and acrosomal integrity (C) of porcine spermatozoa at different time points within a 6 h incubation period. Data represent mean ± SEM of 3 replicates. None of the parameters were significantly different between control and treatment group at any of the evaluated time points (P>0.05)

Evidence for the involvement of a TIMP-3 sensitive, zinc-dependent metalloprotease in sperm passage through the cumulus oophorus during porcine IVF

Here we aimed to assess if the effect of 1,10-PHEN on sperm passage through the cumulus oophorus could be due to inhibition of a zinc-dependent metalloprotease involved in matrix breakdown and remodeling. *In vivo*, tissue inhibitors of metalloproteases (TIMPs) strictly control all active forms of matrix metalloproteases (MMPs). Four related TIMPs (TIMP-1 to TIMP-4) have each the capacity to form a 1:1 complex with all MMPs, but there is variation in the strength of inhibition. Additionally, it has been shown that TIMPs can also inhibit some members of the ADAM and ADAMTS family of metalloproteases. We tested recombinant human TIMP-3 (400 nM) and evaluated the fertilization parameters after IVF of CI and CF oocytes (Fig. 21). The presence of 400 nM TIMP-3 during gamete co-incubation decreased total fertilization rate of CI oocytes ($P<0.01$). In contrast, when CF oocytes were used, there was no significant difference in total fertilization rate between the control group and TIMP-3 ($P=0.166$). Polyspermic fertilization was markedly reduced in both CI and CF oocytes by incubation with TIMP-3 during IVF ($P<0.01$). There was also a tendency towards a lower sperm penetration index (SPI) in presence of TIMP-3, yet the differences were not statistically significant (CI oocytes: 1.5 (control) vs. 1.2 (TIMP-3); CF oocytes: 2.1 (control) vs. 1.4 (TIMP-3)). In conclusion, TIMP-3 reduces the degree of sperm penetration in both CI and CF oocytes, with a negative effect on total fertilization rate in CI oocytes but not in CF oocytes.

TIMP-3 has no significant effect on membrane integrity, motility and acrosomal status of porcine spermatozoa

To exclude a possible negative influence of TIMP-3 on the spermatozoa, membrane integrity, motility and the acrosomal status of spermatozoa were evaluated at different time points during 6 h of incubation. Sperm incubation with 400 nM TIMP-3 did not significantly affect membrane integrity or sperm motility at any of the evaluated time points ($P>0.05$; Fig. 22A + 22B). The acrosomal status of frozen-thawed epididymal spermatozoa in medium supplemented with 400 nM TIMP-3 was not significantly different from that of spermatozoa incubated in standard fertilization medium ($P>0.05$; Fig. 22C). After 1 h of incubation with 1 μM A23187 calcium ionophore, the fraction of spermatozoa that underwent the acrosome reaction was similar in both groups.

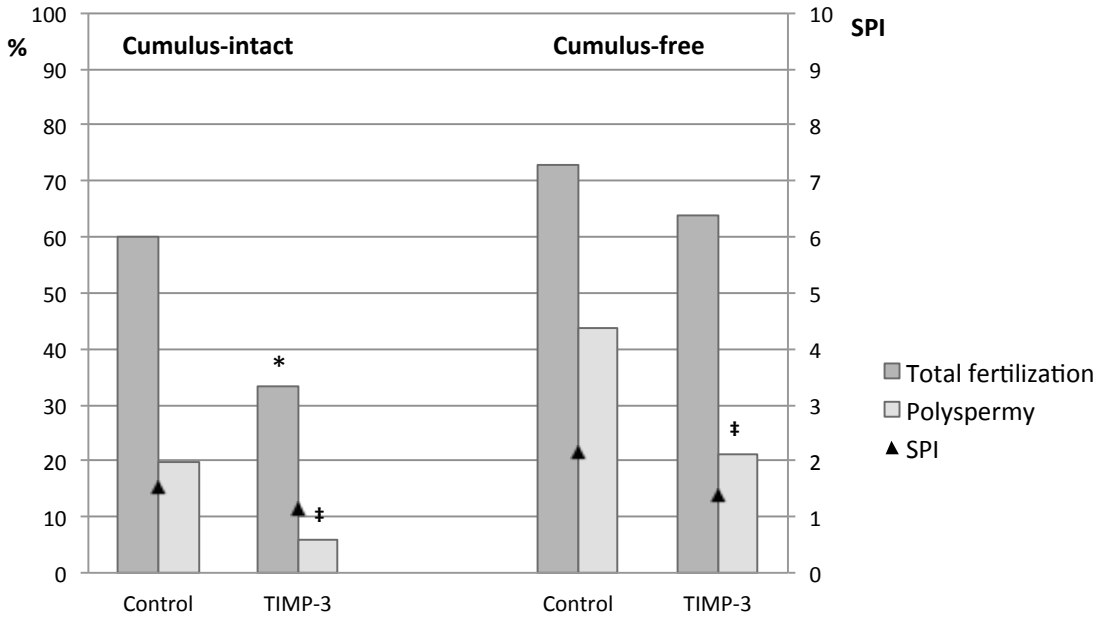


Figure 21: Effect of 400 nM TIMP-3 on total fertilization, polyspermy rate and the sperm penetration index (SPI) of cumulus-intact and cumulus-free porcine oocytes. Data represent mean of 3 replicates. Within each type of oocytes, values significantly different from control ($P<0.05$) are marked with * (total fertilization) or ‡ (polyspermy). There were no significant differences in the sperm penetration index

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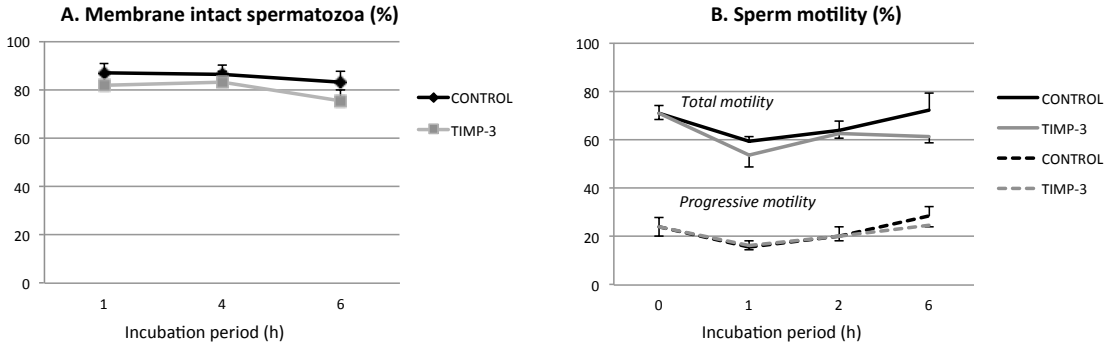

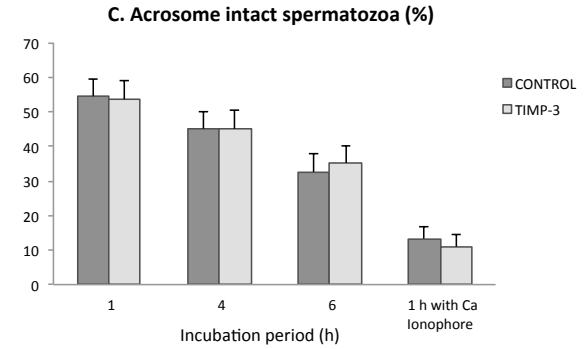

Figure 22: Effect of 400 nM TIMP-3 on membrane integrity (A), motility (B) and acrosomal integrity (C) of porcine spermatozoa at different time points within a 6 h incubation period. Data represent mean ± SEM of 3 replicates. None of the parameters were significantly different between control and treatment group at any of the evaluated time points.

Discussion

The functional significance of proteases for sperm passage through the cumulus matrix is largely unknown. Sperm hyaluronidases on the contrary, in combination with sperm motility, have been considered imperative in this step of the fertilization process (Primakoff and Myles 2002; Kim *et al.* 2008). In the present study we report for the first time on a possible contribution of metalloproteases in sperm passage through the cumulus matrix in pigs. We based our conclusions on several observations: (1) inhibition of zinc-dependent metalloproteases, by means of 1,10-PHEN, induced a strong inhibitory effect on the degree of sperm penetration in CI, but not in CF porcine oocytes; (2) the inhibitory effect of 1,10-PHEN was due to the chelation of metal ions since a non-chelating analogue (1,7-PHEN) did not affect *in vitro* fertilization rates; (3) matrix metalloprotease inhibitor TIMP-3 showed a stronger inhibitory effect on total fertilization and sperm penetration rate in CI oocytes than in CF oocytes, similar to 1,10-PHEN; and (4) the effect of the inhibitors was not mediated by a decrease in sperm quality since sperm motility, membrane integrity and acrosomal status were not altered. Whereas the inhibition profile of 1,10-PHEN is not specific (inhibition of all zinc-dependent metalloproteases via chelation of the catalytic zinc atom), the inhibitory effect by TIMP-3 points to the family of matrix metalloproteases (MMPs) and some members of the ADAM family of metalloproteases which are inhibited by TIMP-3.

Only few studies have looked into sperm-derived metalloprotease activity thus far. Buchman-Shaked *et al.* (2002) described the localization of matrix metalloprotease 2 (MMP2) at the sperm head of human spermatozoa and release of MMP2 from spermatozoa during a short time period after incubation in capacitation medium. The presence of MMP2 on spermatozoa of other species, including the pig, remains putative. Expression of ADAM1 and ADAM2 in porcine spermatozoa, on the other hand, has recently been documented (Fabrega *et al.* 2011). These two ADAMs form a hetero-dimeric complex “fertilin” that was initially isolated at the sperm surface of guinea-pig spermatozoa (Primakoff *et al.* 1987) and participates in sperm-oocyte interaction via binding to integrins (Evans 2001). Yet ADAM1 and ADAM2 are not considered as candidate enzymes for the observed effect of 1,10-PHEN since both members of the ADAM family are enzymatically inactive and therefore unsusceptible for zinc deprivation. From the eight ADAM genes identified in the pig, *ADAM8*, *ADAM9*, *ADAM10*, *ADAM15* and *ADAM17* encode for a protein with metalloprotease activity (Rawlings *et al.* 2012). TIMP-3 has been shown to inhibit ADAM10 and ADAM17 (Amour *et al.* 1998; Amour *et al.* 2000; Loechel *et al.* 2000), but not ADAM8 and ADAM9 (Amour *et al.* 2002).

It is not yet known if TIMP-3 is able to inhibit ADAM15. Especially ADAM10 and ADAM17 are of interest as putative sperm-derived metalloproteases because they mediate cleavage of cell adhesion molecules such as L-selectin, cadherins and also CD44 (Peschon *et al.* 1998; Nagano *et al.* 2004; Nagano and Saya 2004; Maretzky *et al.* 2005). CD44 is present on the surface of cumulus cells of *in vivo* and *in vitro* matured porcine COCs and attaches the cumulus cells to the matrix by interaction with hyaluronic acid (Yokoo *et al.* 2002; Yokoo *et al.* 2007). A downstream effect of CD44 cleavage is cell detachment from extracellular matrix (Nagano and Saya 2004).

One of the hypotheses we derived from the present study is that the TIMP-3 sensitive metalloprotease activity involved in sperm passage through the cumulus oophorus might be directed towards CD44 on cumulus cells (in order to enhance cell detachment and facilitate sperm passage). In that respect, we speculate that a TIMP-3 sensitive ADAM, e.g. ADAM10 or ADAM17, may be present on spermatozoa. These are for now only speculations and will have to be tested in further research. The use of immunofluorescent staining techniques could be a first approach to investigate whether specific ADAMs are present on spermatozoa or not.
Proteases are also abundantly secreted by cumulus cells. For example, urokinase plasminogen activator, tissue plasminogen activator, ADAM17, ADAMTS1 and ADAMTS4 have been shown to be synthesized and secreted by cumulus cells (D'Alessandris *et al.* 2001; Shimada *et al.* 2004; Yamashita *et al.* 2007). The proteolytic activity of ADAMTS1 has been associated with expansion of the cumulus matrix and remodeling of the follicle wall before ovulation (Russell *et al.* 2003; Shimada *et al.* 2004; Brown *et al.* 2010), but has also a critical function in the timely degradation of the cumulus matrix after ovulation. Cumulus-oocyte-complexes of ADAMTS1$^{-/-}$ mice were shown to retain cumulus cells, versican and hyaluronic acid after ovulation (Brown *et al.* 2010). In addition, ADAMTS1$^{-/-}$ females showed a lower *in vivo* fertilization rate. Brown and coworkers proposed that reduced clearance of versican could lead to a more resistant cumulus matrix and inhibition of sperm penetration. In our study, it became clear that the addition of the metalloprotease inhibitor during porcine IVF reduced sperm passage through the cumulus. It is described that porcine cumulus cells increase production of ADAMTS1 in response to gonadotropins, followed by incorporation of ADAMTS1 in the cumulus matrix (Shimada *et al.* 2004). It has been shown that TIMP-3 is able to inhibit ADAMTS1, ADAMTS2, ADAMTS4 and ADAMTS5 (Kashiwagi *et al.* 2001; Rodriguez-Manzaneque *et al.* 2002; Wang *et al.* 2006). Based on these data from literature, it seems theoretically probable that, in our study, inhibition of cumulus-derived

metalloproteases (especially inhibition of ADAMTS1) could have hampered physiological degradation of the cumulus matrix. Inferior matrix disassembly may become evident by the tendency of cumulus cells to stick to the bottom of the culture dish: Shimada *et al.* (2004) noticed a similar phenomenon after culture of porcine COCs in medium with the matrix metalloprotease inhibitor Galardin. We did observe release of cumulus cells from the outer layers of the cumulus oophorus followed by adherence of cumulus cells to the culture dish when COCs were fertilized in medium with the inhibitor 1,10-PHEN. However, the number of cumulus cell layers that surrounded the oocytes after 6 h of gamete co-incubation was not affected by the presence or absence of the inhibitor 1,10-PHEN, whereas the number of spermatozoa that had reached the inner layers of the cumulus oophorus at that time was noticeably lower in the presence of 1,10-PHEN, as shown via fluorescent labeling (Fig. 19). In another experiment, we found that pre-incubation of COCs with 1,10-PHEN during 30 min prior to IVF had no significant effect on fertilization parameters (Table 3), although the level of cumulus-derived ADAMTS1 protein in porcine COCs reaches its maximum at the end of the maturation period (Shimada *et al.* 2004). As a result, we had to reject the hypothesis that in our study metalloprotease inhibitors strongly decreased the degree of sperm penetration due to inhibition of cumulus-derived metalloproteases and hindrance of physiological matrix degradation.

Table 3. Fertilization parameters of cumulus-intact porcine oocytes inseminated with $1.25x10^5$ spermatozoa/ml in standard fertilization medium after 30 min of oocyte pre-incubation in fertilization medium with and without supplementation of 20 μM 1,10-PHEN.

Group	Total fertilization (%)	Monospermy (%)	Polyspermy (%)	Sperm penetration index[1]
Control	76.4	55.0	21.4	1.3
1,10-PHEN	73.2	54.2	19.0	1.5

No. of oocytes examined: control n=104, 1,10-PHEN n=96. Data represent mean of 2 replicates. None of the parameters were significantly different between control and treatment group ($P>0.05$); [1]mean number of penetrated spermatozoa per fertilized oocyte.

Based on our findings, the effect of 1,10-PHEN was clearly restricted to fertilization of CI oocytes. However, TIMP-3 showed a more complex inhibitory effect. TIMP-3 inhibited total fertilization rate in CI and not in CF oocytes, similar to 1,10-PHEN. Yet TIMP-3 reduced the degree of sperm penetration in CI as well as CF oocytes. These results indicate that the general effect of TIMP-3 is a reduction in the number of sperm entering an oocyte. Because the effect is stronger in CI than in CF oocytes, this still points to an inhibitory effect at the level of sperm-cumulus interaction. In addition, TIMP-3 seems to inhibit a subsequent step in the fertilization process. Correa *et al.* (2000) showed that in mice, a TIMP-3 sensitive metalloprotease is involved in sperm-oocyte fusion. Based on the effect of a number of metalloprotease inhibitors, they hypothesized a role for a MMP or ADAM metalloprotease of which the identity still needs to be unraveled (Correa *et al.* 2000). In the pig, ADAM1 and ADAM2 are present on the sperm membrane (Fabrega *et al.* 2011). To answer if the reduction in number of sperm entering CF porcine oocytes by TIMP-3 could be due to inhibition of ADAM1 or ADAM2, the inhibition profile of TIMP-3 needs further investigation.

In conclusion, this study opens new possibilities to improve our understanding of the fertilization process and should provide a basis for further research towards the localization and identification of the metalloprotease involved in sperm passage through the cumulus oophorus. The recognition of key proteases in the fertilization process holds interesting opportunities for porcine embryo production *in vitro* (IVP). Specific inhibitors may be useful as regulators of sperm penetration in porcine IVF, lessening the problem of polyspermic fertilization and increasing IVP efficiency.

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CHAPTER 6

HAMPERED CUMULUS EXPANSION OF PORCINE CUMULUS-OOCYTE-COMPLEXES BY EXCESSIVE ALPHA$_2$-MACROGLOBULIN IS LIKELY MEDIATED VIA INHIBITION OF ZINC-DEPENDENT METALLOPROTEASES

Modified from

HAMPERED CUMULUS EXPANSION OF PORCINE CUMULUS-OOCYTE-COMPLEXES BY EXCESSIVE ALPHA$_2$-MACROGLOBULIN IS LIKELY MEDIATED VIA INHIBITION OF ZINC-DEPENDENT METALLOPROTEASES

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Abstract

Previous work in our laboratory showed a hampered expansion of porcine cumulus-oocyte-complexes (COCs) *in vitro* due to excessive alpha$_2$-macroglobulin (A2M) in maturation medium supplemented with 10% serum (v/v). The present study investigated two hypotheses which could explain the inhibitory effect of excessive A2M: 1) binding of Epidermal Growth Factor (EGF) to A2M, followed by decreased availability of EGF for interaction with cumulus cells; and 2) binding and inhibition of zinc-dependent matrix metalloproteases. In the first experiment, the progression of cumulus expansion was studied during *in vitro* maturation (IVM) under control conditions. Cumulus expansion was evaluated after 22, 36 and 44 h of IVM based on the maximum diameter of the COCs, the proportion of COCs participating in a mucoid cloudy structure and the proportion of COCs with loss of cumulus cells. The first hypothesis was tested by increasing the EGF concentration in the medium (20 and 50 ng/ml vs. 10 ng/ml EGF; experiment 2) and rejected because cumulus expansion did not improve. There even was a tendency towards a lower degree of cumulus expansion in medium with the highest concentration of EGF tested. Next, we investigated the effect of an inhibitor of zinc-dependent metalloproteases, TIMP-3, on cumulus expansion during IVM with and without immuno-neutralization of A2M (experiment 3 and 4). For immunoneutralization of A2M, serum was pre-incubated with A2M antibodies before addition to the maturation medium. TIMP-3 impaired cumulus expansion only during IVM in 10% serum with A2M antibodies. Thus, in the presence of excessive A2M there was no effect of TIMP-3, whereas this inhibitor did have an effect when A2M in the maturation medium was neutralized. These results support our second hypothesis that A2M and metalloprotease inhibitor TIMP-3 share a common target, a zinc-dependent metalloprotease, and direct future research towards the identification of the protease involved.

Introduction

Since the first birth of piglets originating from *in vitro* fertilized oocytes in 1986 (Cheng *et al.* 1986), several studies have confirmed the viability of porcine embryos produced *in vitro* by successful embryo transfer (Mattioli *et al.* 1989; Yoshida *et al.* 1993; Kikuchi *et al.* 2002; Suzuki *et al.* 2006). However, the efficiency of porcine embryo production *in vitro* is still insufficient for large scale implementation (Abeydeera 2002). Further improvement of the *in vitro* maturation (IVM) procedure is needed because the developmental ability of oocytes matured *in vitro* is much lower compared to *in vivo* matured oocytes (Nagai 2001). Effort has been directed towards the development of chemically defined oocyte maturation media, and with success. Porcine oocytes can be matured in a chemically defined medium (Yoshioka *et al.* 2008; Mito *et al.* 2009). However, supplementation of follicular fluid (FF) is still used in the majority of porcine IVM systems, likely because of the well described beneficial effects (Gil *et al.* 2010).

Successful IVM of cumulus-oocyte-complexes (COCs) is characterized by meiotic resumption and progression to the metaphase II (nuclear maturation), migration of organelles, glutathione accumulation and increased activity of Maturation/M-phase promoting factor (MPF) (cytoplasmic maturation) and cumulus expansion (cumulus maturation). Evaluating the degree of cumulus expansion after IVM is a non-invasive way to estimate cumulus cell function and viability. Furthermore, *in vitro* studies have shown a positive correlation between the degree of cumulus expansion and fertilization results in several species, including the pig (Qian *et al.* 2003).

Previous work in our laboratory showed the superiority of autologous FF over serum in supporting cumulus expansion of porcine COCs (Bijttebier *et al.* 2008). A comparative proteome analysis of autologous FF and serum showed thirteen proteins with significantly different abundance levels (Bijttebier *et al.* 2009). In line with findings in human (Jarkovska *et al.* 2010), most of the proteins found to have different abundance levels between porcine FF and serum are participants of the complement cascade, blood coagulation or vascularization (antithrombin III, fibrinogen beta chain, complement component C4, clusterin, angiotensinogen, angiopoietin-like protein 2). As their presence in FF seems to be regulated, these proteins may play a role in the creation of the proper micro-environment for the oocyte during maturation. However, their specific roles are thus far undefined.

We showed before via proteome analysis that alpha$_2$-macroglobulin (A2M), a high molecular plasma protein, was three to four times more abundant in porcine serum compared to FF and

markedly more incorporated in cumulus matrices after IVM in medium with 10% serum compared to medium with 10% FF (Bijttebier *et al.* 2009). After neutralization of A2M in serum by use of polyclonal antibodies, excessive incorporation of A2M in the extracellular matrix was prevented and cumulus expansion was enhanced (Bijttebier *et al.* 2009).

Collectively, our previous data strongly indicated that excessive incorporation of A2M in the extracellular matrix is associated with hampered cumulus expansion, yet the underlying mechanism was still unclear. Alpha$_2$-macroglobulin is a plasma protein with multiple functions as a binding, carrier, and targeting protein (Borth 1992). A broad range of proteases can be inhibited by binding to A2M and steric hindrance of the active site of the enzyme. Growth factors such as platelet-derived growth factor, transforming growth factor-β and epidermal growth factor (EGF) have been found to covalently associate with A2M (Borth 1992; Gettins and Crews 1993). Maturation medium for porcine IVM is commonly supplemented with EGF (1-10 ng/ml) for its positive effect on nuclear maturation, cumulus expansion and blastocyst development (Singh *et al.* 1993; Abeydeera *et al.* 1998a; Abeydeera 2002; Prochazka *et al.* 2003). The objective of the present study was to investigate two hypotheses which could explain the inhibitory effect of A2M on cumulus expansion. Firstly, the possible inhibition of cumulus expansion due to binding of EGF to A2M followed by decreased availability was evaluated. Secondly, we investigated whether excessive presence of A2M hampered cumulus expansion by non-specific binding and inhibition of proteases. More specifically, we focused on the possible inhibition of matrix metalloproteases (MMP), because members of the MMP family have been associated with the COC extracellular matrix and are suggested to be critical for cumulus expansion in porcine COCs (Shimada *et al.* 2004).

Materials and methods

Media

All chemicals used in this study were purchased from Sigma-Aldrich (Bornem, Belgium) unless otherwise stated. The basic medium used for the collection and washing of COCs was a modified HEPES-buffered Tyrode balanced salt solution (HEPES-TM) with 10 µg/ml gentamycin sulfate, 10 mM HEPES and 3 mg/ml BSA. The oocyte maturation medium was the BSA-free 'North Carolina State University' 23 (NCSU23)(Petters and Wells 1993) supplemented with 0.57 mM cysteine, 10 ng/mL Epidermal Growth Factor (EGF), 10 IU/mL eCG (Folligon®, Intervet, The Netherlands) and 10 IU/mL hCG (Chorulon®, Intervet, The Netherlands).

Oocyte collection and in vitro maturation (IVM)

Ovaries of prepubertal gilts (Piétrain crossbred, ± 6.5 months of age) were collected at a local slaughterhouse and transported to the laboratory in physiological saline at 37°C. Cumulus-oocyte-complexes (COCS) were collected by aspiration from follicles with a diameter of 3 to 6 mm. Only COCs with a homogeneous ooplasm and a multilayered cumulus were selected. Subsequently, groups of 80-100 COCs were cultured in 500 µL maturation medium for the first 22 h (39°C, 5% CO2 in air). Afterwards, COCs were transferred to hormone free maturation medium for another 22 h (39°C, 5% CO2 in air). In experiment 1, the oocyte maturation medium was supplemented with 10% (v/v) porcine follicular fluid (pFF) collected from antral follicles (3-6 mm diameter) of prepubertal gilts, representing the standard IVM protocol in our laboratory. In experiment 2, 3 and 4, the maturation medium was either supplemented with 10% (v/v) follicular fluid (FF) or 10% (v/v) serum collected from 3 multiparous sows (Rattlerow-Seghers, Baasrode, Belgium) in the pre-ovulatory stage of the estrous cycle as described by Bijttebier *et al.* (2008). The 3 samples of either FF or serum were mixed and used as a single lot to reduce individual sow effects. Follicular fluid of prepubertal gilts (pFF) and follicular fluid (FF) and serum from sows was centrifuged after collection (10 min, 100 X g) and subsequently the supernatant was aspirated, filtered (0.22µm) and stored at -80°C until use.

Measurement of the cumulus diameter

The diameter of the cumulus oophorus was measured using a stereomicroscope (Leica microsystems, Wild M10), equipped with an eyepiece with a micrometer cross and a x25 objective. The diameter was defined as the maximum distance across the cumulus matrix.

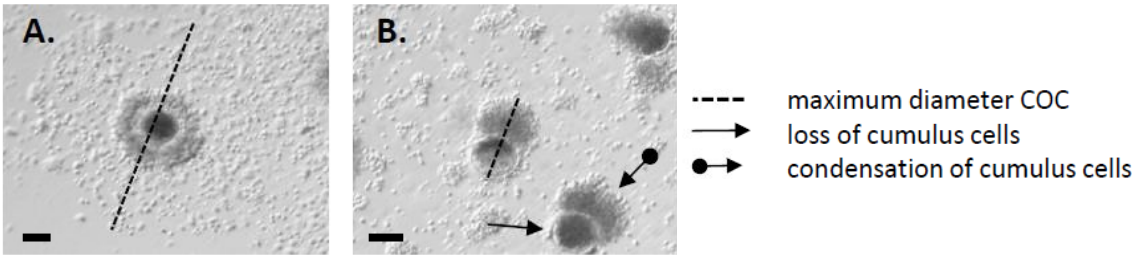

Figure 23. A: COC with expanded cumulus. **B:** COCs showing loss of cumulus cells and condensation of cumulus cells. Bars = 100µM.

Assessment of oocyte maturation

Oocytes were denuded by 2 min vortexing in 0.1% hyaluronidase in HEPES-TM medium. Subsequently, oocytes were washed three times in PBS and fixed overnight with 4% of paraformaldehyde in PBS. The fixed oocytes were stained with 10 µg/ml *bis*-benzimide (Hoechst 33342; Molecular Probes, Leiden, The Netherlands) in PBS for 10 min. Nuclear DNA was visualized under a Leica DMR fluorescence microscope (Leica microsystems, Brussels, Belgium). The meiotic stage of oocytes was assessed according to Hunter and Polge (1996).

Immunoneutralization of alpha$_2$-macroglobulin

For immunoneutralization experiments, FF and serum were pre-incubated with 1:500 polyclonal sheep anti-human A2M or with 1:500 control antibodies (Abcam 8770, Cambridge, MA, USA). Antibodies were diluted 1:50 in NCSU23 medium and then further diluted 1:10 in either FF or serum and incubated during 1 h at 37°C (5% CO2 in air). Antibody-antigen complexes were not removed from the medium.

Experimental design

Progression of cumulus expansion, matrix formation and matrix disaggregation during IVM of porcine COCs

In order to correctly interpret changes in cumulus expansion due to different treatments in this study, we first assessed time-dependent changes in cumulus expansion and matrix formation during IVM using our standard protocol. Groups of 80-100 COCs were cultured in maturation medium supplemented with 10% FF (39°C, 5% CO2 in air). At 0, 22, 36 and 44 h of IVM, 40 COCs were randomly selected for the measurement of the maximum cumulus diameter and the evaluation of the cumulus matrix quality (6 replicates, n = 240 per time point). Condensation and apparent loss of cumulus cells have been associated with matrix disaggregation (Shimada *et al.* 2004). Therefore, we based the evaluation of cumulus matrix quality on two parameters: 1) the percentage of oocytes that participated in the formation of a mucoid, cloudy structure (oocytes outside the cloudy structure were most of the time attached to the bottom of the four-well plate) and 2) the percentage of oocytes with a part of their zona pellucida naked due to loss of cumulus cells. Parameter 1 is positively correlated with cumulus matrix quality, whereas parameter 2 is negatively correlated. Therefore, a good quality cumulus matrix after IVM can be defined as having a high proportion of COCs participating in the mucoid cloudy stucture, and a low proportion of COCs with loss of cumulus cells. We performed three additional IVM experiments in which we evaluated oocyte nuclear maturation after 28, 32, 36, 40 and 44 h of IVM (3 replicates, total n = 450 COCs).

Effect of higher EGF concentrations on cumulus expansion in medium supplemented with 10% serum

In experiment 2, groups of COCs were matured in maturation medium supplemented with 10% serum and increasing EGF concentrations. We included the concentration of 10 ng/ml EGF, which is commonly used in porcine IVM, and a two- and fivefold concentration (20 and 50 ng/ml). Control COCs were matured in 10% FF and 10 ng/ml EGF. The degree of cumulus expansion was evaluated after 22, 36 and 44 h of maturation as described in experiment 1 (3 replicates, total n = 720 COCs).

Effect of TIMP-3 on cumulus expansion in medium supplemented with 10% serum pre-incubated with A2M antibodies

The second hypothesis for the effect of A2M on cumulus expansion was derived from its activity as a non-specific protease inhibitor. Matrix metalloprotease inhibitor tissue inhibitor

of metalloproteases-3 (TIMP-3; T1327, Sigma Aldrich, Bornem, Belgium) was used at an effective concentration as reported by the manufacturer. Inhibitor TIMP-3 was dissolved in deionized water (1000 nM) and stored at -20ºC. The supplementation of TIMP-3 stock solution with deionized water was corrected by the addition of equal volume of maturation medium with twofold concentration of medium components.

Immature COCs were randomly assigned to 6 groups (80 COCs per group) and cultured during the first 22 h in maturation medium with 10% FF (group 1), 10% FF pre-incubated with A2M antibodies (FF + A2M Ab; group 2), 10% serum (group 3), 10% serum with control antibodies (S + CTL Ab; group 4) or 10% serum pre-incubated with A2M antibodies (S + A2M Ab; group 5 and 6). For the second half of the maturation period, COCs were cultured in the same media except for group 6. In this group we added an inhibitor, 400 nM TIMP-3 (group 6). An overview of the different groups and IVM conditions is presented in Table 4. Cumulus expansion and matrix formation were analyzed after 22, 36 and 44 h of IVM as described in experiment 1 (3 replicates, total n = 1440 COCs).

Effect of TIMP-3 on cumulus expansion in medium supplemented with 10% serum without A2M antibodies

In experiment 3, COCs showed a lower degree of cumulus expansion in the presence of TIMP-3 (group 6) compared to COCs matured in maturation medium without inhibitor (group 5). Furthermore, COCs matured in 10% serum with immunoneutralization of A2M reached similar cumulus diameters in the presence of a metalloprotease inhibitor as COCs matured in 10% serum without A2M neutralizing antibodies. The effect of TIMP-3 on cumulus expansion thus appeared similar to the effect of excessive A2M in serum. In order to confirm that the effect of A2M was due to inhibition of the same family of proteases as inhibited by TIMP-3, we performed an IVM experiment in which we compared cumulus expansion in two different media without A2M antibodies: 1) medium with 10% serum, and 2) medium with 10% serum + 400 nM TIMP-3 (3 replicates, total n = 480 COCs). We hypothesized that in case A2M and metalloprotease inhibitors would share the same target (a zinc-dependent metalloprotease), there would be no effect of TIMP-3 on cumulus expansion in the presence of excessive A2M.

Table 4. Overview of the different groups and conditions during IVM of porcine oocytes in experiment 3

Group	**1**	**2**	**3**	**4**	**5**	**6**
0 to 22 h of IVM	10% FF	10% FF	10% Serum	10% Serum	10% Serum	10% Serum
Antibodies[1]	-	A2M	-	CTL	A2M	A2M
A2M[2]	+	**X**	+++	+++	**X**	**X**
22 to 44 h of IVM	10% FF	10% FF	10% Serum	10% Serum	10% Serum	10% Serum
Antibodies[1]	-	A2M	-	CTL	A2M	A2M
A2M[2]	+	**X**	+++	+++	**X**	**X**
Inhibitor[3]	-	-	-	-	-	+ TIMP-3

FF: follicular fluid; A2M: alpha$_2$-macroglobulin; TIMP-3: Tissue Inhibitor of MetalloProteases-3; X: immunoneutralization.

[1] No pre-incubation with antibodies (-) or pre-incubation with either alpha$_2$-macroglobulin (A2M) or control (CTL) antibodies.

[2] Relative presence of A2M in serum and FF, as denoted by +++ and +, was based on a previous study (Bijttebier *et al.* 2009)

[3] No addition of metalloprotease inhibitor (-) or addition of TIMP-3 (+ TIMP-3)

Statistical Analysis

The data of the cumulus diameter measurements at different time points, as shown by the Kolmogorov-Smirnov test ($P<0.01$). The differences between the control group and the treatment groups in the proportion of COCs participating in a mucoid cloudy structure and the proportion of COCs with loss of cumulus cells were also not normally distributed. For that reason, these parameters were analyzed by a non-parametric test, the Mann-Whitney U test (2 groups) or the Kruskal-Wallis test (> 2 groups). Results are presented as median and interquartile range. Oocyte nuclear maturation was analyzed by binary logistic regression. Hypothesis testing was performed using a significance level of 5% (two-sided tests) (SPSS® Version 21.0).

Results

Progression of cumulus expansion and matrix formation during IVM of porcine COCs

The progression of cumulus expansion during maturation of COCs in standard maturation medium is visualized in Figure 24. At 22 h of IVM, COCs within the cloudy structure were quite homogeneous, but with progression of the maturation period the population of COCs became visually bipartite. This observation was underlined by the increase in interquartile range of the cumulus diameters between 22 and 44 h of IVM. The interquartile range increased from 120 µm to 320 µm, the median diameter was 340 µm and 420 µm, respectively. During the maturation period, the diameter of individual COCs either increased until 44 h of maturation (Fig. 23A) or the oocyte was left with few condensed layers of cumulus cells while the cumulus cells of the outer layers had been released into the medium (Fig. 23B). The mean cumulus diameter did not increase significantly between 36 h (441 µm) and 44 h (464 µm) of IVM. On the other hand, matrix quality decreased as shown by the lower percentage of COCs participating in the mucoid cloudy structure (quality parameter 1) as well as a higher percentage of COCs with loss of cumulus cells at 44 h of maturation (quality parameter 2)(Fig. 25). Figure 26 shows the distribution of cumulus diameters of COCs matured for 44 h in maturation medium with 10% FF versus COCs matured in medium with 10% serum (data from preliminary experiment, 3 replicates, total n = 480 COCs). Nuclear maturation of oocytes was completed within 36 h of IVM (Table 5).

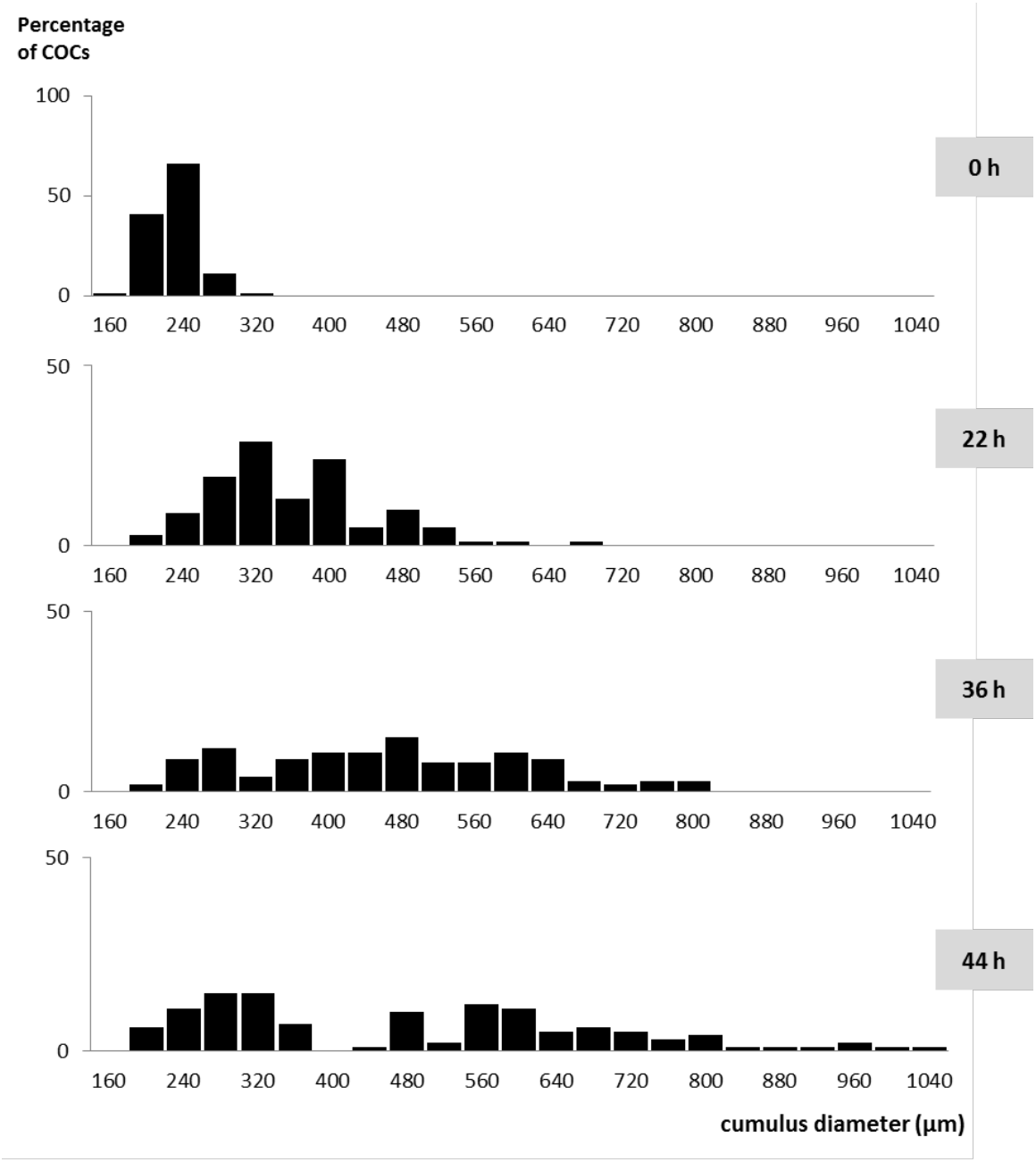

Figure 24. Distribution of cumulus diameter after different durations of IVM of porcine COCs (6 replicates, n = 240 COCS per time point).

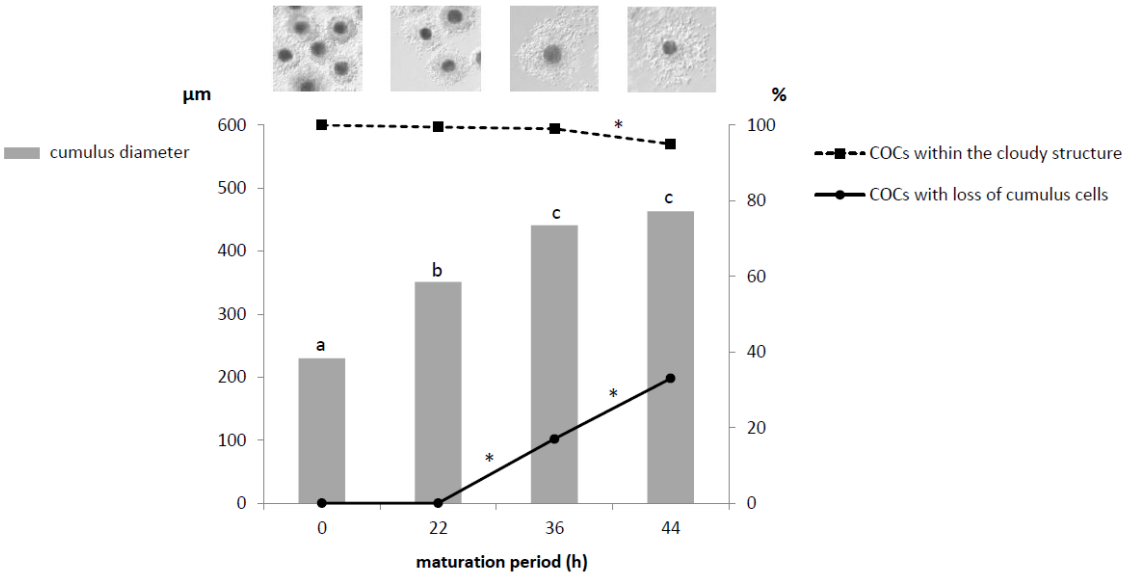

Figure 25. Progression of cumulus expansion, matrix formation and matrix disaggregation during IVM of porcine COCs. Mean cumulus diameter, percentage of COCs within the cloudy structure and percentage of COCs with loss of cumulus cells after 0, 22, 36 and 44 h of maturation in medium with 10% FF. Significant differences between time points are marked with different letters (cumulus diameter) or an asterisk (matrix parameters) (6 replicates, n = 240 COCS per time point).

Table 5. Progression of nuclear maturation of porcine oocytes in a medium supplemented with 10% (v/v) follicular fluid

IVM (h)	No. of oocytes*	GV/GVBD (%)	M I (%)	Ana I (%)	M II (%)
28	87	2.3 ± 2.2	30.9 ± 8.5[a]	18.9 ± 7.8[a]	48.1 ± 7.9[a]
32	85	2.3 ± 2.2	12.9 ± 3.3[b]	4.0 ± 3.9[a,b]	76.4 ± 1.8[b]
36	92	0	4.2 ± 1.5[b]	1.1 ± 1.0[b,c]	94.8 ± 1.8[c]
40	88	0	10.7 ± 1.7[b]	0.6 ± 0.5[b,c]	87.6 ± 1.2[c]
44	92	1.0 ± 0.9	9.7 ± 1.9[b]	0[b,c]	89.3 ± 2.4[c]

*Degenerated oocytes were not included. GV: germinal vesicle, GVBD: germinal vesicle breakdown, M I: Metaphase I, Ana I: Anaphase I, M II: Metaphase II. [a,b,c] Values with different superscripts within the same column differ significantly (P<0.05). (3 replicates, n = 90 COCs per time point)

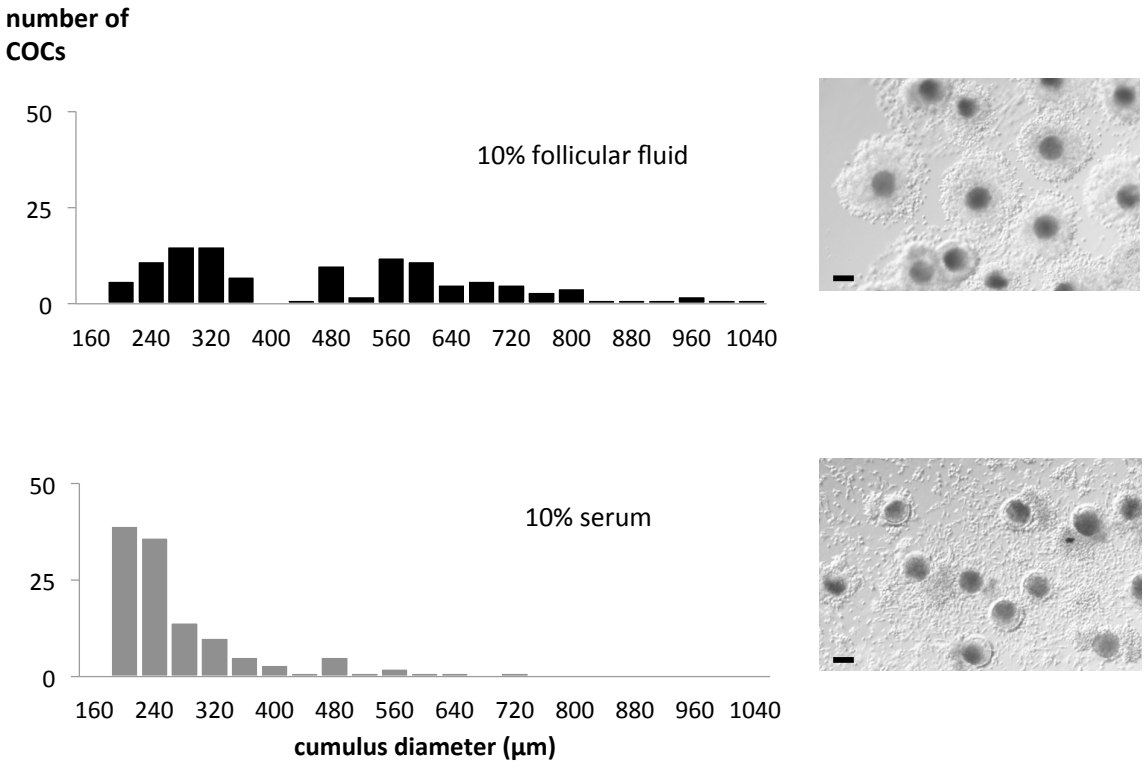

Figure 26. Distribution of cumulus diameter after 44 h of IVM in maturation medium supplemented with 10% FF or 10% serum (data from preliminary experiment, 3 replicates, n = 480 COCs). Bars = 100µM.

Higher concentrations of EGF do not improve cumulus expansion in maturation medium supplemented with 10% serum

Cumulus expansion did not improve by increasing EGF concentrations in maturation medium with 10% serum (Fig. 27). At 44 h of IVM, cumulus diameters (median ± interquartile range) were 260 ± 160 µm (10 ng/ml EGF), 280 ± 80 µm (20 ng/ml EGF) and 240 ± 110 µm (50 ng/ml EGF). The degree of cumulus expansion in the control group (FF + 10 ng/ml EGF) was superior to all other groups at every time point ($P<0.05$). After 44 h of IVM in medium with 10% FF + 10 ng/ml EGF, the median cumulus diameter was 360 ± 340 µm. In addition, the mean percentage of COCs participating in the mucoid cloudy structure at the end of the maturation period was numerically, but not significantly, higher in the control group (93%) than in the groups matured in serum (82%, 77% and 87% for maturation in 10% serum with 10 ng/ml EGF, 20 ng/ml EGF and 50 ng/ml EGF, respectively).

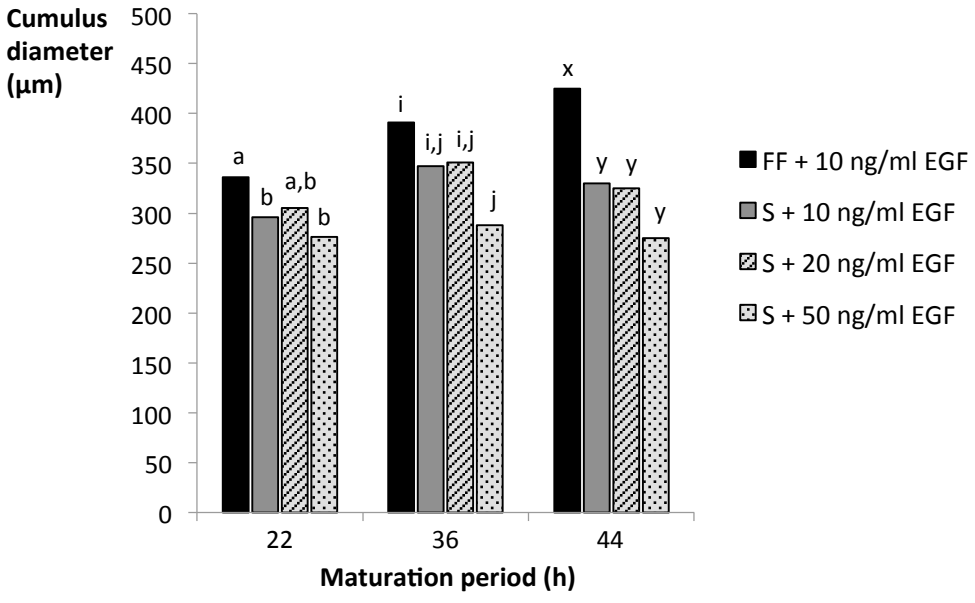

Figure 27. Mean cumulus diameters after 22, 36 and 44 h of IVM in medium supplemented with either 10% follicular fluid (FF) or 10% serum (S), and different concentrations of EGF (10, 20 and 50 ng/ml)). Significant differences between groups per time point are marked with different letters ($P<0.05$). (3 replicates, total n = 720 COCs).

Matrix metalloprotease inhibitor TIMP-3 inhibits cumulus expansion in medium with 10% serum pre-incubated with A2M antibodies

Pre-incubation of serum with A2M antibodies improved cumulus expansion in subsequent IVM of porcine COCs. Already after 22 h, cumulus diameters in 10% serum with A2M Ab (320 ± 120 µm, mean 326 µm) were significantly higher than in 10% serum (280 ± 80 µm, mean 289 µm). After 44 h of IVM, this difference was even more pronounced and mean cumulus diameters were 393 µm and 277 µm, respectively. However, FF remained superior to serum in supporting cumulus expansion during the whole maturation period (cumulus diameter 480 ± 350 µm, mean 483 µm). There was no significant improvement of cumulus expansion when FF was pre-incubated with A2M antibodies before addition to the maturation medium ($P>0.05$, data not shown).

Cumulus expansion was markedly impeded in the presence of TIMP-3 (Fig. 28; experiment 3). Median cumulus diameters of COCs matured in 10% serum pre-incubated with A2M antibodies were 260 ± 160 µm and 320 ± 240 µm for the groups with and without addition of TIMP-3.

The percentage of COCs participating in the mucoid cloudy structure after 44 h of IVM ranged between 71 and 92%, with no significant differences between groups ($P>0.05$). Maturation in 10% serum without A2M antibodies caused a higher percentage of COCs with loss of cumulus cells compared to all other groups ($P<0.05$).

Matrix metalloprotease inhibitor TIMP-3 does not inhibit cumulus expansion in medium with 10% serum without A2M antibodies

The addition of 400 nM TIMP-3 to the maturation medium with 10% serum resulted in a mean diameter of 289 µm after 44 h of IVM, compared to 300 µm in medium with 10% serum without inhibitor (Fig. 28; experiment 4). The median as well as the distribution of cumulus diameters were similar between COCs matured in medium with 10% serum + TIMP-3 and COCs matured in medium with only 10% serum ($P> 0.05$). Median and interquartile range were 280 ± 80 µm and 280 ± 120 µm, respectively. The percentage of COCs participating in the mucoid cloudy structure after 44 h of IVM in control medium differed substantially between replicates, with percentages ranging from 48 to 80%. Within each replicate, there were only small differences (<10%) between both groups.

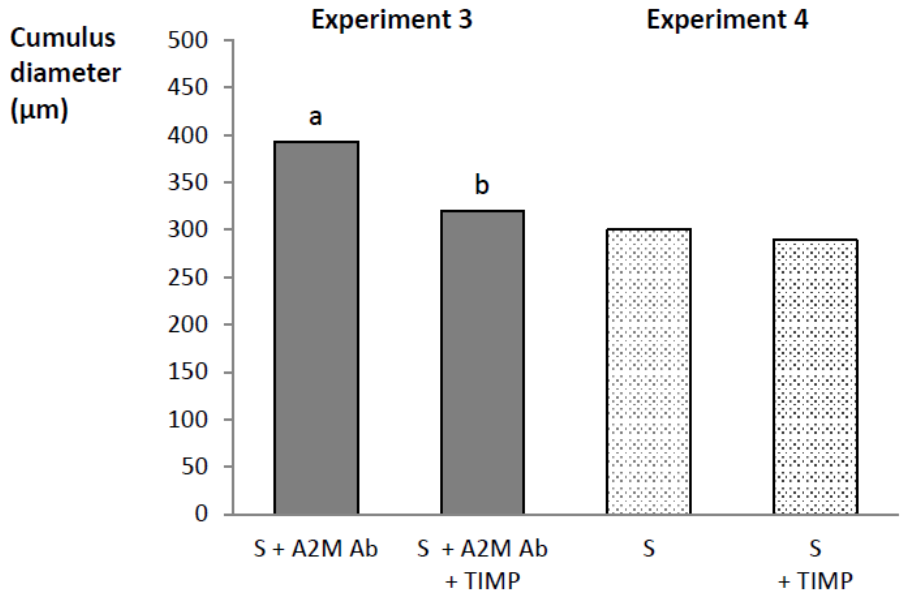

Figure 28. Effect of TIMP-3 on cumulus expansion in maturation medium with 10% serum (S) and with or without A2M antibodies (A2M Ab). Height of the bars represents the mean cumulus diameter of COCs after 44 h of IVM. Significant differences between groups (per experiment) are marked with different letters ($P<0.05$).

Discussion

In vitro maturation of porcine COCs can be performed in various types of media containing FF, serum or fetal calf serum and supplements such as gonadotropins and growth factors to support oocyte maturation and cumulus expansion (Abeydeera 2002). Previous work in our laboratory demonstrated that cumulus expansion is inferior in medium supplemented with porcine serum compared to FF and identified the excessive presence of alpha$_2$-macroglobulin (A2M) in serum compared to FF as an important factor for this distinction (Bijttebier *et al.* 2008; Bijttebier *et al.* 2009). During follicle growth *in vivo*, the amount of A2M in the follicle may primarily be produced by granulosa and theca cells (Ireland *et al.* 2004). Furthermore the concentration of A2M in FF seems to be regulated: the concentration of A2M in bovine follicles was found to be 80-90% lower than in bovine serum or plasma (Andersen *et al.* 1976; Yamada and Gentry 1995) and similarly A2M concentration in porcine FF significantly lower compared to serum (Bijttebier *et al.* 2009).The multiple binding features of A2M enables it to bind and modify most of the growth factors, binding proteins and hormones involved in follicle development (Borth 1992; Ireland *et al.* 2004). For example, A2M induces an increase in estradiol production in bovine granulosa cells, which might be explained by binding and neutralization of inhibin (Ireland *et al.* 2004). Inferior oocyte maturation and/or cumulus expansion in medium supplemented with serum compared to follicular fluid has been mentioned in several studies (Naito *et al.* 1988; Suzuki *et al.* 2006; Bijttebier *et al.* 2009). However, the possible effects of supplementation of A2M, as a component of serum, have not yet been investigated. This might be related to the fact that despite inferior oocyte maturation and cumulus expansion, IVF of oocytes matured in medium with 10% serum can still yield average blastocyst rates (Suzuki *et al.* 2006).

The majority of porcine IVM protocols include a 40 to 44 h maturation period for immature COCs (Funahashi and Day 1993; Prather and Day 1998). Parallel with oocyte maturation, the volume of the cumulus oophorus increases by intercellular deposition of hyaluronic acid and other matrix components produced by the cumulus cells, a process called cumulus expansion. The present study evaluated cumulus expansion based on changes of the maximum COC diameter as well as on two visual parameters, the percentage of COCs within a mucoid cloudy structure and the percentage of COCs with loss of cumulus cells. These parameters have been associated with matrix formation and disaggregation, respectively (Shimada *et al.* 2004). In line with the findings reported by Somfai et al.(2004), the homogeneity of COCs was retained until the second half of the maturation period. After 22 h of IVM, differences in the cumulus

diameter, the number of COCs attached to the bottom of the four-well plate and the condensation and loss of cumulus cells became more pronounced. Moreover, the cumulus diameter did not increase significantly after 36 h of IVM, whereas parameters related to matrix disaggregation became more important. Under the same maturation conditions, nuclear maturation was completed within 36 h of IVM. The importance of cumulus-oocyte crosstalk in the timing of nuclear maturation is well established (Sun *et al.* 2001; Shimada and Terada 2002; Shimada *et al.* 2003). Furthermore, differences in morphology of porcine COCs were related to progression of nuclear and cytoplasmic maturation of oocytes (Somfai *et al.* 2004). Based on experiment 1, we concluded that time-dependent changes in cumulus expansion using our protocol were very similar to previous reports and that the assessment of the cumulus diameter and two visual parameters would be suitable to record the effect of different treatments in further experiments.

Preliminary experiments showed that immunoneutralization of A2M by pre-incubation of serum with A2M antibodies improved cumulus expansion in 10% v/v serum, whereas control antibodies had no effect (data not shown). In addition, pre-incubation of FF with A2M or control antibodies had no positive effect on cumulus expansion. Based on previous reports that 1) many growth factors including epidermal growth factor (EGF) are able to covalently bind to A2M (Borth 1992; Gettins and Crews 1993), and 2) serum tends to mask the stimulatory effects of EGF on cumulus expansion (Wanga and Niwa 1995; Abeydeera *et al.* 1998b), we hypothesized that excessive presence of A2M in serum could hamper cumulus expansion by binding and decreased availability of EGF. We assumed that saturation of EGF binding sites of A2M by extra supplementation of EGF to the medium would increase the amount of non-bound EGF available for interaction with EGF receptors on the cumulus cells. Usually 1-10 ng/ml EGF is used in IVM medium of porcine COCs (Ding and Foxcroft 1994; Abeydeera *et al.* 1998b; Romar *et al.* 2003; Gil *et al.* 2004). In the present study, supplementation of EGF up to 50 ng/ml could not enhance cumulus expansion, in contrast, there was even a tendency towards a decrease in cumulus diameter by higher EGF concentrations. Based on these results, the first hypothesis was rejected. The effect of EGF on maturation of porcine COCs is mediated through cumulus cells (Coskun and Lin 1994) and the responsiveness arises during follicle growth (Procházka *et al.* 1991). The finding that increasing EGF concentrations were not beneficial in medium supplemented with serum is in line with the study of Wanga and Niwa (1995). It is also similar to the findings reported when higher EGF concentrations were tested in medium with 10% FF. For example, Abeydeera et

al. (1998a) showed that an increase of the EGF concentration to 40 ng/ml in medium with 10% FF decreased blastocyst development. In our experiments, we observed that cumulus expansion in relation to EGF concentration was similar in medium with 10% serum compared to medium with 10% FF (data not shown). This suggests that interaction of EGF with receptors was comparable in both media, which further indicates that binding of EGF to A2M did not occur explicitly in medium supplemented with serum.

The second hypothesis for the negative effect of A2M on cumulus expansion was derived from its activity as a non-specific protease inhibitor. More specifically, we focused on the possible inhibition of matrix metalloproteases, since two members of this family of proteases, ADAMTS1 and ADAMTS4, are associated with the COC extracellular matrix and are suggested to play crucial roles during cumulus expansion in COCs (Shimada *et al.* 2004; Brown *et al.* 2010). Furthermore, it has been shown that A2M is able to form a covalent binding complex with ADAMTS1 (Kuno *et al.* 1999).

Cumulus cells synthesize and secrete several proteases, such as urokinase plasminogen activator, tissue plasminogen activator, ADAM17, ADAMTS1 and ADAMTS4 (D'Alessandris *et al.* 2001; Shimada *et al.* 2004; Yamashita *et al.* 2007). Matrix metallo-proteases ADAM17, ADAMTS1 and ADAMTS4 are known to play important roles in remodeling and degradation of extracellular matrix. ADAMTS1 and ADAMTS4 belong to a specific subfamily of the A Disintegrin and Metalloprotease (ADAM) family of proteases because of the presence of trombospondin (TS) motifs (Shiomi *et al.* 2010). The proteolytic activity of ADAMTS1 has been associated with cumulus expansion as well as with timely degradation of the cumulus matrix after ovulation (Russell *et al.* 2003; Shimada *et al.* 2004; Brown *et al.* 2010). For example, COCs of ADAMTS1$^{-/-}$ mice were shown to retain cumulus cells and matrix proteins such as versican and hyaluronic acid after ovulation (Brown *et al.* 2010). Both ADAMTS1 and ADAMTS4 are able to cleave versican, a proteoglycan that contributes to the stabilization of the expanded cumulus matrix (Russell *et al.* 2003). The cleaved N-terminal domain of versican binds to hyaluronic acid, whereas the C-terminal domain has been shown to interact with integrins and to promote cell adhesion (Wu *et al.* 2002; Wight *et al.* 2011). However, there are indications that regulation of ADAMTS4 activity is different from ADAMTS1, and that ADAMTS4 cannot fully substitute ADAMTS1 in the process of cumulus expansion. Inhibition of ADAMTS1 during IVM of porcine COCs, either by suppression of ADAMTS1 synthesis via progesterone-receptor dependent pathways or by blocking enzymatic activity via an inhibitor, resulted in impaired cumulus expansion

and adherence of outer layers of cumulus cells to the culture dish (Shimada *et al.* 2004). Matrix metalloprotease activity *in vivo* is strictly regulated by four Tissue Inhibitors of MetalloProteases, TIMP-1 to TIMP-4. Inhibitor TIMP-3 has been shown to inhibit ADAM17, ADAMTS1 and ADAMTS4 (Kashiwagi *et al.* 2001; Rodriguez-Manzaneque *et al.* 2002; Shiomi *et al.* 2010). Inhibitor TIMP-3 was added to the medium for the second half of the maturation period as it was shown that the level of ADAMTS1 protein in the extracellular matrix of porcine COCs increases from 10 h IVM onwards and reaches its maximum at 40 h IVM (Shimada *et al.* 2004). The effect of TIMP-3 on the progress of cumulus diameters and matrix formation was very similar to the effect of A2M. There was no additional negative effect of the matrix metalloprotease inhibitor on cumulus expansion in medium with 10% serum. We consider these results as a strong indication that the effect of TIMP-3 was due to inhibition of the same type of proteases as inhibited by A2M. Based on the present knowledge on the involvement of metalloproteases during cumulus expansion, we speculate that the zinc-dependent metalloprotease activity inhibited by A2M might be ADAM17, ADAMTS1 and/or ADAMTS4. However, these speculations need to be tested in future research. Isolation of cumulus matrix and co-immunoprecipitation of A2M with its binding partner could be a first approach to confirm an interaction between A2M and an ADAM molecule in the cumulus.

In conclusion, the present study confirmed that A2M is a key factor responsible for the inferior cumulus expansion of porcine COCs in medium with 10% serum compared to 10% FF. Cumulus expansion in maturation medium with 10% serum was not improved by increasing EGF supplementation to the medium and thus the hypothesis that A2M negatively influences cumulus expansion by binding and decreased availability of EGF was rejected. Results indicate that A2M possibly hampers cumulus expansion via inhibition of zinc-dependent matrix metalloproteases. New insights in the effects of individual components of FF and serum will help to optimize chemically defined maturation media for porcine IVM in order to increase the developmental ability of *in vitro* matured oocytes.

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CHAPTER 7

GENERAL DISCUSSION

The general aim of this thesis was to gain more insight in the role of proteases during *in vitro* maturation and *in vitro* fertilization of porcine cumulus-oocyte-complexes (COCs). Elucidation of how proteases contribute to oocyte maturation and fertilization might enable further optimization of the *in vitro* embryo production system in this species. All important players on stage during gamete-interaction, more specifically cumulus cells, oocyte and spermatozoa, have been shown to exert certain protease activity, mainly based on studies in human and mouse. Therefore, proteases represent interesting candidates in order to explore in which step(s) of porcine *in vitro* fertilization they contribute to successful fertilization.

A previous study – investigating the effect of porcine serum and follicular fluid on IVM of porcine COCs – revealed that alpha$_2$-macroglobulin, a non-specific protease inhibitor, hampered cumulus expansion of porcine COCs during IVM in 10% serum. Further investigation of the mechanism underlying this inhibitory effect may elucidate a role for proteases and improve our understanding of how proteolysis is involved in the oocyte maturation process.

PROTEASES AND PORCINE FERTILIZATION

Among the different approaches that can be used to study the fertilization process *in vitro*, incubation of spermatozoa and/or oocytes with enzyme inhibitors or binding ligands inhibiting fertilization has been shown to be a convenient way to study underlying mechanisms of gamete interaction. In this thesis, we performed protease inhibitor experiments to gain insight in the role of proteases during porcine fertilization *in vitro*.

When one or more steps of gamete interaction can be blocked by a protease inhibitor, this evidently indicates the participation of a protease in the fertilization process. The use of inhibitors has thus the advantage to distinguish active proteases from inactive proteins, in contrast with methods such as immunochemistry or proteomics that allow to demonstrate the localization of a protease but generally not its function. More detailed information about the mechanistic class and the role of the protease can be gained by adding protease inhibitors with a small inhibition spectrum to a specific step of the fertilization process. The action of 'specific' inhibitors is seldom exclusively directed to a single protease. Therefore, conclusive results on the identity of a protease will mostly be obtained by biochemical techniques.

Table 6. Overview of the different protease inhibitors tested, their characteristics and the observed effects on spermatozoa and *in vitro* fertilization

Protease inhibitor - characteristics					Effect on spermatozoa			Effect on *in vitro* fertilization*						
Name	Specificity	Mode of action	Molecular weight	Conc.	Membrane/ acrosomal integrity	Motility	Ca ion induced AR	Total fertilization (COC)	Polyspermy rate (COC)	Penetration of			Pre-incubation	
										Cumulus	ZP	oolemma	Sp	COC
AEBSF	Serine	Irrev	Low	100 µM	–	–	↓	↓	↓	–	↓	–	–	↓
E-64	Cysteine	Irrev	Low	250 µM	–§	NT	NT	–§	–§	NT	NT	NT	NT	NT
Pepstatin A	Aspartic	Rev	Low	1 µg/ml	–§	NT	NT	–§	–§	NT	NT	NT	NT	NT
1,10-PHEN	Metallo	Rev	Low	20 µM	–	–	–	↓	↓	↓	–	–	–	–
Phosphoramidon	Metallo	Rev	Low	100 µM	–	–	NT	–	–	NT	NT	NT	NT	NT
STI	Trypsin-like serine	Rev	High	5 µM	–	–	↓	↓	↓	–	↓	–	↓	↓
TIMP-3	Matrix metallo	Rev	High	400 nM	–	–	–	↓	↓	↓	NT	NT	NT	NT
TLCK	Trypsin-like serine	Irrev.	Low	100 µM	–	↓	–§	–	–	NT	NT	NT	NT	NT
TPCK	Chymotrypsin -like serine	Irrev.	Low	100 µM	↓	↓	–§	↓	↓	NT	NT	NT	NT	NT

*Fertilization with epididymal spermatozoa; AR: acrosome reaction; COC: cumulus-oocyte-complex; ZP: zona pellucida; Sp: spermatozoa; Irrev: irreversible; Rev: reversible; - no effect; ↓: significant reduction; §: unpublished data; NT: not tested.

An overview of the protease inhibitors tested ant the observed effects on porcine spermatozoa and *in vitro* fertilization is presented in table 6.

The necessity to accurately test the effect of protease inhibitors on sperm function prior to their use in fertilization studies has been shown in chapter 3. In this study, the effect of different serine protease inhibitors on sperm penetration rate during porcine IVF were interpret and discussed in the light of their effect on sperm quality parameters. Four serine protease inhibitors were included: 4-(2-Aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF), Soybean Trypsin Inhibitor from Glycine Max (STI), N_{α}-Tosyl-L-lysine-chloro-methyl ketone hydrochloride (TLCK) and N_{p}-Tosyl-L phenylalanine-chloromethyl ketone (TPCK). In this way, both a reversible and irreversible mode of action and a small versus high molecular weight of inhibitors were covered. All inhibitors significantly decreased sperm penetration during porcine IVF with fresh diluted semen. Inhibitors AEBSF, TLCK and TPCK (100 μM) decreased total fertilization and polyspermy rate by 50% or more compared to the respective control group. Inhibitor STI (5 μM) had a significant inhibitory effect on total fertilization but not on polyspermy rate. Using ejaculates of the same boars as used for IVF, the possible effect of inhibitors on the following sperm parameters was assessed: membrane integrity, sperm motility, mitochondrial membrane potential, acrosomal integrity and the ability to complete the acrosome reaction in response to treatment with calcium ionophore A23187. Remarkable differences between inhibitors were found.

Co-incubation with serine protease inhibitors AEBSF or STI had no effect on sperm membrane integrity, motility parameters and mitochondrial membrane potential. However, in the presence of AEBSF and STI, a significantly lower proportion of spermatozoa completed the acrosome reaction in response to incubation with calcium ionophore A23187 compared to the control group. Because only acrosome reacted spermatozoa are able to successfully fertilize an oocyte (Tulsiani *et al.* 1998), inhibition of this process is a possible cause for the decreased sperm penetration rate observed in the presence of these inhibitors.

Inhibitor TLCK had no influence on sperm membrane integrity, mitochondrial or acrosomal function but motility of boar spermatozoa was hampered by relatively low concentrations when compared to previous studies in mouse, rat, human and bovine spermatozoa, in which more than tenfold higher concentrations of TLCK were used without an inhibitory effect on motility (de Lamirande and Gagnon 1986). In early *in vivo* studies, a negative effect of protease inhibitors on sperm motility was sometimes mentioned as a possible explanation for the decrease in fertilization rate (Miyamoto and Chang 1973), whereas others reported no

effect. The inhibitor-induced decrease in sperm motility could be considered a “toxic” effect, but there is some evidence for a function of a serine protease in sperm motility (de Lamirande and Gagnon 1986). In the study of De Lamirande and Gagnon (1986), not only inhibitors showed an inhibitory effect on sperm motility of human, mouse, rat and bovine spermatozoa, but also the addition of several substrates for serine proteases. Surprisingly, when we performed porcine IVF with epididymal spermatozoa, there was no effect of 100 μM TLCK on sperm penetration rate. Epididymal spermatozoa are known to have a higher fertilizing potential during IVF than ejaculated spermatozoa, which has been attributed to superior motility and the lack of contact with capacitation-inhibiting factors present in seminal plasma (Rath and Niemann 1997). Since inhibition of sperm penetration rate by TLCK depended on the type of spermatozoa (epididymal vs. ejaculated), further investigations to unravel the underlying mechanism need to focus on intrinsic properties of spermatozoa.

One of the four tested inhibitors, TPCK, most likely decreased sperm penetration due to cytotoxic or pro-apoptotic effects. Even though TPCK is commonly used for inhibition of chymotrypsin and chymotrypsin-like serine proteases (Beynon and Bond 2001), other biochemical effects have been reported including dual effects on cell death (Frydrych and Mlejnek 2008). More specifically, TPCK has been shown to block several features of apoptosis via inhibition of caspases, but also to induce cell death itself or to enhance cell toxicity of other agents. The loss of membrane and acrosomal integrity observed in our study may represent features of cell death only visible with time, whereas loss of mitochondrial membrane potential and sperm motility possibly reflect early stages of cell death. Garner *et al.* (1997) found a high correlation between the percentage of bovine spermatozoa with a high mitochondrial membrane potential and the percentage of viable spermatozoa and similar results have been reported with porcine spermatozoa (Guthrie and Welch 2006).

Advanced technologies such as Computer-Assisted-Sperm-Analysis, flowcytometric analysis and staining with fluorescent probes to evaluate sperm function are now easily available and commonly used. In the majority of previous studies that addressed the effect of serine protease inhibitors during IVF, the effect on sperm function was only checked by visual inspection under a light microscope, and therefore these findings should be interpret with caution. When we compare our findings with previous reports, two important aspects of inhibitor studies are underlined: 1) the range of effective concentrations within the same experimental set up differs between serine protease inhibitors, irrespective of equivalence in inhibitor spectrum; and 2) there are explicit differences between (spermatozoa of) mammalian species in susceptibility towards serine protease inhibitors.

Serine protease inhibitors AEBSF and STI were shown to inhibit sperm penetration of COCs in a dose-dependent way, without disturbance of sperm motility or integrity of the sperm and acrosomal membrane, parameters which are essential for preservation of fertilizing capacity. Subsequently, efforts were made towards identifying the fertilization step(s) at which AEBSF and STI exert their inhibitory effect (chapter 4). To evaluate the effect of inhibitors on sperm penetration through the cumulus, fertilization parameters of cumulus-intact (CI) and cumulus-free (CF) oocytes were compared. The inhibitory effect of both inhibitors was less pronounced in CI oocytes than in CF oocytes. Different from the effect of metalloprotease inhibitors (chapter 5), sperm passage through the cumulus was not hindered by serine protease inhibitors. However, there was a clear difference in the effect of serine protease inhibitors between fertilization of zona-intact and zona-free oocytes. The inhibitor-induced reduction of fertilization in zona-intact oocytes was absent in zona-free oocytes, indicating that sperm penetration of the zona pellucida was the step of the fertilization process which was disturbed. The participation of serine proteases in the fertilization process *in vitro* has been studied previously in several mammalian species, but especially in the human and the mouse (Honda *et al.* 2002; Cesari *et al.* 2010). Based on early studies reporting a decrease in fertilization rates in the presence of protease inhibitors, serine protease activity of spermatozoa has been mainly associated with binding to and penetration of the zona pellucida. Since then, various sperm serine proteases have been detected, on the sperm membrane, the acrosomal membrane and in the acrosomal content (Cesari *et al.* 2010). Unfortunately, it has proven very difficult to unravel the mode of action of each specific protease in order to define its role in fertilization. The sperm-specific serine protease acrosin has been studied most extensively and has been associated with different steps of fertilization, including sperm binding to the zona pellucida and zona lysis in several species including the pig, dispersal of acrosomal content in mouse and guinea pig, and activation of oocyte transmembrane receptors in mouse (Yamagata *et al.* 1998; Smith *et al.* 2000; Tranter *et al.* 2000)

Sperm penetration of the zona pellucida can be divided in three subsequent steps: 1) binding of the sperm cell to the zona pellucida (starting with a loose attachment followed by species-specific tight binding); 2) the acrosome reaction with release of acrosomal enzymes at the surface of the zona pellucida; and 3) zona lysis and sperm penetration of the zona pellucida. The observed inhibition of zona penetration in the presence of serine protease inhibitors can be due to inhibition of one or more of these steps.

Presumed involvement of serine proteases in the first step, sperm binding to the zona pellucida, was supported by our observation that the number of spermatozoa bound to the zona pellucida decreased when AEBSF was added to the fertilization medium. Based on the assessment of sperm motility in the presence of AEBSF it can be concluded with a high degree of certainty that the decrease in sperm-zona pellucida binding was not caused by a negative effect on sperm motility. It has been shown that porcine acrosin contains an effector side that could act as receptor for glycoproteins in secondary sperm binding to the zona pellucida (Tranter *et al.* 2000). Surprisingly, inhibitor STI showed no significant effect on sperm-zona binding, whereas inhibitors AEBSF and STI are both able to inhibit trypsin-like serine proteases such as acrosin. Differences in characteristics of AEBSF (low MW, irreversible inhibition) and STI (high MW, reversible inhibition) may have caused the discrepancy here, for example Müller-Esterl *et al.* (1983) described that low MW inhibitors inhibited porcine acrosin more effectively than inhibitors with a high MW and furthermore, that irreversible inhibitors appeared to perform better than reversible inhibitors. However, this is contradictory to our observations that STI had a stronger inhibitory effect than AEBSF on the acrosome reaction and on IVF after pre-incubation of spermatozoa. Inhibition of the acrosome reaction by serine protease inhibitors has been previously reported in human and cattle, and there is substantial evidence that the activity of a trypsin-like serine is involved (De Jonge *et al.* 1989; Llanos *et al.* 1993; Deppe *et al.* 2008). Whether or not serine protease inhibitors interfered with the acrosome reaction in porcine spermatozoa was not yet reported. Our study demonstrated that serine protease inhibitors AEBSF and STI inhibit the acrosome reaction induced by treatment with calcium ionophore A23187 and points to the involvement of a trypsin-like serine protease downstream of the calcium-induced step of acrosomal exocytosis in porcine spermatozoa. Acrosin has been implicated in the dispersal of acrosomal proteins in mice (Fraser 1982; Yamagata *et al.* 1998) and the cleavage of a matrix protein on acrosin-specific sites followed by release from the acrosome has been reported in guinea pig (Buffone *et al.* 2008). In human and hamster spermatozoa, acrosin inhibitors were able to inhibit the membrane fusion events of the acrosome reaction (Dravland *et al.* 1984; De Jonge *et al.* 1989).

Figure 29 presents a schematic model of sperm-zona pellucida interaction based on the results of the inhibitor experiments with serine protease inhibitors AEBSF and STI.

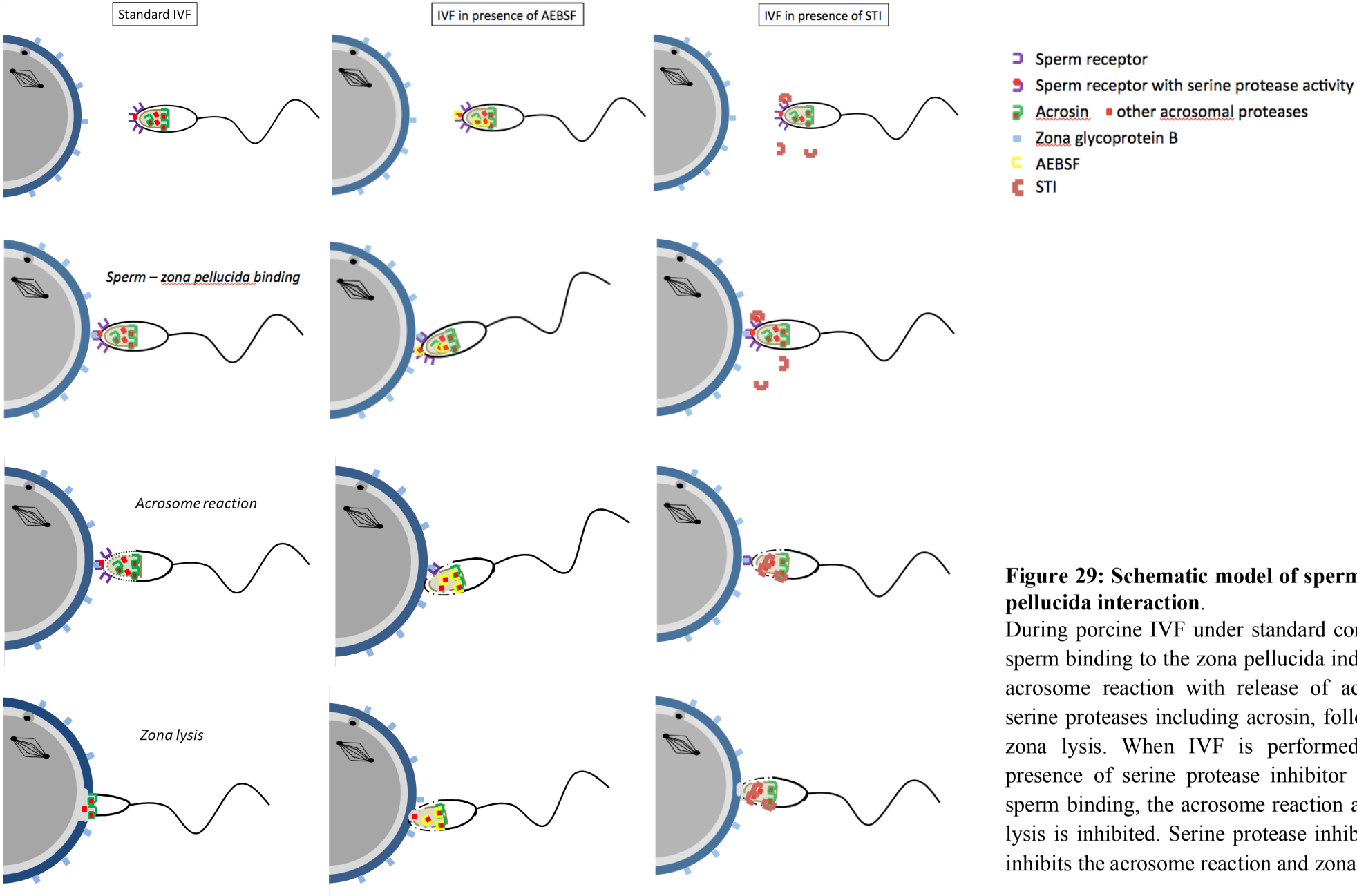

Figure 29: Schematic model of sperm – zona pellucida interaction.
During porcine IVF under standard conditions, sperm binding to the zona pellucida induces the acrosome reaction with release of acrosomal serine proteases including acrosin, followed by zona lysis. When IVF is performed in the presence of serine protease inhibitor AEBSF, sperm binding, the acrosome reaction and zona lysis is inhibited. Serine protease inhibitor STI inhibits the acrosome reaction and zona lysis.

In fertilization experiments with inhibitors, an effect on the acrosome reaction and/or subsequent zona lysis is very difficult to distinguish (Bedford 1998). Given the fact that zona lysis follows sperm binding to the zona pellucida and release of acrosomal content, no conclusions can be drawn from our study towards the possible inactivation of acrosomal proteases because the acrosome reaction was already disturbed by serine protease inhibitors. In most mammalian species, including the pig, only acrosome-reacted spermatozoa are able to penetrate the zona pellucida and fertilize the oocyte (Tulsiani *et al.* 1998). Additionally, it has been described that in porcine IVF stimulation of spontaneous acrosome reaction coincides with higher sperm penetration rates (Funahashi and Nagai 2001). Concordantly, the inhibitory effect of serine protease inhibitors on the acrosome reaction can explain the observed decrease in sperm penetration rate during porcine IVF. The decrease in sperm penetration may have additionally been reinforced by inhibition of acrosomal proteases involved in zona lysis.

Regarding the question how serine protease activity contributes to the acrosome reaction, the use of electron microscopy could be a first approach to differentiate whether the membrane fusion events and/or the dispersal of acrosomal content are altered in the presence of an inhibitor. Since STI and AEBSF are both able to inhibit acrosin but also various other trypsin-like proteases, further investigations are needed to elucidate whether acrosin and/or serine proteases other than acrosin are involved. In continuation of our inhibitor experiments, evaluation of the effect of an acrosin-specific inhibitor or anti-porcine acrosin antibodies on the calcium-ionophore induced acrosome reaction could be a next step. In mice, the role of acrosin and the significance of serine proteases other than acrosin have been investigated via *in vitro* fertilization experiments with spermatozoa from acrosin-deficient male mice. With the continuous progression in genetic modification of pigs, it might become possible on the long term to perform IVF studies with spermatozoa from acrosin-deficient pigs. If serine protease inhibitors AEBSF and STI would have an effect during IVF with acrosin-deficient porcine spermatozoa, this would be a clear indication that serine proteases other than acrosin are involved.

Serine protease inhibitors reduced sperm penetration rate during porcine IVF but what about their usefulness as a tool to improve IVF efficiency? The reduction in polyspermy rate by inhibitor STI coincided with a similar or even stronger decrease in total fertilization rate. Therefore STI is not suitable for improvement of monospermy rate. Inhibitor AEBSF, as tested in a concentration of 100 µM, showed a tendency towards a higher percentage of monospermic fertilization in CI and CF oocytes but this effect was not significant. However,

40 µM AEBSF increased monospermy rate significantly compared to the control group in the dose-response experiment. It would be noteworthy to evaluate whether this positive effect on monospermic fertilization leads to a better embryonic development of oocytes fertilized in the presence of 40 µM AEBSF compared to a control group.

In chapter 5 of this thesis, we investigated the effect of metalloprotease inhibitors during porcine *in vitro* fertilization of cumulus-intact and cumulus-free oocytes. During mammalian fertilization, the sperm cell needs to cross the extracellular layers of the oocyte, the cumulus oophorus and the zona pellucida, before gamete fusion can occur. In many species, including the pig, the cumulus oophorus is still present at the time of fertilization and represents the first site of sperm-oocyte interaction. The cumulus oophorus consists of cumulus cells embedded in a matrix rich in hyaluronic acid. It is generally acknowledged that sperm hyaluronidases and sperm motility enable spermatozoa to pass through the viscous cumulus matrix. In contrast, little is known about the functional significance of proteases in this step of the fertilization process. Three families of metalloproteases are known to play important roles in remodeling and degradation of extracellular matrix in other biological processes: the matrix metalloproteases (MMPs), the "A Disintegrin and Metalloprotease" family of proteases (ADAM) and the ADAM proteases with thrombospondin motifs (ADAMTS) (Shiomi *et al.* 2010). These metalloproteases are 'zinc-dependent' because their cleavage of substrates requires a zinc ion at the zinc binding motif in the active site of the enzyme. The study on the effect of metalloprotease inhibitors during porcine IVF was performed using phosphoramidon, zinc-chelator 1,10-phenanthroline (1,10-PHEN) and tissue inhibitor of metalloproteases (TIMP)-3, which is able to inhibit MMPs and few members of the ADAM family of metalloproteases. Our study showed a strong inhibitory effect of 1,10-PHEN (20 µM) and TIMP-3 (400 nM) on the degree of sperm penetration in cumulus-intact oocytes but not in cumulus-free oocytes, indicating that the metalloprotease inhibitor decreased the number of spermatozoa that penetrated the cumulus oophorus. Additionally, when COCs were co-incubated with mitotracker-labeled spermatozoa in fertilization medium with and without metalloprotease inhibitor, less spermatozoa were observed in the inner layers of the cumulus oophorus of COCs fertilized in medium with metalloprotease inhibitor. Possible negative effects on sperm motility, membrane integrity or acrosomal status were excluded. Therefore, our inhibitor study revealed that in addition to sperm motility and hyaluronidase, also metalloprotease activity plays a prominent role in facilitating sperm passage through the cumulus oophorus during porcine fertilization *in vitro*.

The metalloprotease involved in sperm passage through the cumulus oophorus can originate from spermatozoa and/or cumulus cells. There are only few studies on sperm metalloproteases thus far. The localization of MMP2 on the sperm head of human spermatozoa was described (Buchman-Shaked *et al.* 2002). The presence of MMP2 or other MMPs on spermatozoa of other species, including the pig, remains elusive. More recently, expression of ADAM1 and ADAM2 in porcine spermatozoa has been documented (Fabrega *et al.* 2011), yet these two ADAMs are not considered as candidate enzymes for the observed effect since both members of the ADAM family are enzymatically inactive. From the eight ADAM genes identified in the pig, *ADAM8*, *ADAM9*, *ADAM10*, *ADAM15* and *ADAM17* encode for a protein with metalloprotease activity (Rawlings *et al.* 2012). These ADAMS are capable of mediating cell adhesion and migration via their disintegrin domain (e.g. integrin binding) and via proteolysis of cell adhesion molecules (Reiss and Saftig 2009). Looking to the inhibition profile of TIMP-3, which is more specific than that of zinc-chelator 1,10-phenanthroline, TIMP-3 has been shown to inhibit ADAM10, ADAM15 and ADAM17 (Amour *et al.* 1998; Amour *et al.* 2000; Loechel *et al.* 2000; Maretzky *et al.* 2009), but not ADAM8 and ADAM9 (Amour *et al.* 2002). Especially ADAM10 and ADAM17 are of interest as putative sperm-derived metalloproteases because they mediate cleavage of cell adhesion molecules such as L-selectin, cadherins and also CD44 (Peschon *et al.* 1998; Nagano *et al.* 2004; Nagano and Saya 2004; Maretzky *et al.* 2005). CD44 is present on the surface of porcine cumulus cells and attaches cumulus cells to the matrix by interaction with hyaluronic acid (Yokoo *et al.* 2002; Yokoo *et al.* 2007). Interestingly, cleavage of CD44 leads to cell detachment from extracellular matrix (Nagano and Saya 2004). As literature on sperm metalloproteases is very scarce, we can for now only speculate that a TIMP-3 sensitive ADAM is present on porcine spermatozoa and may facilitate sperm passage through the cumulus by enhancing cell detachment.

Alternatively, the observed effect of metalloprotease inhibitors may result from inhibition of proteases secreted by cumulus cells. Cumulus cells have been described to synthesize and secrete several proteases, including metalloproteases ADAM17, ADAMTS1 and ADAMTS4 (Shimada *et al.* 2004; Yamashita *et al.* 2007). Furthermore, porcine cumulus cells were shown to increase production of ADAMTS1 in response to gonadotropins, followed by secretion of ADAMTS1 into the cumulus matrix (Shimada *et al.* 2004). The protease activity of ADAMTS1 has been associated with expansion of the cumulus matrix and remodeling of the follicle wall before ovulation, and also in the timely degradation of the cumulus matrix after ovulation (Russell *et al.* 2003; Shimada *et al.* 2004; Brown *et al.* 2010). In line with this, it

seems possible that in our study metalloprotease inhibitors could have inhibited physiological degradation of the cumulus matrix and thereby impeded sperm passage through the cumulus oophorus. However, in an additional experiment, we found that pre-incubation of *in vitro* matured COCs with 1,10-PHEN during 30 min prior to IVF had no significant effect on fertilization parameters, whereas it has been documented that the level of cumulus-derived ADAMTS1 protein in porcine COCs reaches its maximum at the end of the maturation period (Shimada *et al.* 2004). This opposes the hypothesis that the reduction in sperm penetration was caused by inhibition of cumulus-derived ADAMTS1 and hindrance of physiological matrix degradation.

Further research should be directed towards the identification of the putative sperm metalloprotease. This metalloprotease is probably bound to the sperm membrane since the acrosomal membrane is exposed after the acrosome reaction, which most spermatozoa only undergo after binding to the zona pellucida. Proteomic analysis of the porcine sperm membrane could provide a limited number of candidate enzymes. Next, antibodies directed against or substrates for these metalloproteases found on the sperm membrane could be tested for their effect on sperm passage through the cumulus.

Polyspermic fertilization can be inhibited with the addition of zinc-chelator 1,10-PHEN but the reduction in total fertilization rate is too strong to be useful for the optimization of IVF efficiency. On the other hand, inhibitor TIMP-3 reduced polyspermic fertilization in CF oocytes with no significant effect on total fertilization. Provided that CF oocytes are used for IVF, TIMP-3 can thus be used to improve the percentage of monospermic fertilization.

In conclusion, this thesis has contributed to better understanding the involvement of proteases during the sequential steps of porcine fertilization *in vitro* (Fig. 30). In first instance, we found that metalloprotease activity facilitates sperm passage through the cumulus, whereas thus far only sperm motility in combination with sperm hyaluronidase were believed to be responsible for paving the way through the cumulus matrix. Second, presumed involvement of serine protease activity in binding of porcine spermatozoa to the zona pellucida has been confirmed. And third, our study demonstrated that trypsin-like serine protease activity plays an important role in the ability of porcine spermatozoa to undergo the acrosome reaction. The critical role of proteases in porcine fertilization has thus been unequivocally demonstrated by our inhibitor experiments.

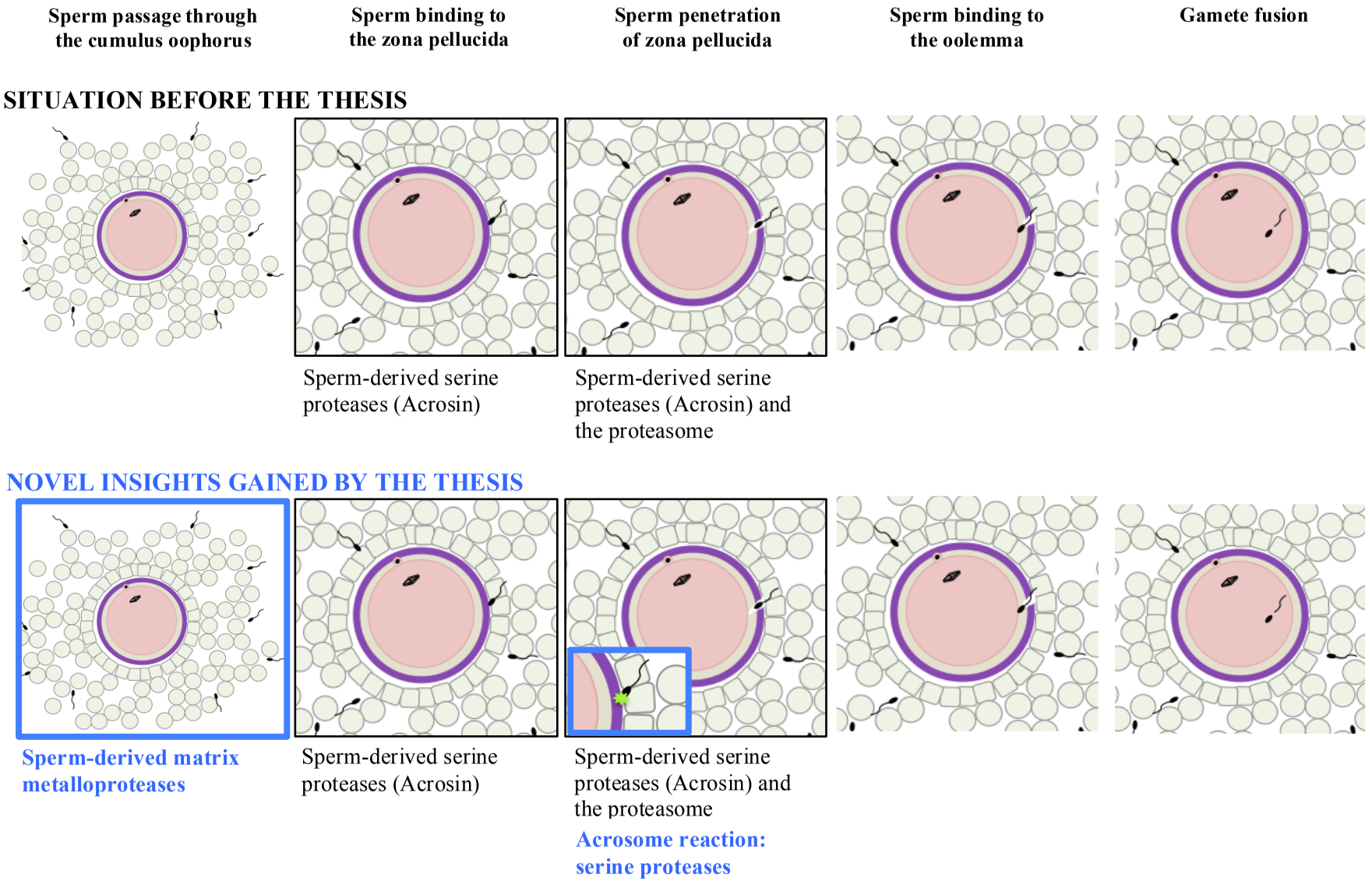

Figure 30: Schematic overview of the involvement of proteases during the sequential steps of porcine fertilization *in vitro*: situation before the thesis and novel insights gained by the thesis

Analogy

There are interesting similarities between the role of proteases in sperm-oocyte interaction and their role in other biological processes with cell invasion, such as neovascularization, inflammation, wound healing and tissue remodeling. Moving cells have been described to upregulate and/or activate different classes of proteases in response to varying substrates. For example, cathepsin L has a critical role in the invasion and function of endothelial progenitor cells in neovascularization (Urbich *et al.* 2005). Secretion of matrix metalloproteases MMP8, MMP9 and MMP25 are believed to support extravasation and migration of neutrophils to the site of inflammation (Faurschou and Borregaard 2003). Following exposure to inflammatory signals, neutrophils release serine proteases cathepsin G and neutrophil elastase, which exert proteolytic activity towards various extracellular matrix components and may facilitate further migration of neutrophils by cleavage of adhesion molecules (Pham 2008). Proteases play also critical roles in tumor invasion and metastasis. Especially two protease systems, the urokinase plasminogen activator/plasmin system and MMPs, are involved in the interaction between tumor cells and their surrounding stromal cells (Steeg 2006). Specific examples are expression of MT1-MMP on the cell surface of invasive tumor cells (Seiki 2003), and activity of MMP-2, together with binding to αvβ3 integrin, which enables mesenchymal cells to invade surrounding tissue (Sato *et al.* 1994; Rupp *et al.* 2008).

Proteases are also associated with host cell invasion by viruses. Analogously to a sperm cell that needs to cross the extracellular layers of the oocyte (the cumulus oophorus and the zona pellucida), a virus has to breach host barriers such as mucus layers, epithelial cells and the basement membrane in order to cause viremia and reach its target cells for infection. Trypsin-like serine protease activity was shown to mediate invasion of porcine pseudorabies virus through the basement membrane of the nasal mucosa (Glorieux *et al.* 2011). Both cellular and viral proteases are described to be involved in the pathogenesis of viral infections (Kaplan *et al.* 1994; Greber *et al.* 1996; Misinzo *et al.* 2008; Glorieux *et al.* 2011). The increased expression of MMP9 and urokinase plasminogen activator by Epstein-Bar virus (Yoshizaki 2002), and MMP-2 and MMP-9 by human papillomavirus (Katori *et al.* 2006), are thought to play a role in cell migration.

PROTEASES AND CUMULUS EXPANSION OF PORCINE OOCYTES

In a second part of this thesis, we investigated two hypothetical mechanisms by which alpha$_2$-macroglobulin (A2M) can exert a negative effect on cumulus expansion. Our first hypothesis was that A2M inhibits cumulus expansion via binding of EGF, followed by decreased availability of EGF for interaction with EGF receptors on cumulus cells (Fig. 31) The number of EGF receptors on cumulus cells is increased by FSH (which is commonly added to porcine oocyte maturation media) and after binding of EGF to its receptor, cumulus expansion is induced (Mattioli *et al.* 1991; Procházka *et al.* 2000). We could merely detect a negative effect during porcine IVM when extra EGF was supplemented to the maturation medium (20 ng/ml and 50 ng/ml vs. 10 ng/ml). Since it was reported that the beneficial effects of EGF are withdrawn, i.e. reduced blastocyst development after IVF, by concentrations higher than 10 ng/ml in the oocyte maturation medium (Abeydeera *et al.* 1997), we consider that the absence of a beneficial effect on cumulus expansion by increased concentrations of EGF suggest that EGF was not captured by A2M molecules. Concordantly, we rejected the first hypothesis.

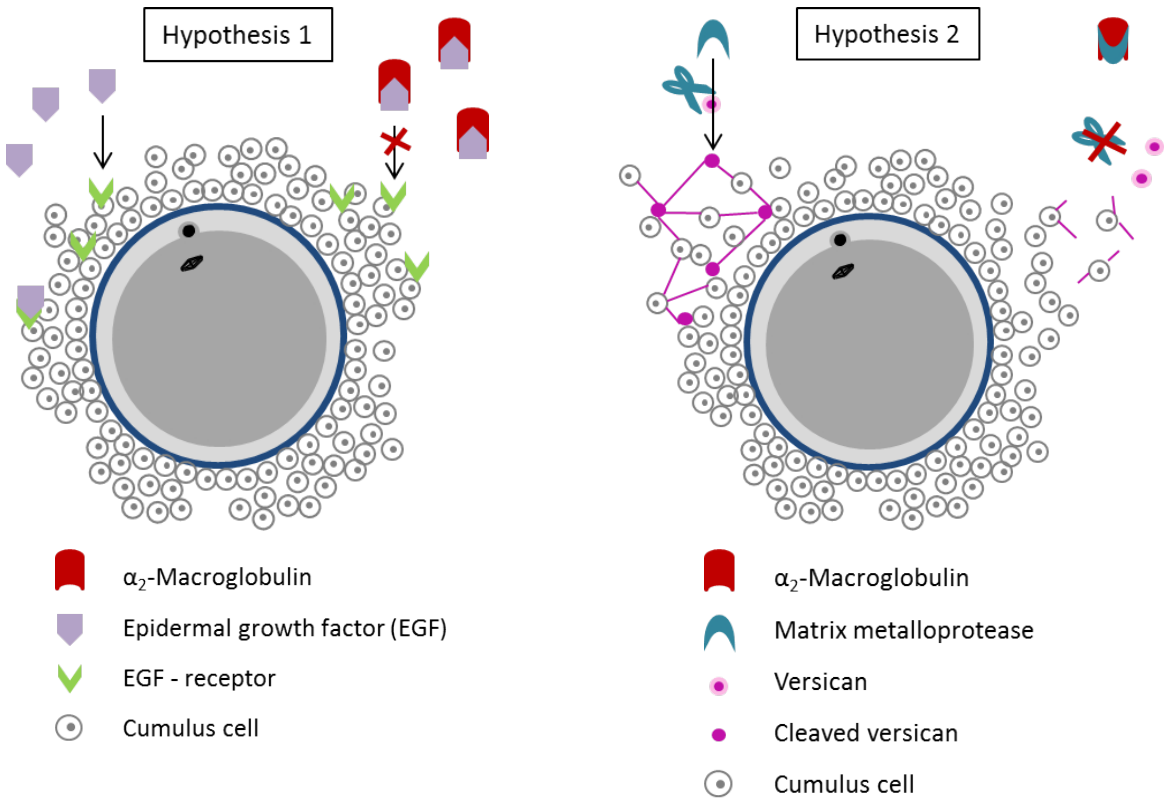

Figure 31: Schematic overview of two hypotheses by which an excessive presence of alpha$_2$-macroglobulin in the oocyte maturation medium may hamper cumulus expansion during porcine IVM.

A second hypothesis for the hampered cumulus expansion in the excessive presence of A2M in the medium, was inhibition of proteases which function in the process of cumulus expansion. The main component of the expanded cumulus matrix is hyaluronan, which is stabilized in a network with hyaluronan-binding proteins such as versican, tumor necrosis factor-stimulated gene-6 protein and serum-derived members of the inter-α-trypsin inhibitor family (Russell *et al.* 2003; Nagyova *et al.* 2004; Nagyova *et al.* 2008). Incorporation of versican into the cumulus matrix requires cleavage by a metalloprotease (Russell *et al.* 2003). In previous studies, inhibition of matrix metalloproteases has been shown to decrease cumulus expansion of porcine cumulus-oocyte-complexes (Shimada *et al.* 2004). Therefore, we focused on the possible interference with cumulus expansion via inhibition of matrix metalloproteases by A2M (Fig. 31, hypothesis 2). Interestingly, supplementation of the *in vitro* maturation medium with tissue inhibitor of matrix metalloproteases (TIMP)-3 only had a significant inhibitory effect on cumulus expansion when A2M was neutralized by A2M antibodies. In contrast, when COCs were matured in maturation medium with 10% serum without immunoneutralization of A2M, there was no effect of this matrix metalloprotease inhibitor, suggesting that serum-derived A2M and exogenously added matrix metalloprotease inhibitor share a common target. To confirm whether the effect of A2M is due to binding and inactivation of matrix metalloproteases, additional experiments are warranted to identify which components of the medium are bound to A2M during *in vitro* maturation. Via immunoprecipitation using a goat-anti-human A2M antibody, A2M could be successfully isolated from cumulus matrixes of porcine COCs matured in maturation medium with 10% serum (Beek, unpublished data). Subsequently, we tried to immunoprecipitate the protein complex of A2M and its binding ligand(s). Mass spectrometry of the protein sample isolated by immunoprecipitation of A2M protein complexes revealed the presence of several other proteins besides A2M, but a matrix metalloprotease was not yet detected. We cannot formally exclude its presence in the sample because detection might have been hindered by the elution of peptides from high abundant proteins, such as A2M and immunoglobulins. Further experiments are thus necessary to confirm our data indicative of an effect of A2M via binding and inhibition of matrix metalloproteases. Furthermore, the inhibitory effect of A2M on cumulus expansion might be due to interactions with more than one component of the maturation medium. Further consideration of the proteins identified by mass spectrometry from the immunoprecipitate with A2M may lead to alternative hypotheses for the effect of A2M on cumulus expansion.

General conclusions

From the results described in the present thesis, the following conclusions can be drawn:

1. Inhibitors of zinc-dependent metalloproteases decrease sperm penetration during porcine IVF by inhibition of sperm passage through the cumulus oophorus. The metalloprotease involved seems to be sperm-derived since pre-incubation of cumulus-oocyte-complexes with inhibitors did not influence fertilization outcome.
2. Serine protease inhibitors can be useful tools to study mechanisms of fertilization on the condition that they are properly tested for side effects. Serine protease inhibitors strongly differ in their effect on fertilizing capacity of porcine spermatozoa. Sperm characteristics that may be affected by these inhibitors include membrane integrity, sperm motility, mitochondrial membrane potential and the ability to undergo the acrosome reaction.
3. During porcine IVF, serine protease activity is involved in sperm-binding to the zona pellucida and the acrosome reaction. Since serine protease inhibitors interfered with the acrosome reaction, the effect on subsequent zona lysis could not be interpreted via inhibitor studies.
4. One protease inhibitor is not able to inhibit fertilization completely. The existence of alternative pathways to successful fertilization is generally acknowledged and also observed in the present thesis. By redundancy of molecules involved in sperm-oocyte interaction, nature has maximized the chance on successful fertilization and survival of a species.
5. From the protease inhibitors tested, AEBSF and TIMP-3 were able to improve IVF efficiency by reducing polyspermic fertilization with only a minor concurrent decrease in total fertilization rate.
6. The negative effect on cumulus expansion of cumulus-oocyte-complexes caused by an excess of A2M present during *in vitro* maturation is not due to binding of EGF, followed by decreased availability of EGF for interaction with EGF receptors on cumulus cells.
7. Inhibition of zinc-dependent metalloproteases hindered cumulus expansion in maturation medium supplemented with 10% serum and A2M antibodies, but did not show an effect during maturation in medium with 10% serum without A2M antibodies, suggesting that the metalloprotease inhibitor and A2M share a common target.

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CHAPTER 8

SUMMARY

Assisted reproductive technologies (ART) are methods used to achieve fertilization and/or pregnancy by artificial or partially artificial procedures. In pig production, the most commonly practiced ART is artificial insemination. Next to that, embryo transfer in pigs is gaining interest because of recent developments improving the feasibility of this technique. Both ARTs are important for exchange of valuable genetics between countries worldwide. Other ARTs such as in vitro production of porcine embryos, the production of cloned embryos by nuclear transfer or the production of transgenic embryos are currently part of fundamental and biomedical research. The domestic pig, *Sus scrofa domestica*, is considered to be a major animal model in human biomedical research. The three major steps of in vitro embryo production, *in vitro* maturation of immature porcine oocytes (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) are described in **chapter 1.2**.

An overview of the current scientific knowledge concerning the involvement of proteases in mammalian fertilization is given **in chapter 1.3**. Proteases have been recognized to play a role in various steps of mammalian fertilization. Serine protease activity of spermatozoa was first associated with sperm penetration through the zona pellucida, and more recently with other fertilization events such as sperm capacitation, dispersion of acrosomal content and activation of oocyte transmembrane receptors. There is also evidence for the involvement of metalloprotease activity in gamete fusion and the subsequent cortical reaction. Proteases thus represent interesting candidates to explore which step(s) during porcine IVF depend on the action of proteases and how the fertilization process is affected by protease inhibition. At this time, porcine IVF is still associated with a high incidence of polyspermic fertilization compromising large-scale embryo production. High polyspermy rates have been associated with failures in the cortical reaction leading to an incomplete “zona block” and with high numbers of spermatozoa near the oocyte when compared to *in vivo* conditions. Specific protease inhibitors might be useful as regulators of sperm penetration in porcine IVF, lessening the problem of polyspermic fertilization and increasing the efficiency of embryo production.

The general aim of this thesis was to gain more insight in the role of proteases during *in vitro* maturation and *in vitro* fertilization of porcine cumulus-oocyte-complexes (COCs) by 1) assessing the inhibitory effect of serine protease inhibitors and metalloprotease inhibitors on fertilization parameters after porcine IVF and 2) testing two hypotheses by which an excess of broad spectrum protease inhibitor alpha$_2$-macroglobulin may hamper cumulus expansion during IVM of porcine oocytes. A more detailed description of the aims of this thesis is given in **chapter 2**.

Among the different approaches that can be used to study the fertilization process *in vitro*, assessment of the inhibitory effect of enzyme inhibitors or binding ligands during different steps of fertilization has been shown to be a feasible method. Previous fertilization studies with serine protease inhibitors did not elaborate on the negative effect of inhibitors on sperm motility, nor provided any quantitative data of possible toxic effects. In **chapter 3**, the effect of different serine protease inhibitors on sperm penetration rate during porcine IVF is evaluated and discussed in the light of their effect on sperm quality parameters. Four serine protease inhibitors were investigated: 4-(2-Aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF), Soybean Trypsin Inhibitor from Glycine Max (STI), N_{α}-Tosyl-L-lysine-chloro-methyl ketone hydrochloride (TLCK) and N_{p}-Tosyl-L phenylalanine-chloromethyl ketone (TPCK). In this way, both a reversible and irreversible mode of action and a small versus high molecular weight protease inhibitor were included. All inhibitors significantly decreased sperm penetration during porcine IVF with freshly diluted semen. Inhibitors AEBSF (100 μM), TLCK (100 μM) and TPCK (100 μM) decreased total fertilization and polyspermy rate by 50% or more compared to the respective control group. Inhibitor STI (5 μM) had a significant inhibitory effect on total fertilization but not on polyspermy rate. Using ejaculates of the same boars as used for IVF, the possible effect of inhibitors on the following sperm parameters was assessed: membrane integrity, sperm motility, mitochondrial membrane potential and acrosomal integrity. Co-incubation with serine protease inhibitors AEBSF or STI had no effect on these sperm parameters. Inhibitor TLCK decreased sperm motility at relatively low concentrations when compared to previous studies in mouse, rat, human and bovine spermatozoa, in which more than tenfold higher concentrations of TLCK were used without an inhibitory effect on motility. One of the four tested inhibitors, TPCK, most likely decreased sperm penetration due to cytotoxic or pro-apoptotic effects. Together the results clearly showed that serine protease inhibitors differ in the way they reduce fertilization rates and underline proper testing of inhibitors before use in fertilization studies.

Serine protease inhibitors AEBSF and STI were shown to inhibit sperm penetration of COCs in a dose-dependent way, without disturbance of sperm motility or integrity of the sperm and acrosomal membrane, parameters which are essential for preservation of fertilizing capacity. Subsequently, efforts were made towards identifying the fertilization step(s) at which AEBSF and STI exert their inhibitory effect (**chapter 4**). To evaluate the effect of inhibitors on sperm penetration through the cumulus, fertilization parameters of cumulus-intact and cumulus-free

oocytes were compared. The inhibitory effect of AEBSF (100 μM) and STI (5 μM) was less pronounced in cumulus-intact (CI) oocytes than in cumulus-free (CF) oocytes. Furthermore, the inhibition of fertilization rate observed in zona-intact oocytes was absent in zona-free oocytes, indicating that sperm penetration of the zona pellucida was the step of the fertilization process which was disturbed. Sperm penetration of the zona pellucida can be divided in three subsequent steps: 1) binding of the sperm cell to the zona pellucida 2) the acrosome reaction with release of acrosomal enzymes at the surface of the zona pellucida; and 3) zona lysis and sperm penetration of the zona pellucida. The number of spermatozoa bound to the zona pellucida decreased when AEBSF was added to the fertilization medium, whereas STI showed no effect on sperm-zona binding. Interestingly, both AEBSF and STI significantly decreased the proportion of spermatozoa that completed the acrosome reaction in response to calcium ionophore A23187, when compared to the control group. Because only acrosome reacted spermatozoa are able to successfully fertilize an oocyte, this inhibition of the acrosome reaction can, in combination with the decreased sperm-zona binding, account for the lower sperm penetration rate observed in the presence of serine protease inhibitors AEBSF and STI.

In analogy with the inhibitor study on serine protease inhibitors, we also investigated the possible effect of metalloprotease inhibitors on porcine IVF (**chapter 5**). In many species, including the pig, the cumulus oophorus represents the first site of sperm-oocyte interaction. Sperm hyaluronidase and sperm motility are generally acknowledged to enable spermatozoa to pass through the viscous cumulus matrix. Little is known about the functional significance of proteases in this step of the fertilization process. Yet three families of zinc-dependent metalloproteases play key roles in remodeling and degradation of extracellular matrix in other biological processes: the matrix metalloproteases, the “A Disintegrin and Metalloprotease” (ADAM) family of proteases and the ADAM proteases with thrombospondin motifs. Therefore, we included three metalloprotease inhibitors, phosphoramidon, zinc-chelator 1,10-phenanthroline (1,10-PHEN) and tissue inhibitor of metalloproteases (TIMP)-3 in our inhibitor study. The two inhibitors of zinc-dependent metalloproteases, 1,10-PHEN (20 μM) and TIMP-3 (400 nM) showed a strong inhibitory effect on the degree of sperm penetration in cumulus-intact oocytes but not in cumulus-free oocytes, indicating that the metalloprotease inhibitor decreased the number of spermatozoa that penetrated the cumulus oophorus. The inhibitory effect of 1,10-PHEN via chelation of metal ions was confirmed by an IVF experiment with a non-chelating analogue (1,7-PHEN) in which fertilization rates of COCs

were not affected. Additionally, when COCs were co-incubated with mitotracker-labeled spermatozoa in fertilization medium with and without 1,10-PHEN, less spermatozoa were observed in the inner layers of the cumulus oophorus of COCs fertilized in medium with the inhibitor of zinc-dependent metalloprotease. Possible negative effects on sperm motility, membrane integrity or acrosomal status were excluded. Together these findings strongly indicate that metalloprotease activity plays a prominent role in facilitating sperm passage through the cumulus oophorus during porcine fertilization in vitro.

Alpha$_2$-macroglobulin, a plasma protein and non-specific protease inhibitor, has been associated with hampered cumulus expansion during IVM of porcine oocytes in medium with 10% serum. **Chapter 6** describes our investigation of two hypothetical mechanisms by which an excessive amount of alpha$_2$-macroglobulin (A2M) in maturation medium can exert a negative effect on cumulus expansion. The first hypothesis was that A2M may inhibit cumulus expansion via binding of Epidermal Growth Factor (EGF), followed by decreased availability of EGF for interaction with EGF receptors on cumulus cells. However, when extra EGF (20 ng/ml and 50 ng/ml vs. 10 ng/ml) was added to oocyte maturation medium with 10% serum, we could not detect a positive effect on cumulus expansion or matrix formation. After 44 h of IVM, cumulus diameters (median ± interquartile range) were 260 ± 160 μm (10 ng/ml EGF), 280 ± 80 μm (20 ng/ml EGF) and 240 ± 110 μm (50 ng/ml EGF). The mean percentage of COCs participating in the mucoid cloudy structure was 82%, 77% and 87% with 10 ng/ml EGF, 20 ng/ml EGF and 50 ng/ml EGF, respectively. As there was no indication that cumulus expansion in medium with 10% serum was influenced by decreased availability of EGF, the first hypothesis was rejected. The second hypothesis for the hampered cumulus expansion in the excessive presence of A2M was derived from its activity as a non-specific protease inhibitor. In previous studies, inhibition of zinc-dependent metalloproteases ADAMTS1 and ADAMTS4 has been shown to decrease cumulus expansion of porcine COCs. Therefore, for the second hypothesis the inhibitory effect of A2M on cumulus expansion was compared with that of a more specific inhibitor of zinc-dependent metalloproteases, TIMP-3. Interestingly, TIMP-3 (400 nM) hindered cumulus expansion in maturation medium supplemented with 10% serum in the presence of A2M antibodies, but did not show an effect during maturation in medium with 10% serum without A2M antibodies or in the presence of control antibodies, suggesting that serum-derived A2M and exogenously added TIMP-3 share a common target. Nevertheless, additional experiments are warranted to confirm whether the effect of A2M is due to binding and inactivation of a matrix metalloprotease.

Finally, in **chapter 7**, the main results of this thesis are summarized and discussed. Protease inhibitors can be useful tools to study mechanisms of fertilization on the condition that they are properly tested for side effects. Sperm characteristics that may be affected by protease inhibitors, as shown by the various effects of different serine protease inhibitors, include sperm membrane integrity, sperm motility, mitochondrial membrane potential and the ability to undergo the acrosome reaction.

During porcine IVF, zinc-dependent metalloprotease activity plays a prominent role in facilitating sperm passage through the cumulus oophorus. The metalloprotease involved seems to be sperm-derived since pre-incubation of cumulus-oocyte-complexes with inhibitors did not influence fertilization outcome. The involvement of serine protease activity in sperm-binding to the zona pellucida was confirmed and furthermore, serine protease activity was shown to be involved in the acrosome reaction.
The negative effect on cumulus expansion in vitro by an excess of A2M present in the maturation medium with 10% serum is not due to a decreased availability of EGF. On the other hand, there is a first line of evidence that the negative effect of A2M on cumulus expansion involves inhibition of zinc-dependent metalloproteases.

SAMENVATTING

Bij geassisteerde voortplanting wordt de bevruchting van een eicel of het tot stand brengen van een zwangerschap door een kunstmatige behandeling ondersteund. In de varkenshouderij is kunstmatige inseminatie de meest toegepaste vorm van geassisteerde voortplanting. Verder is er een toenemende aandacht voor embryotransplantatie vanwege recente ontwikkelingen in het wetenschappelijk onderzoek die de praktische toepasbaarheid van embryotransplantatie verhogen. Beide methoden van geassisteerde voortplanting, kunstmatige inseminatie en embryotransplantatie, spelen een belangrijke rol bij het op wereldschaal uitwisselen van waardevolle genetica. Andere vormen van geassisteerde voortplanting zoals *in vitro* productie van varkensembryo's en het produceren van gekloonde embryo's en transgene embryo's maken nu nog vooral deel uit van fundamenteel en biomedisch onderzoek. Het gedomesticeerd varken, *Sus scrofa domestica*, wordt beschouwd als een belangrijk diermodel voor humaan onderzoek.

De drie hoofdstappen van *in vitro* productie van varkensembryo's, namelijk *in vitro* maturatie van onrijpe varkenseicellen (IVM), *in vitro* fertilisatie (IVF) en *in vitro* cultuur (IVC) worden beschreven in **hoofdstuk 1.2**.

In **hoofdstuk 1.3** wordt een overzicht gegeven van de huidige wetenschappelijke kennis over de rol van proteasen in het bevruchtingsproces bij zoogdieren. Het is algemeen aanvaard dat proteasen betrokken zijn bij verschillende stappen van de bevruchting. Serine proteasen van spermacellen werden in eerste instantie in verband gebracht met de penetratie van de zona pellucida rondom de eicel. Naderhand is hun betrokkenheid bij andere stappen van de bevruchting beschreven, zoals bij de capacitatie van spermacellen, de vrijstelling van acrosomale inhoud tijdens de acrosoomreactie en de activatie van eicel transmembraan receptoren. Er is ook bewijs voor betrokkenheid van metalloprotease activiteit bij de fusie van eicel en spermacel en de daaropvolgende corticale reactie. Op basis van deze informatie zijn proteasen interessante kandidaten om hun rol tijdens het bevruchtingsproces bij het varken verder te onderzoeken, met name hoe de bevruchting beïnvloed kan worden door inhibitie van proteasen die vermoedelijk betrokken zijn bij de bevruchting. Tot op heden resulteert *in vitro* fertilisatie bij het varken in een hoog percentage van polyspermisch bevruchte eicellen, wat embryoproductie op grote schaal verhindert. De hoge graad van polyspermie bij *in vitro* fertilisatie wordt gelinkt met het falen van de corticale reactie en met het hoge aantal spermacellen in de nabijheid van de eicel in vergelijking met de bevruchting onder natuurlijke omstandigheden. Specifieke protease inhibitoren zouden nuttig kunnen zijn om de mate van spermapenetratie tijdens IVF te regelen waardoor het probleem van polyspermische

bevruchting verminderd kan worden en de *in vitro* embryo productie bij het varken efficiënter kan verlopen.
De algemene doelstelling van deze thesis was het inzicht te vergroten in de rol van proteasen tijdens *in vitro* maturatie en *in vitro* fertilisatie van cumulus-eicel-complexen bij het varken door 1) het inhiberend effect van serine protease inhibitoren en metalloprotease inhibitoren op bevruchtingsparameters na IVF te onderzoeken en 2) twee hypothesen te testen aangaande de manier waarop een overmaat aan breedspectrum protease inhibitor alpha$_2$-macroglobuline in het maturatiemedium de cumulus expansie van varkens cumulus-eicel-complexen kan hinderen. In **hoofdstuk 2** worden de doelstellingen van deze thesis meer in detail uitgelegd.

Er zijn verschillende manieren waarop het bevruchtingsproces *in vitro* kan worden bestudeerd waaronder het onderzoeken van een mogelijk effect van enzyme inhibitoren of van bindingspartners tijdens IVF. In eerdere IVF studies met serine protease inhibitoren zijn mogelijke negatieve effecten van deze inhibitoren op de beweeglijkheid van spermacellen nooit uitgebreid aan bod gekomen, noch zijn er kwantitatieve data over mogelijke toxische effecten beschreven. In **hoofdstuk 3** werd het effect van verschillende serine protease inhibitoren op de graad van bevruchting nagegaan en kritisch bekeken in het licht van een effect op kwaliteitsparameters van spermacellen. Vier serine protease inhibitoren werden onderzocht: 4-(2-Aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF), Soybean Trypsin Inhibitor van Glycine Max (STI), N_α-Tosyl-L-lysine-chloro-methyl ketone hydrochloride (TLCK) en N_p-Tosyl-L phenylalanine-chloromethyl ketone (TPCK). Op deze manier werden zowel inhibitors met een reversiebele als irreversiebele werking ingesloten, alsook inhibitoren met een laag versus hoog moleculair gewicht. Alle vier serine protease inhibitoren verminderden de graad van spermapenetratie tijdens IVF. Inhibitoren AEBSF (100 μM), TLCK (100 μM) en TPCK (100 μM) verminderden het percentage totale bevruchting en de graad van polyspermie met meer dan de helft in vergelijking met de controlegroep. Inhibitor STI (5 μM) zorgde voor een significante reductie in percentage totale bevruchting maar niet voor een vermindering van polyspermie. Spermastalen afkomstig van dezelfde ejaculaten als gebruikt voor de IVF experimenten werden ook gebruikt om het effect van deze vier serine protease inhibitoren op belangrijke spermaparameters na te gaan: membraan-integriteit, beweeglijkheid, membraanpotentiaal van de mitochondriën en de integriteit van het acrosoom. Co-incubatie van spermacellen met AEBSF of STI had geen effect op deze parameters. Inhibitor TLCK verminderde de beweeglijkheid van sperma in relatief lage concentraties vergeleken met vroegere studies over spermacellen van muis, rat, mens en rund,

waar er meer dan tienmaal hogere concentraties van TLCK werden gebruikt zonder een remmend effect op beweeglijkheid.
Eén van de vier geteste inhibitoren, TPCK, verminderde de spermapenetratie waarschijnlijk door een cytotoxisch effect of door inductie van apoptose. De resultaten hebben duidelijk aangetoond dat serine protease inhibitoren op verschillende manieren de bevruchtings-percentages kunnen verlagen en benadrukken het belang van een grondige controle op neveneffecten voordat deze inhibitoren gebruikt worden in fertilisatiestudies.

Serine protease inhibitoren AEBSF en STI bleken voor een dosis-afhankelijke reductie in spermapenetratie van cumulus-eicel-complexen te zorgen, zonder daarbij de beweeglijkheid van spermacellen en de integriteit van de spermamembraan en het acrosoom aan te tasten (parameters die essentieel zijn voor de bevruchtingscapaciteit). Vervolgens werden er experimenten uitgevoerd om te onderzoeken tijdens welke stap van het bevruchtingsproces AEBSF en STI hun inhiberend effect op spermapenetratie uitoefende (**hoofdstuk 4**). Om het effect van inhibitoren op spermapenetratie doorheen de cumulus oophorus na te gaan, werden de bevruchtingsresultaten van cumulus-intacte en cumulus-vrije eicellen vergeleken. Het inhiberend effect van AEBSF (100 μM) and STI (5 μM) was minder sterk in cumulus-intacte dan in cumulus-vrije eicellen. Verder bleek dat bevruchting van zona-intacte eicellen geremd werd, maar niet de bevruchting van zona-vrije eicellen. Samen wijzen deze resultaten er op dat spermapenetratie doorheen de zona pellucida de stap van het bevruchtingsproces was die gehinderd werd in aanwezigheid van serine protease inhibitoren. Spermapenetratie van de zona pellucida kan onderverdeeld worden in drie opeenvolgende stappen: 1) binding van de spermacel aan de zona pellucida, 2) de acrosoomreactie met vrijstelling van acrosomale enzymes op het oppervlakte van de zona pellucida, en 3) lyse van de zona pellucida en penetratie van de spermacel doorheen de zona pellucida. Het aantal spermacellen dat gebonden was aan de zona pellucida was significant lager in aanwezigheid van AEBSF terwijl toevoeging van STI aan het fertilisatiemedium geen effect had op sperma-zona binding. Zowel AEBSF als STI verminderden de proportie van spermacellen dat de acrosoomreactie voltooide in reactie op calciumionofoor A23187, vergeleken met de controlegroep. Omdat enkel acrosoom-gereageerde spermacellen een eicel kunnen bevruchten, kan de inhibitie van de acrosoomreactie, samen met de reductie in sperma-zona binding, gezorgd hebben voor de lagere spermapenetratie die werd vastgesteld in aanwezigheid van serine protease inhibitoren AEBSF en STI.

Naar analogie met de studie over het effect van serine protease inhibitoren hebben we ook het mogelijk effect van metalloprotease inhibitoren op IVF bij het varken onderzocht (**hoofdstuk 5**). Bij veel diersoorten, waaronder het varken, is de cumulus oophorus de eerste plaats van interactie tussen spermacel en eicel. Er wordt algemeen van uit gegaan dat het enzyme hyaluronidase in combinatie met beweeglijkheid een spermacel in staat stelt om doorheen de visceuze cumulus matrix te dringen. Er zijn weinig gegevens over het mogelijke aandeel van proteasen in deze stap van de bevruchting. Nochtans zijn er drie families van zink-afhankelijke metalloproteases gekend om hun belangrijke rol in het remodelleren en afbreken van extracellulaire matrix in andere biologische processen: de matrix metalloproteasen, de "A Disintegrin and Metalloprotease (ADAM)" familie van proteasen en de familie van ADAM proteasen met thrombospondin motieven. Daarom werden er drie metalloprotease inhibitoren in onze inhibitorstudie ingesloten: phosphoramidon, zink-chelator 1,10-phenanthroline (1,10-PHEN) en "tissue inhibitor of metalloproteases (TIMP)-3. De twee inhibitoren van zink-afhankelijke metalloproteases, 1,10-PHEN (20 μM) en TIMP-3 (400 nM), toonden een sterke inhibitie van spermapenetratie in cumulus-intacte eicellen maar niet in cumulus-vrije eicellen, indicatief voor een inhiberende werking op de spermapenetratie doorheen de cumulus oophorus. Dat de inhibitie door 1,10-PHEN te wijten was aan chelatie van metaalionen werd bevestigd met een IVF experiment waarin een niet-chelerend analoog, 1,7-PHEN, werd toegevoegd aan het fertilisatiemedium en het bevruchtingspercentage van cumulus-intacte eicellen onveranderd bleef. Na incubatie van cumulus-eicel-complexen met mitotracker-gelabelde spermacellen in fertilisatiemedium met of zonder 1,10-PHEN werden er minder spermacellen waargenomen in de binnenste lagen van de cumulus oophorus van cumulus-eicel-complexen bevrucht in aanwezigheid van de inhibitor. Mogelijke negatieve effecten van de inhibitoren op beweeglijkheid, membraan integriteit en acrosoom status van spermacellen werden uitgesloten. Al deze resultaten samen geven een sterke indicatie dat metalloprotease activiteit een prominente rol speelt in het mogelijk maken van spermapenetratie doorheen de cumulus oophorus tijdens IVF bij het varken.

Enige tijd geleden werd beschreven dat alpha$_2$-macroglobuline, een plasma-eiwit en niet-specifieke protease inhibitor, geassocieerd is met verminderde cumulusexpansie tijdens maturatie van varkenseicellen in medium met 10% serum. **Hoofdstuk 6** beschrijft het onderzoek met betrekking tot twee hypothesen over de manier waarop een overmaat aan alpha$_2$-macroglobuline (A2M) in maturatiemedium een negatief effect kan hebben op cumulusexpansie. De eerste hypothese hield in dat A2M cumulusexpansie zou kunnen

hinderen via het binden van Epidermal Growth Factor (EGF), waarna er minder EGF beschikbaar zou zijn voor interactie met EGF receptoren op de cumulus cellen. Echter, wanneer er extra EGF (20 ng/ml en 50 ng/ml versus 10 ng/ml) werd toegevoegd aan het maturatiemedium met 10% serum was er geen positief effect op cumulusexpansie of cumulus matrix formatie. De cumulus diameter (mediaan ± interkwartielafstand) was respectievelijk 260 ± 160 μm (10 ng/ml EGF), 280 ± 80 μm (20 ng/ml EGF) en 240 ± 110 μm (50 ng/ml EGF) op het einde van de maturatieperiode. Het gemiddeld percentage van cumulus-eicel-complexen dat betrokken was in cumulus matrix formatie was 82%, 77% en 87% met10 ng/ml EGF, 20 ng/ml EGF en 50 ng/ml EGF. Gezien de resultaten niet in de richting wezen van een verminderde cumulus expansie in medium met 10% serum door een lage beschikbaarheid van EGF, werd de eerste hypothese verworpen. De tweede hypothese voor de minder goede cumulusexpansie in aanwezigheid van een overmaat aan A2M was gebaseerd op de rol van A2M als niet-specifieke protease inhibitor. In voorgaande studies is aangetoond geweest dat inhibitie van zink-afhankelijke matrix metalloproteasen ADAMTS1 en ADAMTS4 resulteert in verminderde cumulus expansie van cumulus-eicel-complexen. Daarom werd voor de tweede hypothese het inhiberend effect van A2M op cumulus expansie vergeleken met het effect van een meer specifieke inhibitor van zink-afhankelijke matrix metalloproteasen, TIMP-3. TIMP-3 (400 nM) hinderde cumulusexpansie in maturatiemedium met 10% serum in de aanwezigheid van A2M antistoffen, maar had geen effect in medium met 10% serum zonder A2M antistoffen of in aanwezigheid van controle antistoffen. De bevinding dat TIMP-3 enkel een effect had indien A2M antistoffen aanwezig waren die A2M kunnen neutraliseren, suggereert dat A2M afkomstig van het serum en exogeen toegevoegd TIMP-3 een gemeenschappelijke bindingspartner hebben. Er zijn echter nog bijkomende experimenten nodig om te bevestigen dat het negatieve effect van A2M op cumulusexpansie te wijten is aan binding en inactivatie van een matrix metalloprotease.

Tot slot worden de belangrijkste resultaten van deze thesis samengevat en besproken in **hoofdstuk 7.**

Op basis van de resultaten van deze thesis kunnen volgende besluiten getrokken worden:
protease inhibitoren kunnen een nuttige tool zijn om onderliggende mechanismen van het bevruchtingsproces te onderzoeken (en meer specifiek de rol van proteasen tijdens de bevruchting), op voorwaarde dat ze grondig getest worden op neveneffecten. Er zijn verschillende spermaparameters die beïnvloed kunnen worden, waaronder de integriteit van de membraan en van het acrosoom, de beweeglijkheid van spermacellen en de membraanpotentiaal van de mitochondriën, zoals aangetoond werd via de uiteenlopende effecten van verschillende serine protease inhibitoren op spermacellen van het varken.
Tijdens IVF bij het varken speelt zink-afhankelijke metalloprotease activiteit een belangrijke rol bij de passage van spermacellen doorheen de cumulus oophorus. De betrokken metalloprotease lijkt afkomstig te zijn van de spermacel gezien pre-incubatie van cumulus-eicel-complexen met een metalloprotease inhibitor het bevruchtingspercentage niet beïnvloedde. De betrokkenheid van serine protease activiteit in de binding van spermacellen aan de zona pellucida werd bevestigd en verder werd aangetoond dat serine protease activiteit ook betrokken is bij de acrosoomreactie.
Het negatieve effect van een overmaat aan A2M op cumulusexpansie van cumulus-eicel-complexen in maturatiemedium met 10% serum is niet toe te schrijven aan een verminderde beschikbaarheid van EGF. Aan de andere kant geeft dit onderzoek een eerste aanwijzing dat het negatieve effect van A2M mogelijk te wijten is aan inhibitie van zink-afhankelijke metalloproteasen.

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Josine

CURRICULUM VITAE

Josine Beek werd geboren op 15 mei 1984 te Roermond, Nederland. Na het behalen van het diploma hoger secundair onderwijs aan het Bisschoppelijk College Schöndeln te Roermond (VWO, Profiel Natuur en Gezondheid), begon zij in 2002 met de studie Diergeneeskunde aan de Universiteit Gent. In 2008 behaalde ze aan de Universiteit Gent haar diploma van dierenarts optie varken, pluimvee en konijn, met grootste onderscheiding.

In oktober 2008 startte ze haar doctoraatsonderzoek – ondersteund door een FWO mandaat – aan de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. Het onderzoek was gericht naar de rol van proteasen tijdens in vitro fertilisatie bij het varken. Naast haar doctoraatsonderzoek dat plaatsvond in het IVF laboratorium van de Reproductieve Biologie Unit was Josine Beek ook werkzaam in de kliniek verloskunde rund en als dierenarts binnen de Eenheid Varkensgezondheidszorg. Op 1 oktober 2012 trad zij in dienst van de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde, waar ze als assistent bedrijfs-diergeneeskunde varken betrokken was bij het theoretisch en klinisch onderwijs voor de laatstejaarsstudenten diergeneeskunde. In 2013 behaalde ze het diploma vakdierenarts varken en in 2014 voltooide zij de doctoraatsopleiding aan de Doctoral Schools of Life Sciences and Medicine.
Sinds september 2013 werkt Josine als technical advisor swine bij MSD Animal Health België en is ze verantwoordelijk voor de technische ondersteuning en begeleiding als hulp voor varkensdierenartsen.

Josine Beek is auteur of mede-auteur van verschillende publicaties in internationale en nationale wetenschappelijke tijdschriften en nam actief deel aan diverse internationale en nationale congressen.

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