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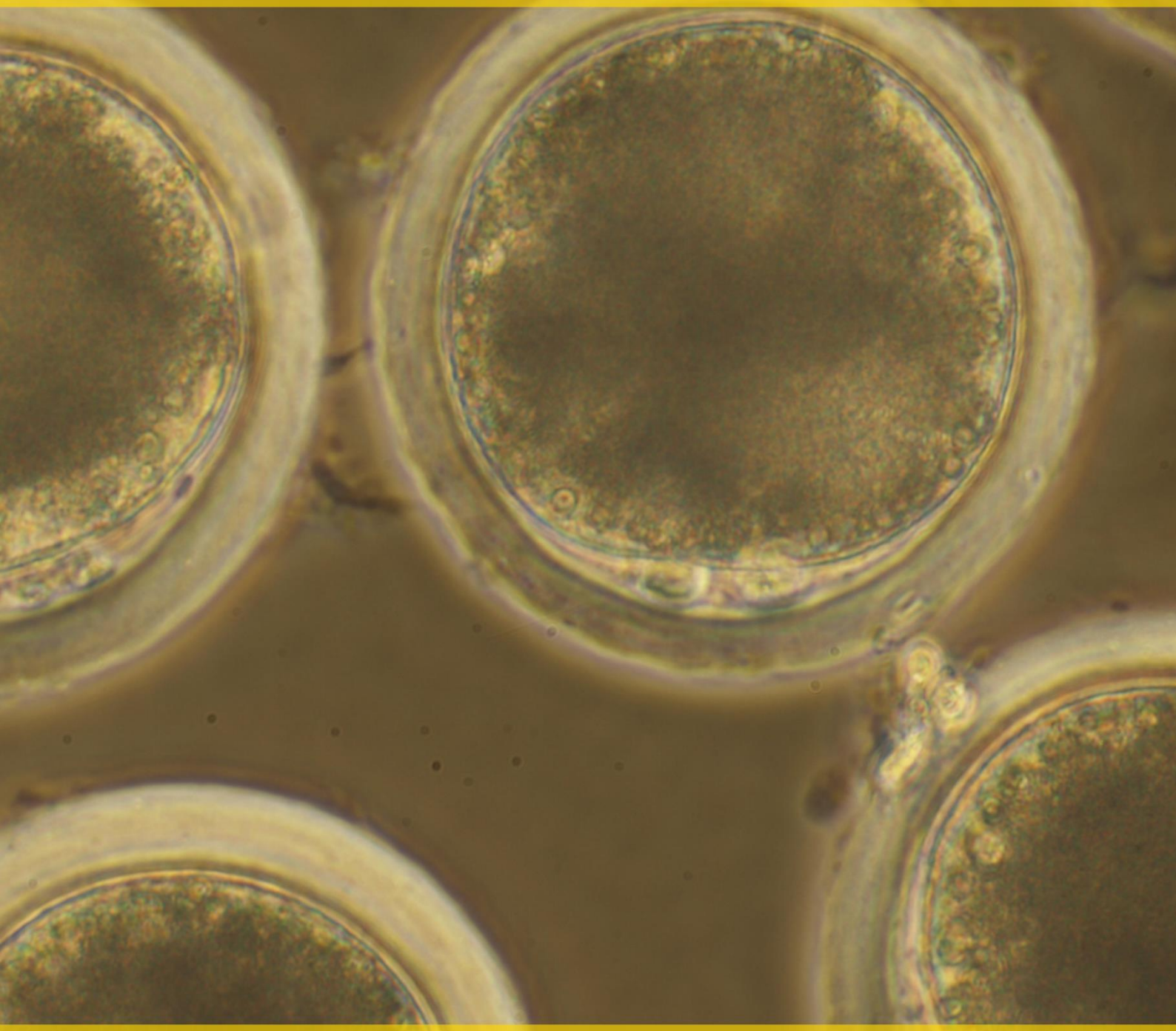

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Abstract

The appliance of *in vitro* fertilisation (IVF) has increased over the last few decades in both human and veterinary medicine. Although widely used in the clinic, IVF is still not very efficient and suffers from various species-dependent problems. *In situ*, sperm is mixed upon ejaculation with seminal plasma and deposited in the female reproductive tract. The seminal plasma is a mixture of fluids secreted by the different accessory sex glands, including the prostate, bulbourethral glands and seminal vesicles, and contains nutrients, steroid hormones, and several (proteolytic) enzymes. Other ubiquitous components of seminal plasma from mammalian species are extracellular vesicles (EVs). These EVs are heterogeneous in their characteristics and originate from the accessory sex glands. Functional studies on EVs indicated roles in a variety of physiological processes, including sperm capacitation, sperm motility activation and the acrosome reaction. However, conflicting outcomes have been reported and the consequences for fertilisation have not yet been elucidated. The aim of the current study was to provide more insight into the effects of pig seminal fluid EVs on *in vitro* fertilisation using the pig as a model species. First, a new method was developed to isolate EVs form boar seminal fluid. Herein, EVs were precipitated from cell-free seminal plasma using polyethylene glycol and subsequently floated to equilibrium density in a discontinuous Nycodenz® gradient. Isolated EVs were then used in a series of *in vitro* fertilisation experiments. Before fertilisation, washed sperm was pre-incubated with isolated EVs under capacitating conditions to allow binding. Sperm was then washed to remove excess non-bound EVs, and co-incubated with matured cumulus-oocyte complexes for 22 hours at varying conditions. Fertilisation was evaluated using fluorescence microscopy. In this setup, EVs reduced the efficiency of fertilisation, suggesting that EVs may have an inhibitory role in fertilisation. These unexpected results may point towards a role in sperm competition, but future experiments are required to further elucidate the role of EVs in fertilisation.

Introduction

Over the last decades, human infertility rates worldwide have increased, and the use of Assisted Reproductive Techniques (ART) has expanded (ESHRE Capri Workshop Group, 2015), approximately 5-10% growth every year (European Society of Human Reproduction and Embryology, 2014). A popular ART is *in vitro* fertilisation (IVF), which is nowadays widely applied as a solution for infertility (Pandian et al., 2012). Also in veterinary medicine, IVF is of considerable interest. In large domestic species, IVF is extensively used for cattle (Perry, 2014), pigs (Kitaji et al., 2015), and sheep and goat breading (Paramio and Izquierdo, 2014). Especially in cattle the production of *in vitro* fertilised embryos has increased over the last few years, with more than half a million IVF embryos produced worldwide in 2013 (Perry, 2014). IVF is also of considerable interest in non-domestic species, especially for the conservation and genetic management of endangered species (Pukazhenthi and Wildt, 2004). Despite its wide appliance in both human and veterinary medicine, IVF still suffers various problems. Depending on the species, problems in IVF involve low fertilisation efficiency (Chastant-Maillard et al., 2010), high occurrence of polyspermy (Chastant-Maillard et al., 2010; Kitaji et al., 2015) and/or low percentages of clinical pregnancy (Kupka et al., 2014). Understanding of the fundamental processes in fertilisation is essential to improve current IVF techniques for both man and animal reproduction.

During ejaculation, sperm is mixed with the secretions from the different accessory sex glands and deposited into the female reproductive tract. The seminal plasma is a complex mixture of fluids secreted by the diverse accessory sex glands and mainly contains nutrients and (proteolytic) enzymes (Druart et al., 2013). Although seminal plasma is considered by many solely as a passive delivery vehicle or a nutritive medium, it has been shown to influence various physiological processes relevant for fertilisation (Mcgraw et al., 2015; Poiani, 2006). Deciphering functions of individual plasma components is challenging due to the molecular complexity of the secretions (Mcgraw et al., 2015). Components of seminal plasma that have gained interest during the last few years, are extracellular vesicles (EVs).

EVs are membrane vesicles that are secreted by many types of mammalian cells under both physiological and pathological conditions (Raposo and Stoorvogel, 2013). Because of their widespread production, EVs can be isolated from various body fluids, including urine (Pisitkun et al., 2004), blood (Caby et al., 2005; Jayachandran et al., 2012), cerebrospinal fluid (Vella et al., 2007), breast milk (Admyre et al., 2007) and semen (Aalberts et al., 2012). Depending on their cellular origin, EVs have been reported to play differing roles in intercellular communication (Raposo and Stoorvogel, 2013). The content of EVs includes a wide variety of proteins, but also mRNA and miRNA, forming integrated packages of targeting information and signalling molecules (Raposo and Stoorvogel, 2013).

Basically, two types of EVs can be distinguished according to their subcellular origin; exosomes and microvesicles (figure 1). Exosomes are formed by the release of intraluminal vesicles of multivesicular bodies (MVBs), whereas microvesicles are formed by regulated outward budding of the plasma membrane (Raposo and Stoorvogel, 2013). In addition, exosomes and microvesicles can often be discerned based on their size, exosomes; 50-100 nm, microvesicles; 100-1000 nm, although this is not a hard criterion (Théry et al., 2009).

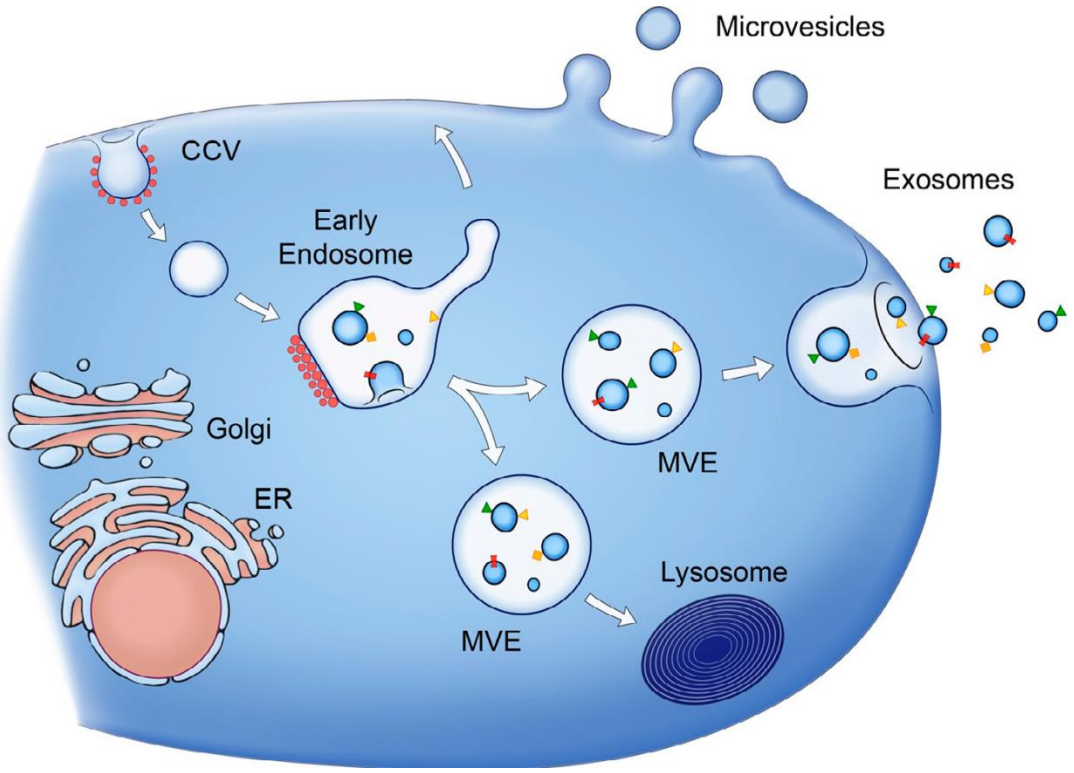

Figure 1. Schematic representation of the formation of exosomes and microvesicles.
Exosomes are formed by inward budding of an early endosome, resulting in intraluminal vesicles and a multivesiclular body (MVE/MVB). The intraluminal vesicles can then be released as exosomes by fusion of MVBs with the plasma membrane. Alternatively, MVBs may fuse with lysosomes, resulting in degradation of their content. Triangles and rectangles represent membrane-associated and transmembrane proteins that are incorporated during the formation of vesicles. Red dots indicate clathrin in clathrin-coated vesicles (CCV) at the plasma membrane, and clathrin coated sheets on endosomes,. The second type of vesicles, microvesicles, are formed by regulated budding from the plasma membrane. From Raposo and Stoorvogel, 2013

EVs were initially characterized in human seminal plasma (Ronquist et al., 1978a; Ronquist et al., 1978b), followed by identification of EVs in seminal plasma of various other species, including rabbits (Davis, 1973), rams (Breitbart and Rubinstein, 1982), bulls (Agrawal and Vanha-Perttula, 1988), stallions (Arienti et al., 1998), boars (El-Hajj Ghaoui et al., 2004), dogs (Zelli et al., 2013) and cats (Polisca et al., 2015). The seminal plasma EVs originate from various accessory sex glands in the male reproductive tract. EVs from distinct accessory sex glands, but also between mammalian species (Aalberts et al., 2014), differ significantly regarding their protein composition (Piehl et al., 2006; Sahlén et al., 2010; Aalberts et al., 2014). Despite their early discovery in the 1970s, the role of seminal EVs in fertilisation remains unclear up to date (Palmerini et al., 2003; Bechoua et al., 2011; Aalberts et al., 2013) .

Both during *in vivo* and *in vitro* fertilisation, ejaculated sperm first have to experience a series of modifications, i.e. capacitation, in order to gain full fertilising capacity (Ferramosca and Zara, 2014). During the process of capacitation, sperm undergoes specific structural and functional changes which allow them to bind to the extracellular matrix of the oocyte. *In vitro*, capacitation can be induced by the presence of extracellular bicarbonate, a cholesterol acceptor and calcium (Harrison and Gadella, 2005). When sperm is incubated in medium with a high concentration of bicarbonate (15 mM; Harrison and Gadella, 2005), intracellular levels of bicarbonate are raised (figure 2). This increase in intracellular bicarbonate rapidly activates a soluble adenylyl cyclase, elevating the concentration of cytosolic cyclic adenosine

monophosphate (cAMP; Bailey, 2010). cAMP on its turn activates cAMP dependent protein kinases (PKA), which are central activators of various capacitation-related events. These include tyrosine phosphorylation, reorganisation of the plasma membrane, and stimulation of sperm motility (Harrison and Gadella, 2005). An increase in protein tyrosine phosphorylation is generally considered as a marker for sperm capacitation (Bravo et al., 2005).

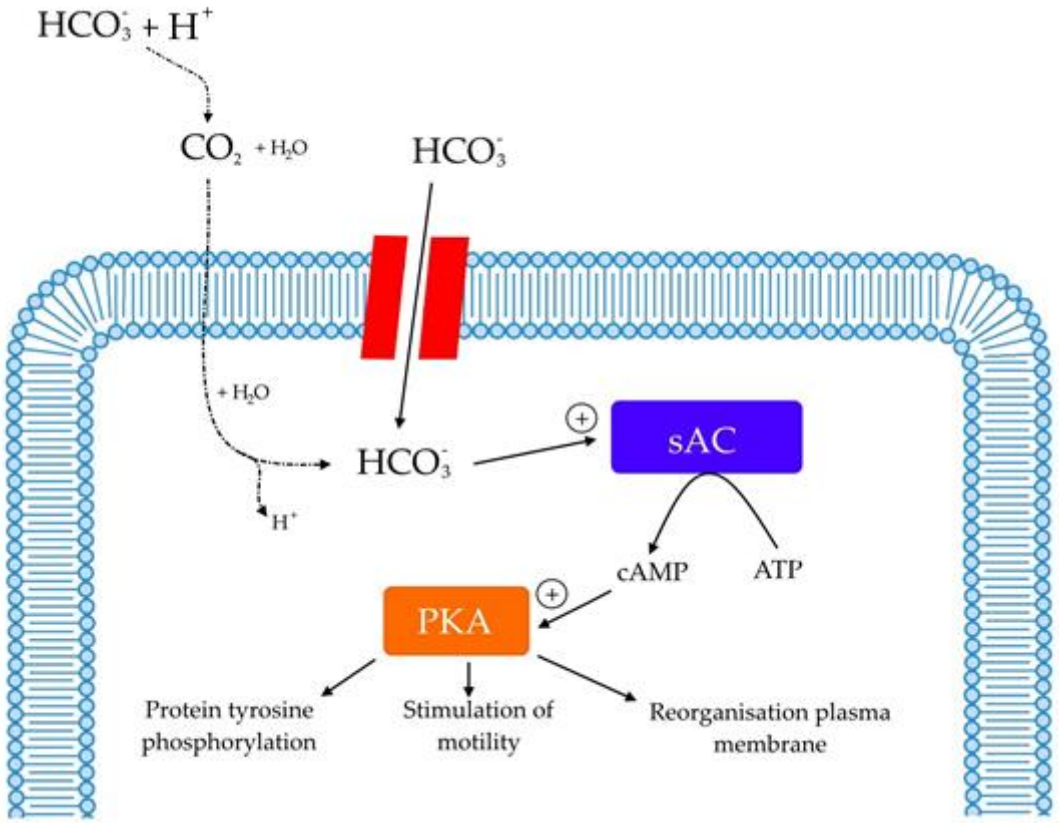

Figure 2. Schematic representation of the signalling cascade induced by bicarbonate in sperm.
Bicarbonate enters the cell via ion-channels (red) and passive diffusion of CO_2 (equilibrium extracellular and intracellular bicarbonate). Intracellular bicarbonate activates soluble adenylyl cyclase (sAC) which catalyses the production of cAMP from ATP. cAMP activates a cAMP dependent protein kinase (PKA), which induces signalling pathways that can be monitored by increased protein tyrosine phosphorylation, increased sperm motility, and reorganisation of the plasma membrane.

Several studies propose seminal plasma EVs as a regulatory factor for *in vivo* sperm capacitation. Regarding the effects of EVs on capacitation, literature discriminates early and late capacitation events. During early capacitation, EVs were reported to increase protein tyrosine phosphorylation (i.e. 5 min; Pons-Rejraji et al., 2011), whereas after a few hours of capacitation (i.e. 3 hours), tyrosine phosphorylation was rather reduced by the presence of EVs (Pons-Rejraji et al., 2011; Bechoua et al., 2011; Aalberts et al., 2013; Piehl et al., 2013), Liposomes with a lipid composition similar to that of EVs mimicked the inhibiting effect of EVs on capacitation, which may indicate that EV lipids are important for EV function(s) (Piehl et al., 2013).

In addition, several reports describe a stimulating effect of EVs on the motility of sperm (Fabiani et al., 1994a; Fabiani et al., 1994b; Carlsson et al., 1997; Arienti et al., 1999; Park et al., 2011). However, the reported effects were inconsistent and hence the effect of EVs on sperm cell motility remains unclear. Where some studies describe EVs to have no effect on

progressive motility of sperm (Bechoua et al., 2011; Piehl et al., 2013), others reported that EVs increase in progressive motility (Fabiani et al., 1994a; Fabiani et al., 1994b).

Following capacitation and binding to either the cumulus-oocyte complex or zona pellucida, sperm undergoes the acrosome reaction. The acrosome is a sperm specific organelle that is formed from the Golgi apparatus during spermiogenesis (Abou-Haila, 2000). The acrosome covers the anterior part of the sperm head and consists of a acid vacuole that is filled with a number of hydrolytic enzymes (Berruti and Paiardi, 2011). Only in the capacitated state, sperm cells can undergo the acrosome reaction, which includes an exocytotic event that leads to the disruption of the acrosome and the release of the hydrolytic enzymes (Fraser, 1998). The acrosome reaction plays a crucial role in the penetration of the zona pellucida and therefore fertility (Berruti and Paiardi, 2011). Both the release of the hydrolytic enzymes from the acrosome and hypermotility are required to allow efficient passage of sperm through the zona pellucida. Only after complete passage of the cumulus cells and the zona pellucida, i.e. when sperm resides in the perivitellin space of the oocyte, it has the potential to fuse with the plasma membrane of the oocyte (Bailey, 2010). *In vivo,* progesterone, released from the antral follicle upon ovulation and presumably produced by the cumulus cells, together with cumulus components stimulates the acrosome reaction (Gadella, 2013). Concerning the possible role of EVs in regulation of the acrosome reaction, literature is rather contradictory. Some reports describe the EVs to have a stimulating effect on the acrosome reaction (Palmerini et al., 2003; Siciliano et al., 2008), whereas others report an inhibiting effect (Pons-Rejraji et al., 2011). There are, however, also reports that claim that EVs have no effect on the acrosome reaction (Piehl et al., 2013).

In conclusion, at this moment there is no clarity about the physiological importance of EVs in semen with respect to regulation of the fertilisation by influencing capacitation and the acrosome reaction (Aalberts et al., 2014).

The aim of the present study was to investigate the effects of pig seminal fluid EVs on porcine *in vitro* fertilisation.

Based on literature, it was expected that seminal fluid EVs may influence, either positively or negatively, on fertilisation. In this study, a new method was established to isolate EVs from porcine semen. Subsequently, standard i*n vitro* fertilisation (IVF) was performed in the presence or absence of purified EVs. To evaluate the effects of EVs on fertilisation, the standard porcine IVF protocol was adapted with respect to different criteria to create more physiological conditions. Fertilisation was determined by fluorescence microscopy. In addition, monospermic and polyspermic fertilisation of the oocytes was discerned. For a schematic overview of the complete study, see figure 3.

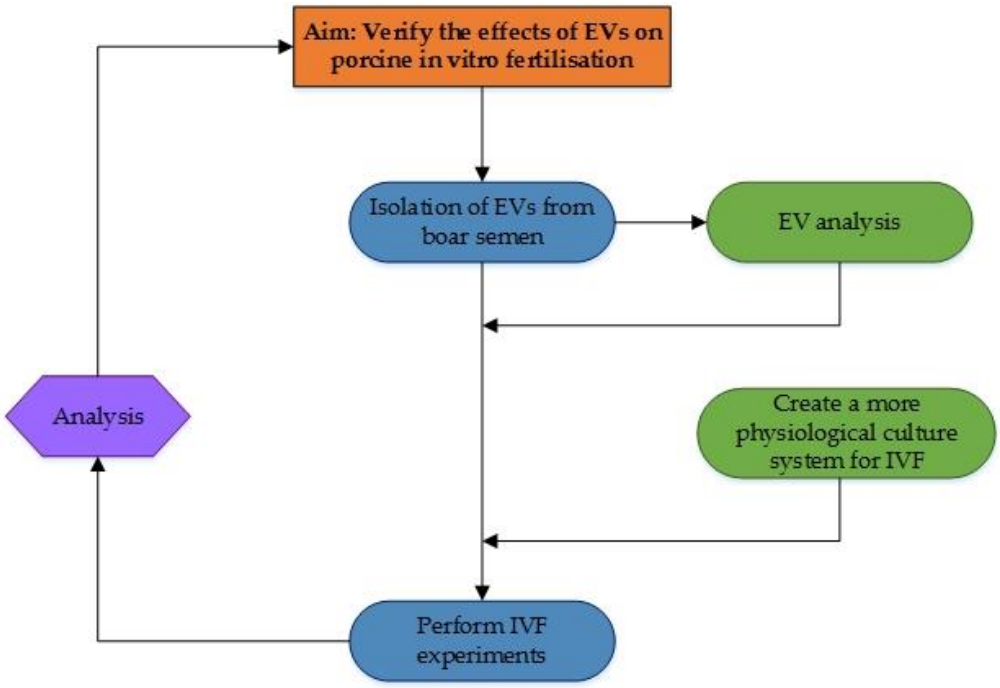

Figure 3. Schematic overview of the study
First, extracellular vesicles were isolated from boar semen. The collected fractions were analysed after which EV-containing fractions were selected. EV-containing fractions were used in IVF experiments to verify their effects on fertilisation. The standard IVF protocol was adapted to a suboptimal, but more physiological culture system. Fertilisation parameters were analysed by fluorescence microscopy.

Embedding in the faculty research program

In this study, the pig has been used as the model system due to local experience at the faculty and easy access to both oocytes and sperm. Yet, as can be stated in the light of One life results of the project can be addressed to fertility problems in both animals and man. "Our research focuses on animals, but also addresses human fertility issues. These studies would not be feasible with human gametes, but shed light on both animal and human reproduction." *Professor Willem Stoorvogel (department Biochemistry & Cell Biology).* The project forms an integral component of the research programs within Fertility & Reproduction as offered by the faculty of Veterinary Medicine since it meets two of its key objectives. The current *in vitro* study, conform one of the key objectives, is able to provide insight into the molecular, cellular, and physiological mechanisms underlying successful reproduction. Consistent with the second objective, results of the project may be imperative to the improvement of current assisted reproductive techniques. In addition, this project is a link to studies on EVs from immune cells, as performed by the department of Biochemistry & Cell biology, and the research on reproduction, by the department of Farm Animal Health and may be of importance for future collaborations.

Materials and methods

Semen collection

Semen for the isolation of EVs was obtained from healthy fertile boars (*Sus scrofa domestica*) housed at an artificial insemination (AI) centre (Topigs Norsvin, Vught, the Netherlands). The sperm-rich fraction was collected on a routine basis according to the standard gloved-hand method (Hancock and Hovell, 1959). During collection, the semen was filtered with a milk filter (nonwoven disk, 200 mm, Universal Filters Inc., New Jersey, USA) to remove the gel fraction of the ejaculate. The semen was collected in a pre-warmed (40 ± 2 °C) plastic container (370 ml, Graham Packaging Company Inc., York, England) without insulation cover cup (Broekhuijse et al., 2012). Immediately after collection, the quality of the sample was determined using Computer Assisted Semen Analysis (CASA, UltiMate™, Hamilton Thorne Inc., Beverly, MA, USA). In addition, the volume of the ejaculate was established.

Semen for *in vitro* fertilisation was obtained from a local AI station (Varkens KI Nederland, Vught, the Netherlands) that produces insemination doses for artificial insemination (AI) of sow herds commercially. Directly after collection, fresh semen was diluted approximately 1:10 (v/v) in Beltsville Thawing Solution (BTS, 0.2 M glucose, 20 mM sodium citrate, 15 mM sodium bicarbonate, 3.36 mM EDTA, 10 mM KCl, 0.1 mg/ml kanamycin, pH 7.4, 300 mOsm) at 25 °C and slowly cooled to and kept at 17 °C for transport and storage. For each experiment, fresh BTS-extended semen of two randomly selected boars, irrespective of breed, was used.

EV isolation using sucrose density gradient centrifugation

Directly after collection of fresh semen at the AI centre, the semen was centrifuged at 750x*g* for 5 minutes at room temperature (4K15, Sigma, Osterode am Harz, Germany) to remove spermatozoa. The supernatant was again centrifuged at 2000x*g* for 20 minutes at room temperature. The supernatant was transported on ice to the laboratory within an hour. At the laboratory, the seminal plasma was centrifuged at 3300x*g* for 60 minutes at 4 °C (slow decelerate, Allegra® X12-R, Beckmann Coulter Inc., Fullerton, CA, USA) to eliminate any residual cell debris. To prevent bacterial growth, 0.02 % (w/v) sodium azide was added to the seminal plasma. This cell-free seminal plasma was then used for EV isolation.

For isolation of EVs from porcine semen, two different methods were used; a modified version of the protocol previously described for isolation of human and stallion EVs (Aalberts et al., 2012; Aalberts et al., 2013), and an alternative method using precipitation with polyethylene glycol 6000. In the protocol of Aalberts et al. (2012), non-membranous particles (e.g. soluble proteins) were separated from EVs by the use of a sucrose block gradient. Briefly, the cell-free seminal plasma (see above) was loaded on top of 6 ml 0.7 M sucrose in phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA) and 0.02% (w/v) sodium azide that was layered on top of 4 ml 2 M sucrose in PBS, 1 mg/ml BSA, 0.02% (w/v) sodium azide. This block gradient was centrifuged at 100.000xg for 80 min at 4 °C (decelerate slow, Optima™ L-90K, Beckmann Coulter Inc., Fullerton, CA, USA). The EVs were collected in 2 ml at the interface of the two sucrose layers, discarding soluble proteins that remained on top. In a subsequent step, EVs were separated from protein aggregates in a linear sucrose gradient. Briefly, solid sucrose was dissolved in the interface fraction to a final concentration of 2.5 M. This was overlaid with a linear sucrose gradient (2.0 to 0.4 M in PBS with 1 mg/ml BSA and 0.02% (w/v) sodium azide) in a SW40 tube and centrifuged at 190.000x*g* for 16 hours at 4 °C (Beckmann Coulter Inc., Fullerton, CA, USA). During ultracentrifugation EVs floated upward into the sucrose gradient. Fractions of 1 ml were collected from the bottom of the tube

(total of 13 fractions) and the density of each fraction was determined using a refractometer (Atago Co. Ltd., Tokyo, Japan). Fraction densities were calculated from the refractive index using the following formula; density (g/ml) = (2.6488 * refractive index) - 2.5263 (Association of Agricultural Chemist, 1965)

To concentrate the collected interfaces, the EV-containing interfaces were pooled and suspended in 28 ml PBS containing 1 mg/ml BSA. This pooled material was centrifuged in a SW28 tube at 100.000x*g* during 60 minutes at 4 °C (Optima™ L-90K, Beckmann instruments Inc. Fullerton, CA) and the pellet was resuspended in 1 ml PBS containing 1 mg/ml BSA and 0.02% (w/v) sodium azide. The sample was designated concentrated sucrose interface and stored at 4 °C.

EV isolation using PEG precipitation and Nycodenz® gradient centrifugation

Alternatively, EVs were precipitated from cell-free seminal plasma (see above) using polyethylene glycol 6000 (PEG 6000, Merck Chemicals Ltd., Beeston, Nottingham, England). Hereto, a solution of 50% (w/v) PEG6000, 400 mM NaCl, 0.02% (w/v) azide was prepared and gently mixed with the seminal plasma to obtain final concentrations of 10% (w/v) PEG6000, 80 mM NaCl, 0.02% (w/v) azide. The resulting mixture was then placed on a rocker overnight at 4 ˚C to allow aggregation of EVs and proteins. Subsequently, the formed aggregated were pelleted at 1500x*g* for 30 minutes at 4 °C (Allegra® X12-R, Beckmann Coulter Inc., Fullerton, CA, USA). The supernatant was removed from the pellet of approximately 3.5 ml. The pellet, containing EVs and protein aggregates was resuspended in 60% (w/v) Nycodenz® (Axis-Shield PoC,Oslo, Norway), 0.5 M EDTA, 0.02% (w/v) azide using a dounce homogenizer, to a final of 50% Nycodenz®, 10 mM EDTA, 0.02% (w/v) azide. For complete disassembly of aggregates, the suspension was placed on a rocker overnight at 4 ˚C. Next, EVs were separated from the precipitated proteins and remaining PEG6000 by floatation in a Nycodenz® block gradient. Hereto, 15 ml of the 50% Nycodenz®/pellet suspension was loaded on the bottom of a SW28 tube (Beckmann Coulter Inc., Fullerton, CA, USA) and overlaid with 10 ml 40% (w/v) Nycodenz® containing 0.02% (w/v) azide followed by 11 ml PBS containing 0.02% (w/v) azide. The Nycodenz® block gradients were centrifuged at 100.000x*g* for 4 hours at 4 ˚C (Optima™ L-90K, Beckmann instruments Inc. Fullerton, CA). After centrifugation, fractions of 2 ml were collected from the top (total of 18 fractions) and analysed for total protein content (see below). In addition, densities of all fractions were determined using a refractometer (Atago Co. Ltd., Tokyo, Japan). Densities were calculated from the refractive index using the following formula; density (g/ml) = (3.242 * refractive index) - 3.323 (AXIS-SHIELD PoC AS, 2011)

SDS-PAGE and Western blotting

From each sucrose and Nycodenz® fraction 7.5 µl was mixed with 7.5 µl SDS-sample buffer to a final concentration of 2% SDS (w/v), heated at 100 °C for 5 minutes and loaded onto 12.5% gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The Bio-Rad Precision Plus Protein™ All Blue Standard (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used as a molecular weight marker. Proteins were separated by electrophoresis for 75 min at 40 mA. For total protein detection, gels were stained with the fluorescent SYPRO Ruby Protein Gel Stain (Bio-Rad Laboratories Inc., Hercules, CA, USA) on a rocker at 4 °C for a minimum of 16 hours. To maintain an accurate fluorescent signal, gels were kept in the dark during all procedures. After staining, gels were rinsed in 10% (v/v) methanol, 7% (v/v) acetic acid for 60 minutes at 4 °C and subsequently, washed two times in MilliQ (Merck Millipore,

Billerica, MA, USA) for 5 minutes. Fluorescent proteins were detected with the Bio-Rad ChemiDoc™ (Bio-Rad Laboratories Inc., Hercules, CA, USA).
For Western blotting, samples, either sucrose interface, concentrated sucrose interface, sucrose gradient fractions or Nycodenz® fractions, were separated by 12.5% SDS-PAGE as described above. As an internal control, sucrose interface collected from seminal plasma pooled from four vasectomized men, prepared and provided by Sacha Grisel (Grisel, 2015), was used. Next, proteins were transferred from the gel to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA) and blots were blocked in PBS containing 5% (w/v) no fat containing dry milk (Nutricia, Zoetermeer, the Netherlands) and 0.1% (v/v) Tween-20 (MP Biomedicals, Santa Ana, CA, USA. After blocking, blots were probed with primary antibodies in the same buffer for a minimum of 16 hours at 4 °C. All antibodies used are listed in table 1. Next, the blots were washed five times (5 minutes each) in PBS containing 5% (w/v) no fat containing dry milk, 0.1% (v/v) Tween-20 and incubated with horse radish peroxidase (HRP) conjugated secondary antibody (in the same buffer) for 60 minutes at room temperature. To remove unbound secondary antibody, blots were washed twice for 5 minutes in 5% (w/v) nonfat dry milk containing 0.1% (v/v) Tween-20, three times 5 minutes in PBS containing 0.1% (v/v) Tween-20 and three times 10 minutes in PBS (all steps at room temperature). Protein bands labelled with horseradish peroxidase were visualised by chemiluminescence using SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific™, Waltham, MA, USA) and detected with the Bio-Rad ChemiDoc™ (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Dialysis of EV-containing Nycodenz® fractions for in vitro fertilisation
To prevent interference with the IVF culture system, Nycodenz®, EDTA and azide were removed from the EV-containing Nycodenz® fractions by extensive dialysis. Hereto, EV-containing fractions with a density of 1.07 and 1.16 g/ml were pooled and loaded into a Slide-A-lyzer™ G2 Dialysis Cassette (molecular weight cut-off 2 kDa, Thermo Fisher Scientific™, Waltham, MA, USA). The cassette was floated vertically in 2 l PBS and dialyzed at 4 °C under constant stirring for 53 hours. The dialysis buffer was changed five times (fresh PBS) at regular intervals. After 53 hours, the EV solution was collected from the membrane and either directly used (‘*fresh*’) or snap frozen in aliquots of 200 µl and stored at -80 °C until use to maintain biological activity of the EVs.

To verify the efficiency of dialysis for the removal of Nycodenz®, independent samples containing 40% (w/v) Nycodenz®, 0.5 M EDTA, 0.02% (w/v) sodium azide were dialyzed for 21, 53 or 72 hours respectively. During the 21 and 72 hours dialysis, buffer was refreshed three times at regular intervals with 2 l PBS. Buffer changes for the 53 hours sample were identical to those of the EV sample (five times). After dialysis, a dilution series of 10% to 0.5% Nycodenz® was prepared and absorbance was measured at 350 nm using a SoftMax® Multi-well Plate reader (Molecular Devices, Sunnyvale, CA, USA) to construct a calibration curve. Absorbance of the dialysis samples was measured at 350 nm and converted to percentage of Nycodenz® by SoftMax® Pro software 5.4 (Molecular Devices, Sunnyvale, CA, USA).

Table 1. Primary/secondary antibodies and the dilution from manufacturer stock as used in this study

Primary antibody	***Clone***	***Manufacturer***	***Dilution***	***Secondary antibody***
CD9 *Mouse anti-human*	HI9a	BioLegend San Diego, CA, USA	1:1000	Goat anti-mouse HRP (1:10.000, Jackson ImmunoResearch Laboratories Inc., Grove, PA, USA)
CD9 *Mouse anti-human*	MM2/57	AbD Serotec Kidlington, England	1:100	Streptavidin-Biotinylated HRP (1:1000, GE Healthcare, Little Chalfont, England)
CD63 *Mouse anti-human*	H5C6	BD Biosciences Franklin Lakes, NJ, USA	1:500	Goat anti-mouse HRP (1:10.000, Jackson ImmunoResearch Laboratories Inc., Grove, PA, USA)
CD63 *Rat anti-mouse*	NVG-2	BioLegend San Diego, CA, USA	1:100	Rabbit anti-rat HRP (1:5000, Dako, Glostrup. Denmark)
PSCA *Mouse anti-human*	7F5	Santa Cruz Biotechnology Inc. Dallas, Texas, USA	1:500	Goat anti-mouse HRP (1:10.000, Jackson ImmunoResearch Laboratories Inc., Grove, PA, USA)
CRISP3 *Rabbit anti-human*	-	Novus Biologicals Littleton, CO, USA	1:250	Goat anti-rabbit HRP (1:5000, Dako, Glostrup. Denmark)
TSG 101 *Mouse anti-recombinant mouse*	C-2	Santa Cruz Biotechnology Inc. Dallas, Texas, USA	1:200	Goat anti-mouse HRP (1:10.000, Jackson ImmunoResearch Laboratories Inc., Grove, PA, USA)
TSG 101 *Goat anti-mouse*	M-19	Santa Cruz Biotechnology Inc. Dallas, Texas, USA	1:100	Rabbit anti-goat HRP (1:5000, Nordic-MUbio, Susteren, The Netherlands
Flotillin 1 *Mouse anti-rat*	18	MD Biosciences GmbH Zürich, Switzerland	1:1000	Goat anti-mouse HRP (1:10.000, Jackson ImmunoResearch Laboratories Inc., Grove, PA, USA)
GLIPR2 *Rabbit anti-human*	-	Aalberts et al., 2012	1:5000	Goat anti-rabbit HRP (1:5000, Dako, Glostrup. Denmark)

Media for in vitro oocyte maturation and fertilisation

All chemicals for the preparation of culture media were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless indicated otherwise. For *in vitro* maturation, media were similar to those used by Schoevers et al., 2010. The basic oocyte maturation medium (OMM) was M199 (Gibco™, Paisley, Scotland) supplemented with 2.2 mg/ml $NaHCO_3$, 10% porcine follicular fluid (PFF), 1 mM pyruvate and 100 μM cysteamine. The maturation medium used during the first 22 hours of maturation, designated OMM I, consisted of OMM supplemented with 0.05 IU/ml recombinant human FSH (Organon, Oss, the Netherlands). For the second 22 hours of maturation, OMM without FSH, designated OMM II, was used. Media for oocyte maturation were equilibrated at 38.5 ˚C with 5% CO2 in humidified atmosphere for at least 2 hours before use.

For *in vitro* fertilisation either the standard IVF medium, modified Tris-buffered medium (mTBM), or an alternative medium, Tyrode's albumin lactate pyruvate (TALP) medium, was used. Standard IVF medium consisted of 113.1 mM NaCl, 3 mM KCl, 20 mM Tris, 11 mM glucose, 7.5 mM $CaCl_2$, 5 mM sodium pyruvate, 1 mM caffeine and 1 mg/ml BSA fatty acid free (Kidson et al., 2004). The alternative medium consisted of 114.1 mM NaCl, 3.2 mM KCl, 5 mM glucose, 8 mM $CaCl_2$, 25.07 mM $NaHCO_3$, 1.1 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.35 mM NaH_2PO_4, 1.85 ml/L 60% syrup sodium lactate, 2 mM caffeine, 1 mg/ml polyvinylpyrrolidone (PVP) and 3 mg/ml BSA fatty acid free (Coy et al., 2008). All media for IVF were equilibrated at 38.5 °C in a humidified atmosphere with 5% CO_2 for at least 16 hours before use.

Oocyte collection and in vitro maturation

Ovary collection and cumulus-oocyte complex isolation were implemented as described in Schoevers et al., 2003. Briefly, ovaries from prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory in an insulated container (± 30 °C) within 2 hours after slaughter. At the laboratory, the surrounding tissues (i.e. oviduct, uterus horns) were dissected and the ovaries were rinsed under running tap water (± 30 °C). Subsequently, the ovaries were placed in a beaker with pre-warmed saline (30 °C) supplemented with 1% (v/v) penicillin/streptomycin (final 10 units/ml, Gibco™, Paisley, Scotland) and kept at 30 °C until the moment of follicle aspiration. Cumulus-oocyte complexes (COCs) were aspirated from 3 to 6 mm antral follicles with a 18 G winged infusion needle set attached to a 50 ml sterile conical tube (Greiner Bio One International GmbH, Kremsmünster, Austria). After aspiration, follicular contents were allowed to sediment at room temperature and the sediment was washed three times in Tyrode's lactate-HEPES medium (Bavister et al., 1983) supplemented with 0.1% (w/v) polyvinylpyrrolidone (PVP)

Subsequently, follicular contents were searched for cumulus-oocyte complexes using a stereomicroscope on a heated stage (16x magnification, 37.5 ˚C, Olympus, Shinjuku, Tokyo, Japan). Only COCs that were surrounded by an intact cumulus of two or more layers of cumulus cells and containing oocytes of equal size were selected (Kidson et al., 2004). Selected COCs were collected in HEPES-buffered M199 (room temperature, Gibco™, Paisley, Scotland) and washed twice in OMM I . The COCs were matured by culturing in groups of 50-60 COCs per well in 4-well plates (Thermo Fisher Scientific™, Waltham, MA, USA) in OMMI for 22 hours at 38.5 ˚C and 5% CO2. After 22 hours of culture, COCs were washed twice in OMM II. Subsequently, the COCs were transferred to equilibrated OMM II medium and cultured in groups of 50-60 for another 22 hours at 38.5 ˚C and 5% CO2. After the second maturation period, all COCs were used for *in vitro* fertilisation.

Standard in vitro fertilisation

For standard *in vitro* fertilisation either intact COCs or denuded oocytes were used. In order to obtain denuded oocytes, COCs were gently pipetted after maturation, thereby removing the expanded cumulus cells from the oocyte. Oocytes were then washed twice in 1 ml standard IVF medium (mTBM) and placed in groups of 40 to 50 in 500 µl equilibrated mTBM in wells of 4-well plates. Intact COCs were directly transferred to mTBM for washing steps (twice in 1 ml) and subsequently placed in 500µl equilibrated mTBM. Both intact COCs and denuded oocytes were incubated in mTBM at 38.5 °C in 5% CO_2 for at least 30 minutes before the addition of sperm.

For preparation of sperm, 2 ml of BTS-extended semen of two boars (total 4 ml) was added to 4 ml equilibrated mTBM (room temperature) in a 15 ml conical tube (Greiner Bio One International GmbH, Kremsmünster, Austria). Subsequently, the tube was centrifuged at 700x*g* for 5 minutes at room temperature (5804-R centrifuge, Eppendorf, Hamburg, Germany). After centrifugation, the supernatant was removed and the pellet resuspended in 7.5 ml mTBM (room temperature). This was repeated twice. After the third centrifugation step, the pellet was resuspended in 2 ml mTBM. The sperm concentration of this suspension was determined using a Bürker-Türk counting chamber and adjusted to $1x10^6$ spermatozoa/ml with mTBM. Sperm suspension was then added to the denuded oocytes or COCs to a final ratio of 500 spermatozoa/oocyte. Sperm and oocytes were co-incubated for 22 hours at 38.5 °C with 5% CO_2 in humidified atmosphere.

IVF in the presence of EVs

Fifty microliter of freshly dialysed EV-sample was added to matured COCs before addition of the sperm. The COCs were shortly (approximately 1 minute) co-incubated with the EVs, after which spermatozoa were added (ratio: 500 spermatozoa/COC). The standard IVF procedure was followed as described above.

IVF with washed spermatozoa pre-incubated with EVs

To obtain spermatozoa free of endogenous EVs, sperm was washed. Briefly, fresh BTS-extended semen from two randomly selected boars was pooled in a 15 ml conical tube (Greiner Bio One International GmbH, Kremsmünster, Austria) (2 ml of each boar) and mixed with 4 ml modified BTS (205 mM glucose, 20.4 mM NaCl, 5.4 mM KCl, 15.0 $NaHCO_3$, 3.35 mM EDTA). The tube was centrifuged at 700x*g* for 5 minutes at room temperature (5804-R centrifuge, Eppendorf, Hamburg, Germany), the supernatant was removed and the pellet was resuspended in 7.5 ml modified BTS. Subsequently, the sample was centrifuged for 5 minutes at 700x*g*, room temperature, and the pellet was then resuspended in 7.5 ml of IVF medium. This suspension was centrifuged once more and the pellet was resuspended in 2 ml IVF medium. When indicated, 200 µl dialysed EVs (from stored stock at -80 ˚C) or 200 µl PBS as a control was added to the sperm suspension. The tubes were placed at 38.5 ˚C in humidified atmosphere with 5% CO2 for 2 hours to induce sperm capacitation and to allow binding of the EVs. After 2 hours of incubation, the tubes were centrifuged for 5 minutes at 700x*g* (room temperature), the pellet was resuspended in 7.5 ml IVF medium and the tube was centrifuged as described above. Subsequently, the pellet was resuspended in 2 ml IVF medium. The sperm concentration of this resuspension was determined by using a Bürker-Türk counting chamber and adjusted to $1x10^6$ spermatozoa/ml. For *in vitro* fertilisation either mTBM or TALP medium was used. Hereafter, the standard IVF procedure was followed as described above.

IVF in the absence of caffeine

In order to perform IVF without caffeine, caffeine was omitted from TALP medium. Spermatozoa were washed and pre-incubated in medium without caffeine. Next, matured COCs were co-incubated with EV pre-incubated sperm in TALP medium without caffeine. The standard IVF procedure was followed as described above.

IVF in the presence of progesterone

After addition of EV pre-incubated sperm, the oocytes and spermatozoa were co-incubated for 30 minutes at 38.5 °C and 5% CO_2. Next, 0.5 µl of a stock of 260 µg/ml progesterone (Sigma-Aldrich Corporation, St. Louis, MO, USA) in dimethyl sulfoxide (DMSO) was added. The control group was treated with 0.5 µl DMSO. The oocytes and spermatozoa were incubated for 22 hours at 38.5 °C and 5% CO_2 in humidified atmosphere.

Analysis of oocytes

After co-incubation of COCs or denuded oocytes and spermatozoa for 22 hours, ethidium homodimer (ETH, live-dead stain, Thermo Fisher Scientific, Waltham, MA, USA) was added to the wells to a final concentration of 1.6 µM and incubated at 38.5 °C in 5% CO_2 in humidified atmosphere for 2 hours. Subsequently, the COCs were gently pipetted to remove remaining cumulus and sperm. The oocytes were then washed three times in PBS with 0.05% polyvinyl alcohol (PVA) and fixed in 4% (w/v) paraformaldehyde at 4 °C for a minimum of 24 hours. After fixation, the oocytes were stained in 0.2 µg/ml 6-diamidino-2-phenylindol (DAPI, Sigma-Aldrich Corporation, St. Louis, MO, USA) in PBS/0.05% PVA at room temperature for a minimum of 5 minutes. Oocytes were mounted between a glass slide and a cover slip, and viewed using a fluorescent microscope (BH2-RFCA, Olympus, Shinjuku, Tokyo, Japan) at 200x magnification. Unfertilised oocytes were scored according to their nuclear maturation pattern (metaphase I, metaphase II, germinal vesicle) or were classified as degenerated. Degeneration was scored mainly based on their morphological appearance. In addition, oocytes that were positive for ETH (positive = red) were also classified as degenerated. However, oocytes that met the morphological criteria for degeneration, as described above, were not always positive for ethidium homodimer. Therefore, morphological changes were mainly used to classify degeneration. Fertilised oocytes were classified according to the number of encountered pronuclei (monospermy vs. polyspermy) as well to their developmental stage (zygote, 2 cells, 4 cells). For detailed classification parameters for the scoring of oocytes and examples, see figure 7 (results section). The total number of matured oocytes was determined as the number of oocytes that reached metaphase II plus the number of fertilised oocytes. Oocytes in germinal vesicle stage, metaphase II and degenerated oocytes were classified as immature. Fertilisation was determined as the percentage of matured oocytes that was fertilised.

Statistical analysis

Statistical analysis of fertilisation parameters was carried out using Chi Square. Differences of $p < 0.05$ were considered significant. Analyses were done using the statistical analysis program R (R version 3.2.2).

Results

Isolation of EVs from porcine seminal plasma

EVs were isolated from porcine seminal plasma either by using an established protocol (Aalberts et al., 2012), employing sucrose density gradients, or by PEG6000 precipitation and subsequent separation on Nycodenz® gradients. For sucrose gradient fractionation, EVs were first collected by downward displacement into a sucrose block gradient, separating the majority of soluble proteins from sedimenting EVs (data not shown, see Aalberts et al., 2012 and materials and methods section). EVs were then separated from remaining soluble proteins and sedimented protein aggregates by upward displacement into a continuous sucrose density gradient. As expected, the sucrose gradient fractions displayed an almost linear decline in density (figure 4A). Low molecular weight proteins predominantly remained at the bottom three fractions of the gradient (figure 4B). EVs were expected to float upwards as a consequence of their relatively low buoyant density. Proteins were scattered throughout the gradient, indicating a heterogeneity in size (influencing the rate of floatation) or equilibrium density of the floating proteins (Aalberts et al., 2012). BSA was added throughout the gradient to prevent non-specific binding of EVs to the plastic tube, and is visible in each fraction as a ~60 kDa band.

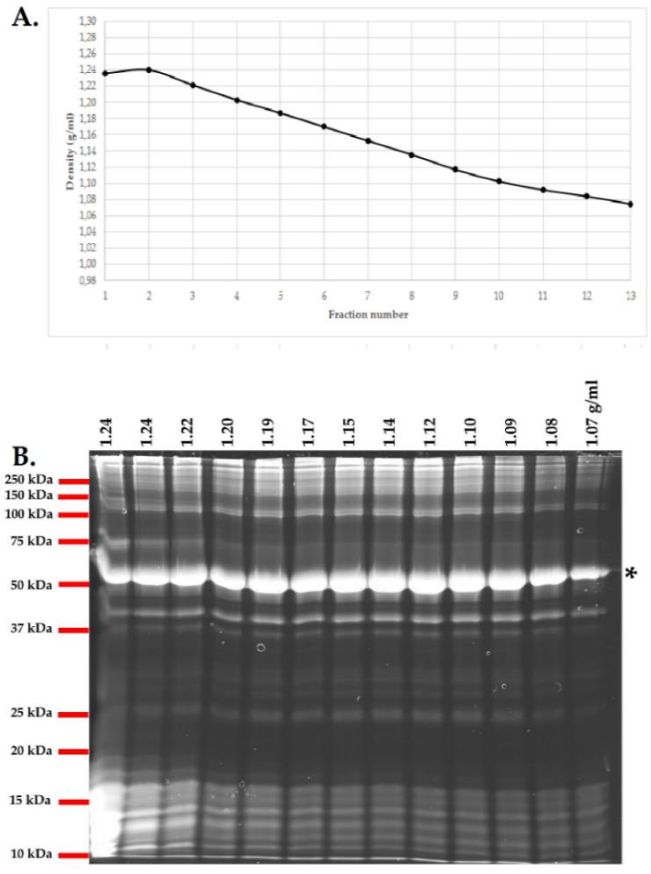

Figure 4. Densities of sucrose gradient fractions (A), and SDS-PAGE analysis of these fractions (B).
EVs were collected from seminal plasma by ultracentrifugation and then separated by sucrose density gradient fractionation. Densities were determined by refractometry. Proteins were stained in gel using SYPRO Ruby Protein Gel Stain . The major band that can been seen in all sucrose fractions between 50 and 75 kD indicates BSA (aterisk), which was added to the sucrose to prevent aggregation of the EVs. Molecular weight markers are indicated in M_r x 10^{-3}.

Although the former isolation protocol has been shown to be successful for diverse applications, a novel EV isolation protocol was developed for two main reasons. First, EVs in sucrose gradients were very diluted and pelleting by ultracentrifugation (after dilution of the sucrose) has the disadvantage of EV aggregation (Aalberts et al., 2012). Second, contact with sucrose is suggested to be detrimental for biological functions of EVs in other cell systems (personal communication W. Stoorvogel). As an alternative approach, EVs were first precipitated from cell-free seminal plasma using PEG. EV aggregation occurs as a consequence of chelation of their water mantle by PEG. In addition to EVs, also many soluble proteins aggregated at this condition. Formed aggregates readily pelleted at low speed centrifugation (1500x*g* for 30 minutes). The EV-containing pellet was resuspended in 60% (w/v) Nycodenz®. EVs disaggregated and proteins dissolved as a consequence of the strong dilution of PEG. Notably, EDTA facilitated complete disassembly of the aggregates (data not shown). Next, EVs were separated from the now solubilized proteins and remaining PEG by floatation into a Nycodenz® block gradient (figure 5A), with the loading sample covering almost half of the centrifugation tube. During ultracentrifugation, EVs were expected to migrate to the interface between 40% Nycodenz and overlaid PBS (fraction 6-7; optical density of 1.07 and 1.16, figure 5B). Soluble proteins were not expected to migrate during centrifugation and showed an expected even distribution between the bottom 8 fractions. The majority of these soluble proteins have a low molecular weight.

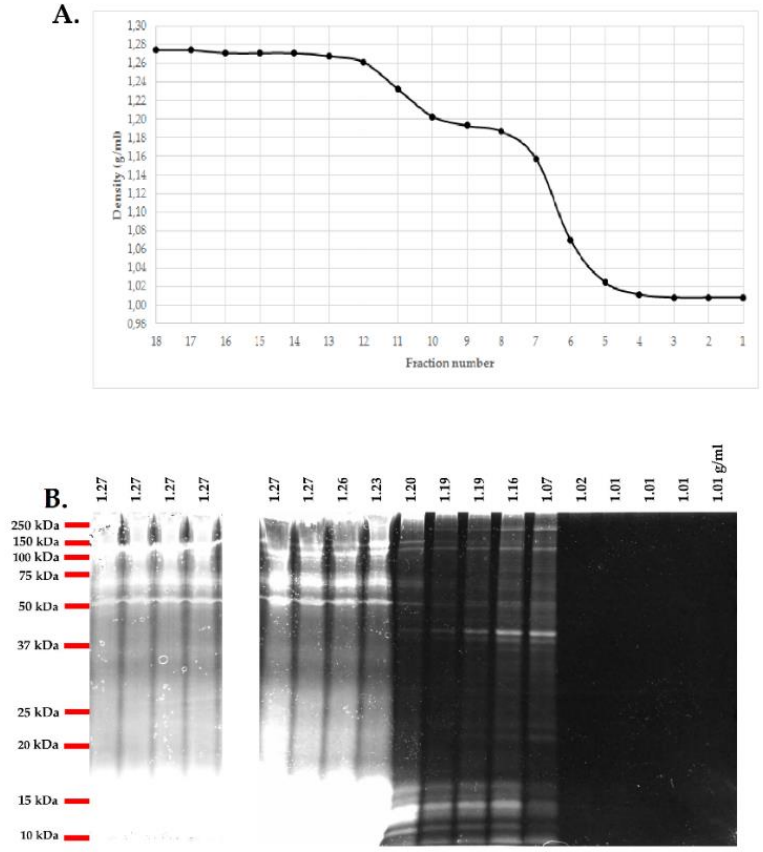

Figure 5. Density and visualisation of proteins in Nycodenz® fractions of semen samples.
EVs were collected from seminal plasma by PEG precipitation and subsequent flotation in a Nycodenz® block gradient.. Densities were determined by refractometry (A). Proteins were visualized by SYPRO Ruby Protein Gel

Stain (B). Nycodenz® samples were run on two separate gels, as indicated by the white bar. Molecular weight markers are indicated in $M_r \times 10^{-3}$.

The EV-containing Nycodenz® fractions with a density of 1.07 and 1.16 g/ml were pooled and extensively dialyzed (see M&M section and supplementary figure) to successfully remove azide, EDTA, and Nycodenz®, to prevent their interference with the IVF experiments (see below).

Western blotting

With the aim to detect EV marker proteins, EV-preparations were loaded on SDS-PAGE and Western blotted using various primary antibodies (table 1, materials & methods). Only two antibodies, raised against rodent equivalent proteins, showed cross-reactivity with porcine proteins (see table 2 and figure 5). One of the Tumor susceptibility gene 101 (TSG101) directed antibodies, TSG101 (M-19), detected a faint band at the expected molecule weight of 45 kDa in EV-samples that were highly concentrated by ultracentrifugation (figure 6A). Flotillin 1 migrates at approximately 48 kDa and was also detected in porcine EVs (figure 6B). Both markers could, however, not be detected in Nycodenz® gradient fractions, probably because the concentration of EVs was too low (data not shown). Instead a background band at 250 kDa in the bottom fractions (table 2, indicated by (+*)) was noted, which was most likely due to cross reactivity with PEG (data not shown).

Table 2. Tested antibodies for EV-detection by Western blotting

Tested primary antibodies are shown in the first column, with between brackets the specific clone. SSC indicates the result in a porcine sample whereas HSA represents an human sample. Western blotting was performed on either concentrated sucrose interface, sucrose fractions, Nycodenz® fractions or an human sucrose interface. A negative result is represented in the table as a (-) whereas a positive result is indicated by a (+). A positive result that is not at expected molecular weight for the antibody is represented by (+*). When Western blotting is not performed on a certain sample, this is indicated by Not Done (ND).

	SSC ***Sucrose interface***	***SSC*** ***Concentrated sucrose interface***	***SSC*** ***Fractions linear sucrose gradient***	***SSC*** ***Nycodenz® fractions***	***HSA*** ***Sucrose interface***
CD9 (HI9a)	-	-	ND	ND	+
CD9 (MM2/57)	-	-	ND	ND	+
CD63 (H5C6)	-	ND	ND	ND	+
CD63 (NVG-2)	-	ND	ND	ND	+
PSCA (7F5)	ND	-	ND	-	+
CRISP 3	ND	ND	ND	-	-
TSG101 (C-2)	-	-	ND	ND	+
TSG101 (M-19)	-	+	ND	ND	+
Flotillin 1 (clone 18)	+	+	-	+*	+
GLIPR2	-	ND	ND	ND	-

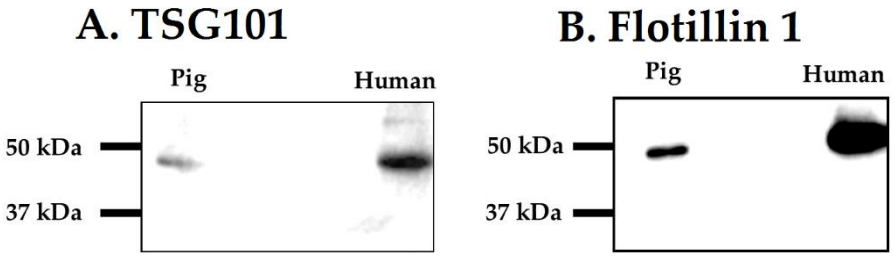

Figure 6. Detection of TSG101 (A) and Flotillin 1 (B) on porcine and human EVs by Western blotting.
EVs were collected from porcine seminal plasma by a sucrose block gradient and subsequently concentrated by ultracentrifugation. Control samples consisted of EVs collected in a similar manner from human seminal plasma. Molecular weight markers are indicated in $M_r \times 10^{-3}$

Standard in vitro fertilisation using denuded oocytes and cumulus-oocyte complexes

Standard IVF was performed with denuded oocytes and COCs from the same batch of isolated COCs. After maturation, randomly picked COCs were denuded and the remaining COCs were left intact. Both groups were fertilised with the same batch of semen and cultured in parallel. In the group of denuded oocytes, 95% of the matured oocytes was fertilised and 60% of the fertilised oocytes showed more than two pronuclei, indicative for polyspermy (see table 3 and figure 7). From the matured intact COCs, 65% was fertilised and 20% of the fertilised oocytes showed polyspermy. Oocytes that are fertilized within the oviduct are thought to be still surrounded by cumulus cells. It cannot be excluded that sperm associated EVs play a role in the penetration of cumulus cells, and hence it was decided to proceed with COC rather than denuded oocytes. Also, to be able to measure both positive and negative effects of EVs on fertilisation, suboptimal IVF conditions are preferred, consistent with those found for COCs. Therefore, it was decided to use COCs for further experiments.

Table 3. Effect of denudation of oocytes on *in vitro* fertilisation

	Denuded	***COC***
Total number of oocytes	53	42
Matured (%)	79	74
Fertilised of matured(%)	95	65*
Of fertilised:		
Monospermy(%)	20	60*
Polyspermy (%)	60	20*
Zygote (%)	10	10
2/4 cells (%)	10	10

* Values indicate significant differences ($p < 0.05$) within a single experiment

Results suggest that the use of COCs instead of denuded oocytes leads to an decrease of fertilisation, accompanied with an increase in monospermy and a decrease in polyspermy.

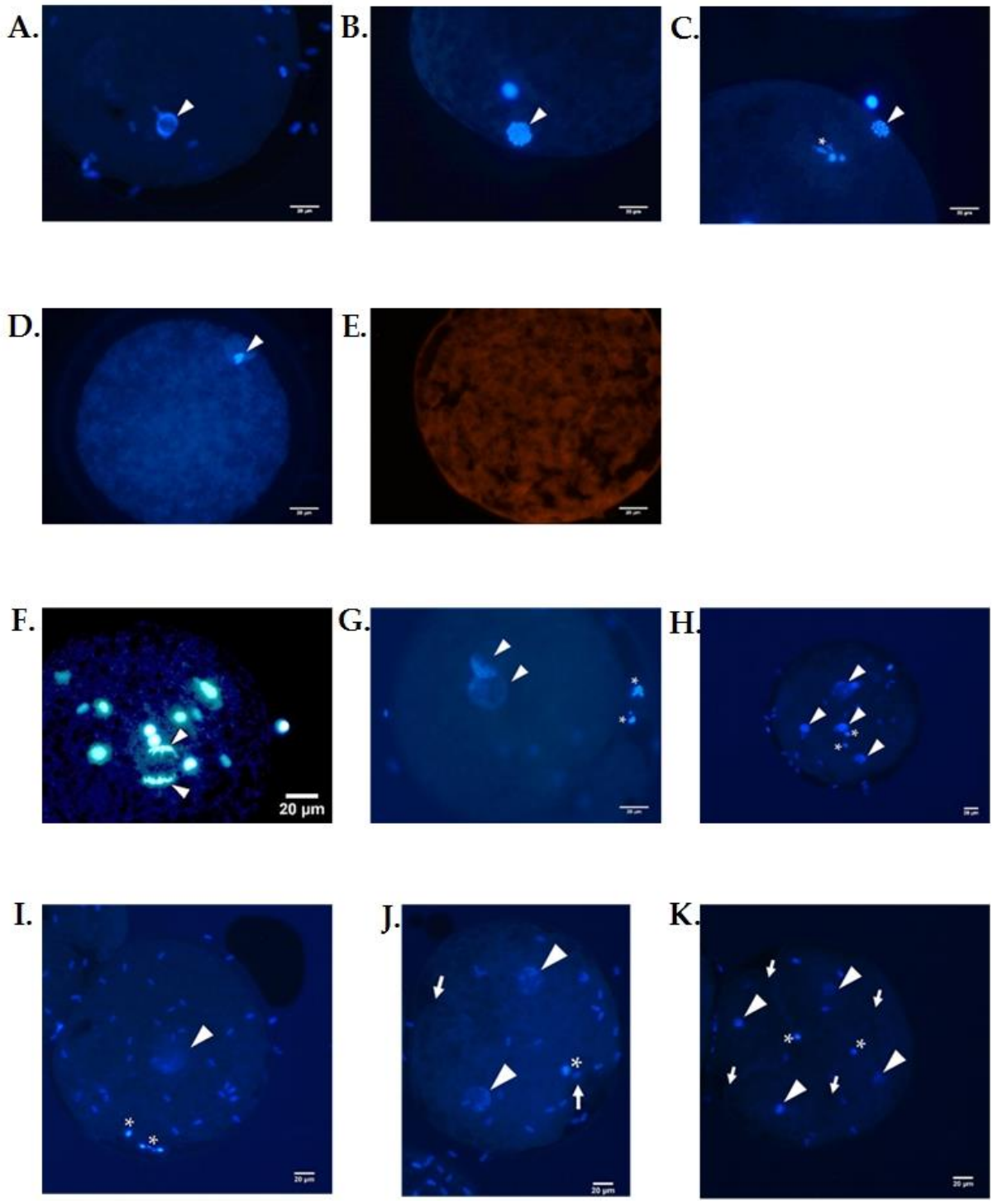

Figure 7. Classification of oocytes using epifluoresence (DAPI and EHD)
A. Germinal vesicle stage: Chromatin is arranged in a strong fluorescence nucleus which appears to be surrounded by nuclear membrane (gives the nucleus a "halo"- appearance, arrow head). No polar bodies present.
B. Metaphase I: Homologous chromosomes are arranged in the metaphase I plate (presence of metaphase I plate, arrow head), no polar bodies present. Metaphase I plate can be distinguished from metaphase II plate by its size (metaphase I; twice as big).
C. Metaphase II: Presence of metaphase II plate (arrow head), one polar body is present (asterisk).
D. Degenerated oocyte: Oocytes with granular cytoplasm, chromatin is strongly condensed (arrow head) and/or fragmented. Sometimes the cytoplasm is full of blister-like structures.
E. Degenerated oocyte: In addition to the morphological criteria (D), oocytes that are positive for ethidium homodimer (live-dead stain, positive = red) are also considered as degenerated.

F. Telophase II: Penetrated sperm head induces segregation of the sister chromatids by the meiotic spindle. Oocytes display a segregating metaphase II plate and/or chromatin arranged at two poles (arrow heads). Since meiosis II is not finished, only one polar body can be observed (not visible in the picture).
G. Monospermy: Oocytes containing one decondensed sperm head or two pronuclei (arrow heads, one female and one male pronucleus). Two polar bodies are present (aterisks).
H. Polyspermy: Oocytes containing more than one decondensed sperm head or more than two pronuclei (arrow heads). Two polar bodies are present (asterisks).
I. Zygote: Syngamy between two pronuclei (arrow head), and the presence of two polar bodies (asterisks).
J. 2-cells stage: Two blastomers are separated by a membrane (arrows, nucleus; arrow head). Two polar bodies (asterisks) can be observed, however, one polar bodies have already been broken down.
K. 4-cells stage: Four blastomers are separated by a membrane (arrows, nucleus; arrow head). Two polar bodies (asterisks) can be observed.
Oocytes in panels A. to E. are considered unfertilised, oocytes in panels F. to K. are scored as fertilsed.
Bars, 20 μm.

Effects of the presence of excess EVs during in vitro fertilisation at standard conditions

In situ, it is very unlikely that significant amounts of seminal EVs will reach oocytes in the oviduct. To study potential effects of a redundant presence of non-sperm bound EVs during fertilisation, isolated EVs were added to matured COCs just before sperm was added. No EVs were added to the control group. As can be seen in table 4, addition of EVs during IVF led to a significant increase in the percentage of degenerated oocytes (71% of all oocytes) when compared to the control (38%). Interestingly, this effect seemed to be rather specific on the oocyte, since no effects on cumulus cell morphology or sperm penetration were observed (data not shown). In addition to these differences in degeneration, fertilised oocytes in the vesicle-group showed small condensed pronuclei (figure 8A) whereas oocytes in the control group showed more expanded and round pronuclei (figure 8B).

Table 4. Effect of the presence of excess seminal plasma EVs on oocytes during standard *in vitro* fertilisation

	Control	***EVs***
Total number of oocytes	63	65
Matured (%)	56	28
Fertilised of matured (%)	91	78
Degeneration (%)	38	71*

* Values indicate significant differences ($p < 0.05$), in a single experiment
Results suggest that the addition of seminal fluid EVs during IVF (without pre-incubation of sperm), induced oocyte degeneration

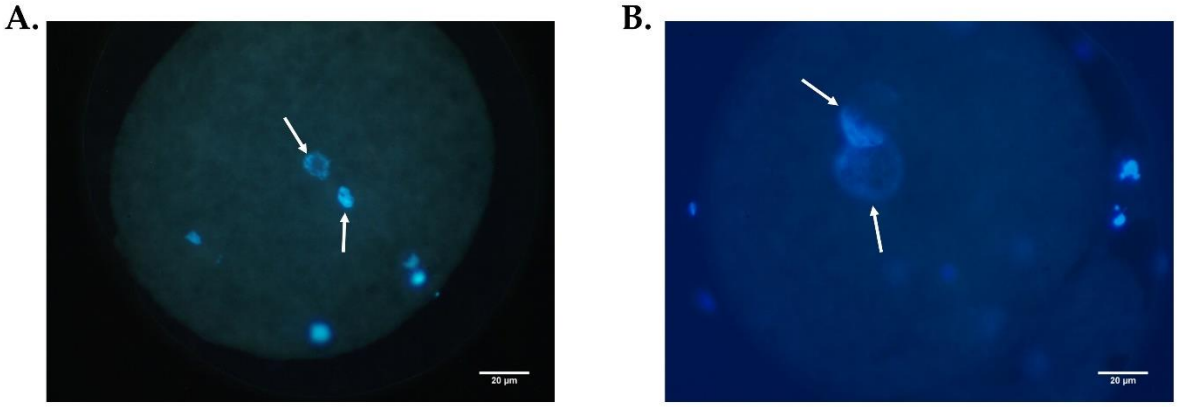

Figure 8. Fertilised oocyte in the EV-group (A) and the control group (B). The two ponuclei are indicated by the white arrows. Bars, 20 µm.

In the next experiment a physiologically more relevant experiment was performed in which washed spermatozoa were first pre-incubated with EVs for 2 hours at capacitating conditions, then washed again to remove excess EVs, and added to mature COCs. Direct effects of EVs on the COC were thus eliminated by washing unbound EVs from sperm samples prior to IVF. In the control group, PBS was added to the sperm and incubated for 2 hours before washing and IVF. In this experiment, no apparent effect on oocyte degeneration was observed (see table 5). Based on these results, it was decided to pre-incubate sperm with EVs in all of the following IVF experiments. Interestingly, pre-incubation of sperm with EVs led to a twofold reduction in fertilisation efficiency as compared to the control group (19% of the matured oocytes was fertilised in the EV-group vs. 38% in the control group).

Table 5. Effect of seminal plasma EVs on in vitro fertilisation using pre-incubation of sperm and EVs

	Control: PBS	***EVs***
Total number of oocytes	75	71
Matured (%)	60	61
Fertilised of matured (%)	38	19*
Degeneration (%)	24	15

* Values indicate significant differences ($p < 0.05$), in a single experiment
Results suggest that EV pre-incubated sperm have a reduced fertilisation efficiency.

Effect of caffeine on in vitro fertilisation

Possibility, established and generally applied IVF conditions may have earned their success by using experimental conditions that bypass otherwise required physiological conditions that apply to *in situ* fertilisation. For example, caffeine is added to artificially increase intracellular cAMP concentrations. To determine the effect of caffeine in porcine IVF, matured COCs were fertilised in medium with or without caffeine. Tris is a weak base that neutralizes the pH of intracellular compartments. To exclude effects of Tris, from here on experiments were performed with Tyrode's albumin lactate pyruvate (TALP) medium, which is bicarbonate buffered instead of Tris buffered IVF medium. For both the caffeine containing and caffeine lacking groups, sperm was pre-incubated with EVs for 2 hours and subsequently washed,

before being added to COCs. In the group where caffeine was omitted from the IVF medium only 5% of the matured oocytes was fertilised, whereas 62% was fertilised in the group with caffeine (table 6), confirming that caffeine facilitates IVF.

Table 6. Effect of caffeine on *in vitro* fertilisation with EV-associated sperm

	+ caffeine	***- caffeine***
Total number of oocytes	84	91
Matured (%)	82	72
Fertilised of matured (%)	62	5*
Degeneration (%)	12	20

* Values indicate significant differences ($p < 0.05$), in a single experiment
Results suggest that omitting caffeine from the IVF leads to a decrease in fertilisation

Effect of caffeine, progesterone and seminal plasma EVs on in vitro fertilisation
To test effects of EVs in combination with other parameters on fertilisation, an IVF experiment with four different conditions was performed (table 7). In this experiment, all conditions included TALP medium, isolated COCs from the same batch and pool of semen mix of the same boars. Table 7 summarizes the results. Fertilisation decreased significantly when caffeine was omitted from the IVF medium (group 1; 74% of matured oocytes vs. group 4; 19%) while both EVs and progesterone were present. Fertilisation efficiency seemed to be slightly higher in the absence of progesterone, although this difference was not significant (group 1 vs. 2) and the presence of caffeine and progesterone as compared to the same condition with P4. Most interestingly, pre-incubation of sperm with EVs followed by IVF in the absence of caffeine and in the presence of progesterone decreased the percentage of fertilisation as compared to the control group in which sperm was pre-incubated with PBS (group 3 vs. 4).

Table 7. Effect of caffeine, EVs and progesterone on oocytes after *in vitro* fertilisation

	1 ***+ caffeine*** ***+ EVs*** ***+ progesterone***	***2*** ***+ caffeine*** ***+ EVs*** ***- progesterone***	***3*** ***- caffeine*** ***- EVs*** ***+ progesterone***	***4*** ***- caffeine*** ***+ EVs*** ***+ progesterone***
Total number of oocytes	52	45	49	52
Matured (%)	67	56	55	71
Fertilised of matured (%)	74^{a}	88^{a}	41^{b}	19^{c}
Degeneration (%)	23	20	22	15

^{a,b,c} different superscripts differ significantly ($p < 0.05$), in a single experiment
Results suggest that omitting caffeine from the IVF leads to a decrease in fertilisation. In addition, fertilisation was reduced by pre-incubating sperm with EVs.

Discussion

Isolation of EVs from porcine seminal plasma

In the current study EVs were isolated from porcine seminal plasma using two different methods. The first method consisted of a modified version of the protocol used by Aalberts et al. for the isolation of EVs from human and stallion seminal plasma (Aalberts et al., 2012; Aalberts et al., 2013). Briefly, the modified method of Aalberts et al. consisted of the separation of non-membranous particles, like protein aggregates, and EVs from cell-free seminal plasma by ultracentrifugation into a sucrose block gradient. Next, the EV-containing interface from the sucrose block was floated upward into a linear sucrose density gradient. The alternative method involved EV precipitation using PEG6000, and subsequent upward flotation into discontinuous Nycodenz® gradient.

Although the isolation method from Aalberts et al. was successful for the isolation of EVs from both human and stallion seminal plasma, it also comes with some disadvantages. The method is time consuming and the use of the sucrose gradients generates practical difficulties when dealing with relatively large volumes of seminal plasma, and the volume of boar semen is in the range of 200 to 300 ml (Sancho and Vilagran, 2013).

A method that can be used to efficiently isolate particles from large amounts of fluid is precipitation with PEG. PEG is traditionally used in virology to efficiently collect viruses from large amounts of fluid (Yamamoto et al., 1970) and is still a commonly used method for the purification of labile viruses (Taylor and Shah, 2015). PEG precipitation is not only used in virology, but also to separate differentially precipitating proteins (Kumar et al., 2011), DNA (Paithankar and Prasad, 1991), RNA (Rosas-Cárdenas et al., 2011), membrane vesicles from brush borders (Basivireddy and Balasubramanian, 2003; Prabhu and Balasubramanian, 2001), and other types of EVs (Taylor and Shah, 2015).

Although PEG precipitation is a very efficient way of collecting various particle types, its use in biological fluids also has some disadvantages. Since PEG precipitation is effective for a wide range of particles, use of PEG in biological fluids not only leads to the precipitation of the particles of interest, but also to the precipitation of other, contaminating particles and proteins (Taylor and Shah, 2015). In this study, PEG precipitation was performed on seminal plasma, which in addition to EVs also contains a large amount of proteins (Druart et al., 2013). In order to remove contaminating proteins and other particulate matter in the precipitate from the EVs, various subsequent isolation steps can be used. The post-isolation step used in the current research consisted of upward floatation of EVs into a discontinuous Nycodenz® gradient. Nycodenz® is the monomeric form of the popular density medium Optiprep® (Ford et al., 1994), and is well-known as a density medium since the 1980s (Rickwood et al., 1982; Ford et al., 1994). Nycodenz® is nearly iso-osmotic, non-toxic and metabolically inert (Ford and Rickwood, 1983) in cell cultures, and therefore no direct effects of the density medium on biological functions of the EVs are expected. In addition, Nycodenz® can be removed by subsequent dialysis (Rickwood et al., 1982), preventing interference of Nycodenz® with the culture system.

Total protein pattern staining for sucrose fractions with a density of 1.07 to 1.20 g/ml revealed similarities with the 1.07 to 1.19 g/ml Nycodenz® fractions, indicating that both methods isolated the same EVs from porcine seminal plasma. The fraction densities of 1.07 to 1.19 g/ml are consistent with the equilibrium densities for EVs from seminal plasma of other species (Aalberts et al., 2012; Aalberts et al., 2013). The protein pattern observed for EVs in sucrose and Nycodenz® fractions resembled that of boar seminal plasma EVs isolated by

others (Piehl et al., 2013). In the study by Piehl et al., proteomics revealed that the major band between 37 and 50 kDa corresponded to the major cytoskeletal component actin. Other bands corresponded to enzymes, intracellular chloride channels and several porcine seminal plasma proteins.

In this study, two ubiquitous EV-marker proteins were detected in porcine seminal plasma derived EVs, namely Tumour Susceptibility gene 101 (TSG101; (Razi and Futter, 2006) and flotillin 1 (Otto and Nichols, 2011). TSG101 is a cytosolic protein (Wagner et al., 1998) and is amongst others involved in the formation of multivesicular bodies (MVBs; Razi and Futter, 2006). TSG101 is commonly used as a general exosomal marker (Théry et al., 2009), and was recently shown by proteomics to be present in human seminal plasma EVs (Chiasserini et al., 2015). The current study for the first time shows the presence of TSG101 on porcine seminal plasma EVs. Flotillin 1 is tightly associated with the inner leaflet of the plasma membrane, endosomes, phagosomes, lysosomes and exosomes. The function of flotillin 1 in various cellular processes is still somewhat unclear. Suggested functions include involvement in endocytosis and a role as scaffold for membrane rafts (Otto and Nichols, 2011). In this study, flotillin 1 was detected at the expected molecular weight of approximately 48 kDa in both porcine and human seminal plasma EVs. The detection of flottilin 1 in the human EV-sample is in agreement with literature in which proteomics revealed the presence of this protein on human seminal plasma EVs (Poliakov et al., 2009). This study was the first to detect flotillin 1 on porcine seminal plasma EVs. Flotillin 1 nor TSG101 could be detected by Western blotting in Nycodenz® or sucrose gradient fractions, presumably as a consequence of detection limitations. For future experiments it is therefore recommended to concentrate the EV proteins in gradient fractions, for example by trichloroacetic acid (TCA) precipitation (Koontz, 2014), prior to Western blot detection.

Others have analysed porcine seminal plasma content by the presence of aminopeptidase activity (Siciliano et al., 2008; Piehl et al., 2013). In future experiments, porcine seminal plasma EVs may also be assessed for the presence of dipeptidyl peptidase IV (DPP4) or aminopeptidase N activity. DPP4, also known as CD26, is a peptidase that specifically cleaves dipeptides from proteins that have proline or alanine in their sequence as penultimate amino acid (Aalberts et al., 2013). It plays a role in various physiological processes, and in seminal plasma it has been suggested to play a role in the fusion of EVs with sperm (Arienti et al., 1997a). So far, there is no literature on the presence of DPP4 on porcine seminal plasma EVs. Aminopeptidase N, also known as CD13, is a protease that has a preference for the N-terminal of neutral amino acids (Mina-Osorio, 2008). It is known to play a role in various *in vivo* processes and, although it is not specifically present on seminal plasma EVs, CD13 was found on human (Arienti et al., 1997b), stallion (Aalberts et al., 2013) and porcine (Siciliano et al., 2008) seminal plasma EVs. Of course additional validation of the presence of EVs should be provided by ultrastructural visualisation using electron microscopy (El-Hajj Ghaoui et al., 2004; Piehl et al., 2006; Siciliano et al., 2008).

In conclusion, the current research has shown that EVs can be isolated from seminal plasma using two different methods. The first method was previously used for the isolation of EVs for human and stallion seminal plasma and, in this study, also demonstrated to isolate EVs for porcine seminal plasma. The alternative method is novel, and uses a combination of PEG precipitation and subsequent floatation in a discontinuous Nycodenz® gradient.

Effects of EVs on in vitro fertilisation

To evaluate effects of seminal plasma EVs on porcine *in vitro* fertilisation, standard *in vitro* fertilisation conditions had to be slightly adapted to mimic a more physiological environment. *In vivo*, porcine oocytes are fertilised in the oviduct while surrounded by cumulus cells (Romar et al., 2005), whereas oocytes for porcine IVF are generally denuded before fertilisation (Lin et al., 2015). In this study the effect of denudation on fertilisation was verified by comparing standard *in vitro* fertilisation in cumulus-oocyte complexes and denuded oocytes. Denudation of the oocytes prior to IVF increased the efficiency of fertilization. The increased fertilisation by denudation was, however, accompanied by an increase in polyspermy. These results are in in agreement with experiments performed by others and indicate a barrier function of the cumulus cells for the penetration of sperm (Gil et al., 2004; Bijttebier et al., 2008). Since the presence of cumulus cells represents a more physiological situation and creates a suboptimal IVF system, COCs were used for all further experiments.

The addition of EVs during standard IVF resulted in oocyte degeneration. The observed degenerative effect of EVs on oocytes were rather specific since no effects on cumulus cell morphology and sperm penetration were observed (data not shown). The calculated concentration of EVs that were added to the COCs was similar to that in the originating ejaculate. However, these are still non-physiological conditions, as only very little seminal plasma is considered to pass the cervix and into the oviduct. Our lab has demonstrated previously in an equine model system that EVs bind to capacitating sperm in an environment with a neutral or slightly alkaline pH, supporting the hypothesis that *in vivo* seminal plasma EVs first bind to spermatozoa and subsequently travel bound to the sperm into the oviduct towards the cumulus oocyte complex (Aalberts et al., 2013). According to this hypothesis, EVs may bind to sperm when deposited in the cervix (e.g. neutral pH; Smiljaković et al., 2008) and hence COCs *in vivo* would never experience "free" EVs, but only to sperm pre-exposed to seminal plasma EVs prior to their entry in the oviduct. To mimic this situation, *in vitro* washed sperm was pre-treated with purified seminal plasma EVs at capacitating conditions, which is expected to induce EV binding to the sperm surface (Aalberts et al., 2013). At these conditions, oocyte degeneration was not induced. This demonstrates that possibly high concentrations of free seminal plasma EVs were responsible for oocyte degeneration. Interestingly, the pre-incubation of sperm with EVs led to a decrease in fertilisation efficiency as compared to the control, suggesting an inhibitory role of seminal plasma EVs on fertilisation. When the effect of EVs was verified in a more physiological IVF culture system (e.g. omitting caffeine and supplementing progesterone), again the inhibitory effects of EV pre-treated sperm on IVF efficiency were noted. Apparently, the inhibitory effect of EVs occurred independently from progesterone and caffeine.

Protein kinase A stimulating agents, bicarbonate (activating the soluble adenylate cyclase; (Bailey, 2010) and caffeine (a phosphodiesterase inhibitor; (Funahashi and Romar, 2004), both support the typical capacitation dependent rise of cAMP in sperm. In the current study, the more physiologically bicarbonate buffered TALP medium (Bailey, 2010) was compared with the Tris buffered standard IVF medium. The fertilisation efficiency in TALP medium, EV pre-incubated sperm in the presence of caffeine, was clearly higher than when IVF was performed in the same conditions for mBTM. In agreement of previous studies (Funahashi and Romar, 2004; Martecikova et al., 2010), IVF was almost completely abolished in the absence of caffeine, which was not restored by the addition of EVs. Supplementing progesterone during co-incubation of EV pre-treated sperm and COCs in medium without caffeine resulted in a similar

decrease in fertilisation, indicating that the cAMP dependent signalling increased by caffeine administration is required for pig IVF.

The above described effects of seminal plasma EVs on pig IVF efficiency are preliminary (n=1 for each experiment) and should be confirmed in multiple replicated experiments in the future. In general, such experimental repeats are hampered by the fact that COCs from different batches of ovaries obtained from the slaughterhouse vary quite dramatically in quality, as noted by the efficiency of oocyte maturation ranging from 28% to 82%. Therefore, in each experiment negative and positive control conditions must be compared/validated together with the experimental conditions of interest, limiting (due to the laborious IVF procedures involved) the number of distinct conditions that can be tested in each IVF experiment. Due to this limitation, unfortunately, it was not possible yet to test the effect of caffeine in the absence of EVs but in presence of progesterone. Likewise, the effect of EVs in absence of progesterone and caffeine and the omission of all three have not been compared. Nevertheless, such additional experiments are required to substantiate conclusions on the effects of EVs on porcine IVF.

In addition, binding of EVs to equine sperm was established by confocal microscopic detection of biotinylated EVs (Aalberts et al., 2013). Whether boar EVs bind to sperm needs to be confirmed in future experiments using a similar approach. In addition, this can be confirmed by recruitment of aminopeptidase activity, or by electron microscopy.

In conclusion, it was shown experimentally that capacitation of sperm in the presence of EVs interfered with their IVF potential. This effect was independent of the presence of caffeine and progesterone and also not influenced by the use of Tris/bicarbonate buffered media (mTBM vs. TALP). These results are in contradiction to the current view that seminal plasma EVs promote fertility (Aalberts et al., 2014) and suggest a possible role for EVs in sperm competition. Competition between subsequently mating males in the context of reproduction is a well-known phenomenon that is widely preserved amongst animal classes. However, competition does not only include the struggle between males to mate with a female, but also includes the competition of their gametes within the female reproductive tract, also called sperm competition (Stockley, 2004). Current theories addressing sperm competition mainly describe the role of the sperm component of the ejaculate (e.g. numbers of sperm in the ejaculate, size of the flagellum or the role of deformed sperm; Stockley, 2004; Dhole and Servedio, 2014). However, also the non-sperm component of the ejaculate is suggested to have significant effects on sperm survival and fertilising capacity (Dhole and Servedio, 2014). In boars, the largest accessory sex glands are the seminal vesicles. The major secretory products of the seminal vesicles are spermadhesins (Strzezek, 2002), which are specific proteins that have been shown to inhibit sperm capacitation (Vadnais and Roberts, 2010) and sperm interactions with the oocyte (Caballero et al., 2005). These results suggest an inhibiting role of spermadhesins on fertility, which might be beneficial in sperm competition. Proteomics on boar seminal plasma EVs also identified the presence of spermadhesins in boar exosomes (Piehl et al., 2013), supporting the hypothesis that porcine seminal plasma EVs may be involved in sperm competition too. However, final proof for this scenario and evidence for the *in situ* functioning of EVs requires additional experiments.

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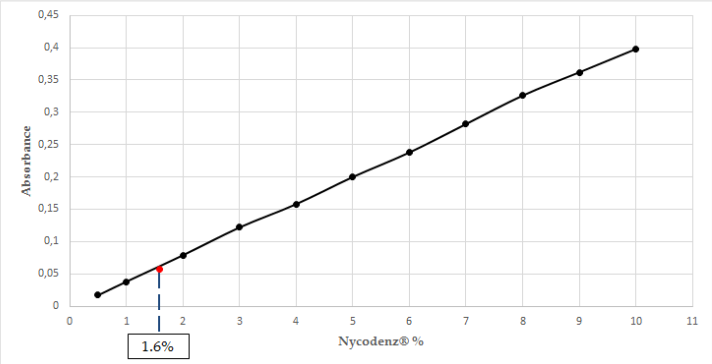
Supplementary data

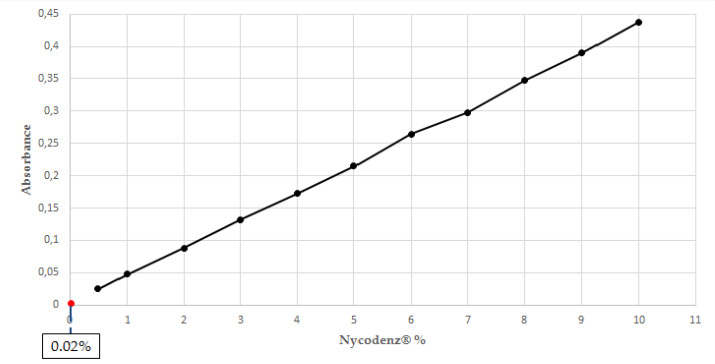
Extensive dialysis of independent samples containing 40% (w/v) Nycodenz®, 0.5 M EDTA, 0.02% (w/v) sodium azide.

Absorbance, measured at 350 nm, of dialysis samples containing 40% (w/v) Nycodenz®, 0.5 M EDTA, 0.02% (w/v) sodium azide after 21 (A), 53 (B) and 72 (C) hours of dialysis. Amount of buffer changes differed between samples and is specified per sample. Based on the calibration curve (10% - 0.5% Nycodenz®, black line), the percentage of Nycodenz® of the samples was calculated (text box). The samples are represented in the graphs as a red dot.

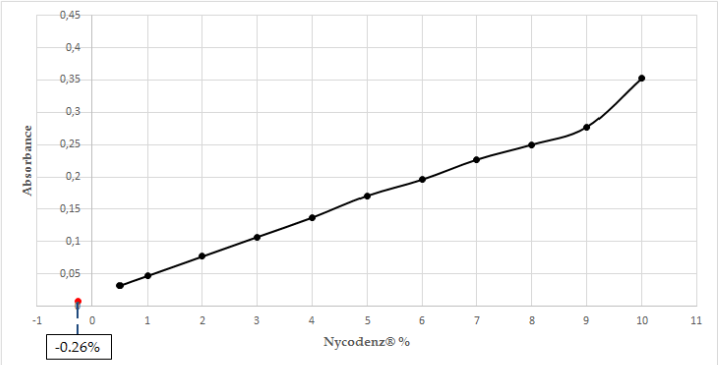
A. 21 hours dialysis, 3x buffer change



B. 53 hours dialysis, 5x buffer change



C. 72 hours dialysis, 3x buffer change



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Courses

Introductory Statistics Course

Provided by the faculty of Veterinary Medicine

When
8/12/2014 - 9/12/2014

Short description
This course is designed as an introductory course for Modern Methods in Data Analysis (see later). During this two-day course, students are introduced to the use of R and R studio for statistical computing. In addition, statistical methods that are thought during the MSc Veterinary Medicine are reviewed.

Personal experience
It was very useful to refresh all the statistical methods and models before the start of Modern Methods in Data Analysis since my knowledge of statistics usually gets quite blurred after a long time not using it. Also, the introduction of R and R studio during this two-day course was helpful since none of us had used it before. Altogether, this course made it easier for me to follow the course Modern Methods in Data Analysis and enabled me to work with R/R studio.

Modern Methods in Data Analysis

Provided by MSc Epidemiology, University of Utrecht, Utrecht, the Netherlands

When
5/1/2015 - 23/1/2015

Short description
The main purpose of this course is to learn the student to use statistical methods to study the association between (multiple) determinants and the occurrence of an outcome event. The course takes three weeks to complete and represents 4.5 ECTs. During the first two weeks, the course includes a mixture of lectures and seminars. Lectures, in the morning, are used to introduce and explain different statistical methods. Examples of introduced statistical methods and/or models are the likelihood theory, logistic regression, resampling methods and longitudinal data analysis. After the lectures in the morning, seminars are given in the afternoon. The seminars include computer exercises that designed to provide the students more insight into the use of the introduced statistical methods and/or models, as well as to make students familiar with the use of statistics in R and R studio for statistical computing. The last week of the course will be used for students to practise and to complete their understanding of the taught methods/models (by self-study). In order to receive the course certificate and ECTs, an obligatory exam has to be taken. The exam will be at the end of week 3 of the course.

Personal experience

Since I have never had classes in statistics in secondary school, statistical methods and models have always been somewhat hard to understand for me. During my BSc in Veterinary Medicine, we were provided by some short courses in basic statistics, but still applying statistics to more extensive data was quite hard for me. Joining this course really helped to increase my understanding of diverse statistical methods and introduced me to new, in research essential, statistics. The combination of lectures in the morning and computer assignments in the afternoon was very illustrative and forced students to actually understand the statistical methods/models. Working with R and R studio for statistical computing was initially somewhat though, but eventually helped me to understand what I was really doing with the statistical models (in contrast to the commonly used SPSS Statistics, IBM). The teachers were very helpful and they were able to formulate suitable answers to individual questions. The lecture handouts and assignments plus answers were self-explanatory and made it easy for me to recap things at home. The obligatory exam was so-called "open book" and was doable if you joined all lectures and seminars. In conclusion, this course helped me to understand and apply the most important statistic in medical research, therefore making it less scary to do statistic on my own data.

English for Academic Purposes

Provided by Babel talen, Utrecht, the Netherlands

When

20/4/2015 – 29/6/2015
9 sessions

Short description

Current Bachelor's and Master's programmes require students to be skilful in both English writing and speaking. However, teaching students how to structure their papers, to apply desirable style and remains somewhat neglected. The course English for Academic Purposes is specifically designed to provide students the necessary language skills that are essential in current BSc and MSc programmes. In the course both writing and presenting skills will be taught by a mixture of individual assignments, work in pairs, small groups and plenary discussions. Subjects that will be covered in the writing part include grammatical structures, academic vocabulary, punctuation, signalling words and style. To practise academic writing, students have to submit self-written sections of an academic paper (on a topic related to your own discipline). During the presenting sessions, aspects like signalling language, the structure of a presentation, interaction with the group, pronunciation and fluency will be addressed. After this course, the students will have increased fluency, vocabulary and self-confidence and hence be better in expressing themselves in (academic) English.

Personal experience

One of my main objectives to join this course was to learn how to write your own research papers. For writing this course offers a very useful book; Science Research Writing for non-native speakers of English by Hilary Glasman-Deal. This book offers a stepwise oversight on how to write an academic paper and shows the reader how easy writing one can be. This is

the first book that has really given me a complete oversight of the structure of a publication and about how this structure should be applied during writing. Especially, the latter, in my opinion, is given too less attention in most books. Another positive aspect of the writing part of this course includes the submission of self-written sections. By writing the sections, the course allows you to practise the structure of a paper and to increase your academic vocabulary. By receiving individual feedback from the teacher, you readily improve your writing skills during the course of the sessions. Although instantly, I went to this course specifically for the writing part, I found out that even when you are quite experienced in presenting, this course can help you to improve certain aspects of your presenting. The course improved both the fluency of my presenting as well as the interaction with the group during your presentation (i.e. look at the whole group, not at one person). To conclude, joining this course increased my academic writing skills by expanding my insight in the structure of an academic paper, the use of signalling words and easing the use of academic vocabulary. Furthermore, the course helped me to improve my presenting skills.