Sperm binding to ZP2-coated beads improve the efficiency of porcine *in vitro* fertilisation

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Abstract

The role of specific zona pellucida (ZP) glycoproteins in gamete interaction has not yet been elucidated in many species. A recently developed 3D model based on magnetic sepharose beads (B) conjugated to recombinant ZP glycoproteins (B_{ZP}) and cumulus cells (CB_{ZP}) allows the study of isolated ZP proteins in gamete recognition studies. The objective of this work was to study the role of porcine ZP2, ZP3 and ZP4 proteins in sperm binding, cumulus cell adhesion and acrosome reaction triggering. ZP protein-bound beads were incubated with fresh ejaculated boar spermatozoa and isolated cumulus cells for 24 h. The number of sperm bound to the beads, the acrosomal shrouds (presence of acrosomal content) on the bead's surface, and the acrosome integrity (by means of PNA-FITC lectin) in bound and unbound sperm were studied. Finally, *in vitro* matured porcine oocytes mixed with B_{ZP2} were inseminated *in vitro* using fresh sperm and fertilisation results evaluated. Over 60% of beads had at least one sperm bound after 2 h of coincubation. ZP2-beads (B_{ZP2}) and cumulus-ZP2-bead complexes (CB_{ZP2}) reached the highest number of sperm per bead, whereas B_{ZP3} and B_{ZP4} models showed the highest number of unbound reacted sperm cells and acrosomal shrouds. Fertilisation efficiency and monospermy rate increased when oocytes were fertilised in the presence of B_{ZP2}. We, therefore, conclude that in pigs, it is mainly ZP2 that is involved in sperm-ZP binding whereas ZP3 and ZP4 induce acrosome reaction. Using magnetic sepharose ZP2-bound beads might be a valuable tool to improve the fertilisation rate in pigs.

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Introduction

The zona pellucida (ZP) is an extracellular matrix composed of three to four glycoproteins, named ZP1, ZP2, ZP3 and ZP4 depending on the species (Goudet *et al.* 2008). It surrounds mammalian oocytes from oogenesis until a fertilised egg reaches the blastocyst stage (Wassarman & Litscher 2018). Sperm-ZP binding is the first event during fertilisation and allows sperm to penetrate through the matrix to reach the oocyte's plasma membrane and fuse. Once a single spermatozoon fertilises the oocyte, the ZP participates in the blockage of polyspermy (Yanagimachi 1994). In addition, the ZP is involved in other crucial mechanisms such as stabilising intercellular connections between an oocyte and its corona radiata cells to ensure proper nutrition and the protection of the preimplantation embryo (Wassarman & Litscher 2018).

Recent studies in mice and human determined the role of specific ZP glycoproteins in gamete interaction and showed that successful fertilisation requires sperm binding to ZP2 (Avella *et al.* 2014). Furthermore, in studies performed in genetically edited mice, it has been demonstrated that the sperm

binding domain of ZP2 glycoprotein is cleaved by ovastacin, an enzyme exocytosed after fertilisation to prevent polyspermy (Gahlay *et al.* 2010, Burkart *et al.* 2012). However, the specific role of ZP glycoproteins in other mammalian species remains controversial. Moreover, there is a dearth of knowledge for livestock species like pigs, where the incidence of polyspermy after *in vitro* fertilisation remains a substantial problem (Tanihara *et al.* 2014). In the pig, the ZP is comprised of the ZP2, ZP3, and ZP4 glycoproteins (Hedrick & Wardrip 1987), and ZP4 has been shown to orchestrate the sperm-ZP binding prior fertilisation (Yonezawa *et al.* 2012). This proposal is based on sperm binding assays performed with solubilised and purified ZP proteins or recombinant proteins expressed in non-mammalian cells (Yonezawa *et al.* 2012). Although the role of the ZP as an inducer of the sperm's acrosomal reaction has been described in multiple studies, consensus on which protein is directly responsible for the acrosomal content exocytosis in mammals is still missing. The controversy is partly due to species differences. For example, recent studies have shown that the mouse spermatozoa initiates the acrosome reaction in response to some stimuli

other than or in addition to ZP binding (Jin *et al.* 2011, Hirohashi & Yanagimachi 2018). ZP2, however, has been reported to bind only to acrosome reacted sperm in human and mouse (Gupta 2018).

In addition to the ZP, the granulosa cells comprising the cumulus oophorus at the time of ovulation also play an important role during fertilisation (Yanagimachi 1994). Their contributions are two-fold: creating the microenvironment required for fertilisation and secreting chemoattractants. Secondly, preventing changes in the oocyte to ensure successful fertilisation (Tanghe *et al.* 2002). Cumulus cells have demonstrated to be a useful tool to develop *in vitro* models such as the capillary-cumulus model (Hong *et al.* 2004) to select sperm for ICSI (Rijsdijk & Franken 2007, Franken & Bastiaan 2009). Their role in gamete interaction cannot be ruled out although cumulus cells are routinely removed from COCs in porcine *in vitro* fertilisation (IVF) systems (Romar *et al.* 2019).

New molecular techniques are necessary to overcome our current limited knowledge on gamete interaction and particularly on sperm-ZP binding in mammals. To date, the available techniques such as double or triple transgenic animals are time-consuming to generate, expensive, and often not practicable to apply in far animals. A deeper insight into the molecular regulation of sperm-oocyte interactions could improve the success rate of assisted reproductive technologies (ART). This would be particularly beneficial for porcine ART as the fertilisation rate after IVF rarely exceeds 45% (Romar *et al.* 2019).

Recently, we developed a new molecular assay to study the role of single proteins involved in sperm-oocyte recognition. The assay uses magnetic sepharose beads (B) conjugated to recombinant ZP glycoproteins (ZP2, ZP3 and ZP4). This assay can easily be applied to different mammalian species and it has been shown to be stable over time and scalable. In this study, we used three ZP glycoprotein-specific assays: B_{ZP2}, B_{ZP3} and B_{ZP4}. The specific type of ZP glycoproteins covers a spherical 3D surface, thus mimicking the oocyte's shape and allowing sperm binding (Hamze *et al.* 2019). The assay has been successfully used to study the fertilisation ability of bovine sperm with minor modifications such as conjugating the sepharose beads to JUNO protein (Hamze *et al.* 2020). In this study, the models furthered knowledge on porcine gamete interaction by studying its potential to bind boar sperm cells and trigger their acrosome reaction, the binding of cumulus cells to the different models, and its use in conventional IVF systems to increase its efficiency. From these studies, we hypothesise that sepharose beads coated with porcine ZP proteins and cumulus cells are equivalent – although artificial – to the female cumulus-oocyte complex and a useful tool in studying sperm-oocyte interactions in pig. Thus, the objectives of this study are (1) to investigate the direct role of each ZP protein-bound bead to decoy

sperm, (2) to corroborate the sperm binding ability to ZP protein-bound beads by using complexes cumulus:ZP protein-beads, (3) to analyse the acrosomal status of sperm cells bound to ZP protein-bound beads, and (4) to study the likely modulating effect of ZP protein-bound beads when added to the porcine IVF system.

Materials and methods

All chemicals were purchased from Sigma-Aldrich Química, unless otherwise indicated. This study was carried out in strict accordance with the recommendations in the Guiding Principles for the Care and Use of Animals (DHEW Publication, NIH, 80-23). The protocol was approved by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain (Project Code: AGL2015-70159-P).

Recombinant zona pellucida protein expression and conjugation to magnetic sepharose beads

The expression and characterisation of the recombinant proteins as well as its conjugation to the magnetic sepharose beads (His Mag Sepharose Excel™; GE Healthcare) was performed as previously described (Hamze *et al.* 2019). His Mag Sepharose excel is a magnetic IMAC medium precharged with nickel ions, which are strongly bound to polyhistidine affinity-tagged proteins. The medium enables an efficient and simplified workflow for capturing, purifying, and screening histidine-tagged proteins. Briefly, the expression plasmids encoding the porcine ZP proteins ZP2 (UniProt P42099), ZP3 (UniProt P42098), and ZP4 (UniProt Q07287) and carrying a histidine tag (6x) at the N-terminus of ZP2 (38–43 aa), ZP3 (41–46 aa) and ZP4 (22–27 aa) were amplified and purified using Library Efficiency DH5α™ Competent cells (Thermo Fisher Scientific) and the GenEluted Plasmid Kit, respectively. Chinese Hamster Ovary cells (CHO cells, ECACC, The European Collection of Authenticated Cell Cultures) were transiently transfected using lipotransfectin (Solmegeas, Madrid, Spain). One microgram of each plasmid-DNA encoding one of the three ZP glycoproteins and 2 µL of lipotransfectin were dissolved in a final volume of 200 µL Opti-MEM reduced-serum medium (Gibco-Invitrogen) and incubated for 15 min at room temperature (RT). The mix was then added to 2 mL Opti-MEM and overlaid on growing CHO cells prior to incubation at 37°C, 5% CO₂ and 95% humidity for 48 h. Proteins secreted by the CHO cells during the incubation time were harvested and concentrated in a 10,000 Da Vivaspin® Turbo 4 centrifuge (Sartorius, Goettingen, Germany). The recovered proteins were eluted in 20-mM sodium phosphate buffer (pH 7.4) in a final volume of 200–300 µL. To coat the sepharose beads, beads were washed twice in 500 µL washing buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4) and 500 µL binding buffer. Ten microlitres of washed sepharose beads were then mixed with the protein solution of each ZP glycoprotein in 500 µL binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.1% Tween 20, pH 7.4) and incubated overnight at 4°C. The next morning, the protein-coated beads (B_{ZP2}, B_{ZP3} and B_{ZP4}) were washed twice with 20 mM sodium phosphate buffer (pH 7.4) and stored at 4°C until further use. Control beads (B_{Ctrl})

were generated using this protocol with the exception that the beads were incubated in the supernatant of non-transfected CHO cells. Successful protein expression and conjugation were confirmed before each experiment by gel electrophoresis and Western blot using anti-Flag antibody for ZP2 (1:1000, v/v); anti-ZP3 antibody, kindly donated by Dr Hedrick, for ZP3 (Berger *et al.* 1989) (1:2000, v/v); and V5 Epitope Tag Antibody for ZP4 (1:2000, v/v) (Thermo Fisher Scientific). All antibodies were diluted in TBST 1x, 1% BSA. Visualisation was carried out by chemiluminescence (Pierce ECL-Plus, Thermo Fisher Scientific).

Sperm collection and processing

Semen was hand-gloved collected from mature fertile stud boars routinely used in a commercial pig farm located close to the laboratory (1–2 years old, Landrace x Large White; CEFU, SA, Pliego, Murcia, Spain). Once in the laboratory, the semen was diluted in Beltsville Thawing Solution (BTS; 1:5, v/v; Pursel *et al.* 1973) and used within 24 h after collection. We used heterospermic samples from two different boars for all experiments described throughout this study. The sperm samples were prepared using the double centrifugation method described previously by Hamze *et al.* (2019) and displayed a $\geq 80\%$ motility and sperm movement quality of ≥ 4 (on a scale of 0 to 5). Briefly, the diluted semen was centrifuged at 300 g for 3 min, the supernatant collected, and subject to an additional round of centrifugation at 800 g for 5 min. The sperm pellet was resuspended in modified TALP medium (Tyrode Albumin Lactate Pyruvate medium: 114.06 mM NaCl, 3.20 mM KCl, 0.50 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10.00 mM Na-Lactate, 0.35 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 5.00 mM glucose, 25.07 mM NaHCO_3 , 2.00 mM caffeine, 8.00 mM Ca-lactate- $5\text{H}_2\text{O}$, 0.1% PVA, 0.17 mM kanamycin, 0.003 mM red phenol (Rath *et al.* 1999) and supplemented with 1 mM sodium pyruvate, 0.3% BSA, and 50 $\mu\text{g}/\text{mL}$ gentamycin (IVF-TALP). Sperm concentration was adjusted for each experiment.

Sperm- B_{ZP} binding assay and assessment of sperm acrosome reaction

Groups of 50–55 beads conjugated to recombinant ZP proteins (B_{ZP2} , B_{ZP3} , and B_{ZP4}) were washed twice prior to co-incubation with 200,000 sperm/mL in 500 μL IVF-TALP medium at 38.5°C , 20% O_2 , 5% CO_2 , and under saturated humidity. Acrosome integrity of sperm not bound to beads was evaluated just before and 3 min after sperm preparation, as well as at 0.5, 1, and 2 h after incubation with the beads. Sperm- B_{ZP} complexes were assessed after 2 h of co-incubation: sperm- B_{ZP} complexes were aspirated from the incubation well, washed three times in PBS supplemented with 0.1% PVA (polyvinylalcohol) (PBS-PVA), and fixed in glutaraldehyde (0.5%, v/v in PBS). The fixed complexes were then stained for 30 min with 0.01 mM Hoechst 33342 and 4 $\mu\text{g}/\text{mL}$ fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC; Matas *et al.* 2010) before mounting them on microscope slides and imaging under an epifluorescence microscope (Leica). The following parameters were assessed and recorded: number of sperm bound to each bead, acrosome status of each sperm bound to

bead (green stained acrosome equals reacted sperm), number of acrosomal shrouds (deposition of acrosomal content) on the beads' surface. The percentage of beads with at least one sperm bound ($B_{ZP}\text{SB}\%$), the mean number of sperm per bead (S/B_{ZP}), and the percentage of acrosome reacted sperm among total bound sperm was calculated.

Unbound spermatozoa remaining in the incubation well were aspirated and stained after 0.5, 1, and 2 h of incubation following the same protocol as used for the staining of the sperm- B_{ZP} complexes. Acrosome integrity was assessed in 200 cells per incubation period. Data for sperm- B_{ZP} complexes were collected from four and acrosome integrity of unbound sperm were collected from three replicates.

Incubation of B_{ZP} with cumulus cells and further insemination

Following 44 h in vitro maturation of porcine cumulus-oocyte complexes (COCs) as previously described (Coy *et al.* 2010), cumulus cells were removed from the COCs by repeated up-and-down pipetting in IVF-TALP medium. While oocytes were removed, the medium containing cumulus cells was centrifuged twice at 1200 g for 10 min. The cell pellet was resuspended in IVF-TALP medium and the number of cumulus cells determined using a hemocytometer. Groups of 50–55 B_{ZP} were washed twice in IVF-TALP medium before incubation with 2500 cumulus cells per B_{ZP} in 500 μL IVF-TALP medium at 38.5°C , 20% O_2 , 5% CO_2 under saturated humidity for 24 h. The number of cumulus cells per B_{ZP} was chosen based on the reported number of cumulus cells per porcine COC (Campos *et al.* 2001, Munakata *et al.* 2016). After 24 h the presence of cumulus mass plugs around the B_{ZP} (CB_{ZP}) were confirmed under a stereomicroscope. To retain only the cumulus cells tightly attached to the beads, CB_{ZP} complexes were pipetted up-and-down three times in IVF-TALP medium. The CB_{ZP} were then co-incubated with 200,000 freshly prepared sperm/mL at 38.5°C , 20% O_2 , 5% CO_2 under saturated humidity for 2 h. CB_{ZP} complexes with bound sperm were fixed in glutaraldehyde (0.5%, v/v in PBS) and stained with 0.01 mM Hoechst 33342 and 4 $\mu\text{g}/\text{mL}$ fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC) for 30 min. Once mounted on slides, CB_{ZP} complexes were imaged under an epifluorescence microscope and the images analysed. Three replicates were analysed. The percentage of beads with at least one sperm bound ($\text{CB}_{ZP}\text{SB}\%$) and the total number of sperm bound to B_{ZP} (TS/CB_{ZP}) were recorded.

Evaluation of protein-bound beads and oocytes by field emission scanning electron microscopy

To learn more about the nature of cumulus cell adhesion and sperm binding to the sepharose beads, we performed field emission scanning electron microscopy (FESEM). Groups of 50–100 B_{ZP} , CB_{ZP} and CB_{ZP} (the difference between the two CB_{ZP} ?) co-incubated with sperm and in vitro matured and partially denuded porcine COCs were fixed in 2% glutaraldehyde (Dulbecco's PBS w/o Ca-Mg (PBS-w/o)) at 4°C for 2 h followed by three washes in PBS-w/o. Samples were then sent to the Electron Microscopy Service Section of the

University of Murcia for further preparation and imaging using the scanning electron microscope ApreoS (Thermo Fisher Scientific).

Evaluation of protein-bound beads and oocytes by confocal microscopy

Groups of 30–40 live sperm- B_{ZP} complexes were washed three times for 10 min in PBS-w/o and stained with primary antibodies for 1 h at RT. Antibody dilutions for each glycoprotein were as follows: anti-FLAG for ZP2 (1:200, v/v), anti-ZP3 for ZP3 (1:200, v/v), and V5 Epitope Tag for ZP4 (1:200, v/v). Secondary antibodies were anti-rabbit IgG-FITC conjugate (1:100, v/v) for ZP2 and ZP4, and Alexa Fluor 488 Donkey anti-goat IgG (1:200, v/v) for ZP3. The stained sperm- B_{ZP} complexes were then fixed in 4% paraformaldehyde and stained with 0.01 mM Hoechst 33342 for 30 min.

To determine whether cumulus cells adhere to the B_{ZP} complexes, groups of 30–40 CB_{ZP} and in vitro matured partially denuded COCs were first fixed in 4% paraformaldehyde for 30 min. Samples were then permeabilised for 20 min in PBS-PVA and 1% Triton X-100 and stained with rhodamine-phalloidin (1:50 (v/v) in PBS-PVA; Invitrogen) and 0.01 mM Hoechst 33342 for 30 min at RT. All samples were placed onto Gene Frame chamber slides (25- μ L; Advanced Biotechnologies, Leatherhead, UK) and covered with a coverslip. Images were taken with a Leica TCS SP8 confocal microscope and image analysis was performed with LAS X Core software (Leica Microsystems).

In vitro maturation of porcine COCs

In vitro maturation of cumulus-oocyte complexes (COCs) was carried out as described by Coy *et al.* (2010). Briefly, gilts' ovaries (Landrace x Large-White) were collected within 30 min of slaughtering and transported in saline supplemented with 100 mg/mL kanamycin sulphate at 38.5°C. Once in the laboratory, the ovaries were washed once in 0.04% cetrimide solution and twice in saline. Antral follicles (3–6 mm in diameter) were aspirated and COCs collected under a stereomicroscope. COCs with dark granulated cytoplasm and several layers of cumulus cells were washed twice in PBS-PVA, and twice in NCSU-37 maturation medium (Petters & Wells 1993). NCSU-37 maturation medium consists of 108.73 mM NaCl, 25.07 mM NaHCO_3 , 4.78 mM KCl, 1.19 mM KH_2PO_4 , 1.19 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.70 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5.55 mM glucose, 1.00 mM glutamine, 12.00 mM D-sorbitol, 0.1 IU/l penicillin, and 0.0125 mM streptomycin and is supplemented with 0.57 mM cysteine, 1 mM dibutyl cAMP, 5 mg/mL insulin, 50 mM β -mercaptoethanol, 10 IU/mL equine chorionic gonadotropin (eCG; Folligon, Intervet International BV, Boxmeer, Holland), 10 IU/mL hCG (Veterin Corion, Divasa Farmavic, Barcelona, Spain), and 10% porcine follicular fluid (v/v). The 50–55 washed COCs were transferred into 500 μ L of maturation medium that has been equilibrated for 3 h at 38.5°C and 5% CO_2 . COCs matured in vitro at 38.5°C, 20% O_2 , 5% CO_2 and saturated humidity for 22 h followed by two washing steps and an additional 22 h incubation in maturation medium without dibutyl cAMP and hormones.

Simultaneous incubation of in vitro matured COCs and B_{ZP}

Following 44 h of in vitro maturation, the COCs were partially denuded by pipetting and washed twice in IVF-TALP medium. Groups of 25–30 COCs were transferred to a four-well culture plate containing 250 μ L equilibrated IVF-TALP medium and 25–30 B_{ZP} or B_{Ctrl} . A control sample consisted of 50–55 partially denuded COCs without beads. Heterospermic sperm samples were diluted in IVF-TALP medium to adjust the concentration at 10,000 sperm/mL that were added to each well containing partially denuded COCs and beads in a final volume of 500 μ L. Sperm, oocytes, and beads (B_{ZP} or B_{Ctrl}) were co-cultured at 38.5°C, 20% O_2 , 5% CO_2 , and saturated humidity for 18–20 h. After coincubation, the beads were aspirated from incubation well, washed thrice in PBS-PVA, fixed and stained as described below for the putative zygotes. The putative zygotes were mechanically stripped of cumulus cells and sperm, fixed with glutaraldehyde (0.5%, v/v in PBS-PVA), and stained with 0.01 mM Hoechst 33342. Oocytes and beads (B_{ZP} or B_{Ctrl}) were analysed under an epifluorescence microscope (Leica). Sperm penetration was scored when at least one spermatozoon was found within the ooplasm. Once a sperm enters the ooplasm, its DNA gradually decondenses until the male pronucleus is formed. The sperm head can be (1) compact without signs of nuclear expansion keeping its paddle-shape; (2) partially decondensed, swollen and loss of its typical paddle-shape but has not yet reached the spherical shape of the male pronucleus; (3) fully decondensed in form of the male pronucleus with its spherical shape and pronuclear envelope. These are all normal configurations and represent a developmental continuum. Hoechst staining does not allow to distinguish between the male and female pronucleus because the sperm tail is not stained. We, therefore, assumed the presence of a male and female pronucleus every time zygotes displayed two Hoechst-stained pronuclei. If the metaphase or chromatin structures from an oocyte's nucleus were missing but one pronucleus visible, we counted this pronucleus as female and the result of a spontaneously activated oocyte. Based on our experience, it is extremely rare to see an oocyte's metaphase plate together with a fully formed male pronucleus. Therefore, we defined the variable male pronucleus formation (MPNf) rate as zygotes with at least two visible pronuclei. The total number of spermatozoa per penetrated oocyte included both the number of MPN and decondensed heads. Zygotes were considered monospermic regardless of whether one pronucleus and one sperm head or two pronuclei were visible. The final efficiency of IVF (% output) was calculated as the number of zygotes with two pronuclei per total number of oocytes subjected to in vitro fertilisation. In the bead samples, the number of sperm bound per bead, percentage of beads with at least one sperm bound ($B_{ZP}SB$), and the mean number of sperm per bead (S/B_{ZP}) were calculated. A total of five replicates were analysed.

Statistical analysis

Data are presented as mean $\pm$ S.E.M. and all percentages were modelled following a binomial model of variables and arcsine transformation to achieve a normal distribution in the data set.

We used one-way ANOVA to analyse all parameters of interest unless otherwise noted. Data revealing significant effects were subject to Tukey's test and were considered statistically significant if the P value was <0.05 . All statistical analyses were performed using the Systat v13.1 software (Systat Software, Inc San Jose, CA, USA).

Results

ZP2-coated beads (B_{ZP2}) bind the highest number of sperm cells

To learn whether sperm preferentially interacts with one of the three porcine ZP glycoproteins, we performed the sperm-ZP binding assay. Magnetic sepharose

beads conjugated with ZP2 (B_{ZP2}), ZP3 (B_{ZP3}) and ZP4 (B_{ZP4}) porcine recombinant glycoproteins allowed the sperm-ZP bead binding assay (Fig. 1A). Electrophoresis and Western blots confirmed the presence of the recombinant proteins conjugated to the beads (100 kDa for B_{ZP2} , 55 kDa for B_{ZP3} and 65 kDa for B_{ZP4}) (Fig. 1B) and the uniform distribution of protein over the entire bead's surface for the three models was confirmed by confocal microscopy, as well as a slight deformation of the proteinaceous surface caused by the transformation of the kinetic energy of the spermatozoa at binding (Fig. 1C).

The number of porcine sperm bound to B_{ZP} was recorded as the percentage of B_{ZP} with at least one

Figure 1 Sperm- B_{ZP} binding assay. (A) Schematic representation of sperm- B_{ZP} binding assay. Recombinant ZP glycoprotein coated beads (B_{ZP}) and beads incubated with non-transfected CHO cells media (B_{Ctrl}) were coincubated with double centrifuged porcine sperm for 2 h. Beads with sperm attached were observed under epifluorescence microscope after being fixed (0.5% glutaraldehyde) and stained (0.01 mM bisbenzimidazole). (B) Electrophoresis and Western blot of ZP2, ZP3 and ZP4 proteins conjugated to magnetic beads. Media after conjugation (lane 1), proteins eluted from the beads after conjugation (lane 2) and media before conjugation (lane 3). Anti-Flag antibody was used for ZP2, anti-ZP3 for ZP3 and V5 Epitope Tag antibody for ZP4. (C) Confocal microscopy images of B_{Ctrl} and B_{ZP2} showing uniform coating of beads with recombinant ZP glycoproteins and sperm stained with bisbenzimidazole. The uniform distribution of the protein over the bead's surface was observed in the three models, but only B_{ZP2} is shown as an example. Scale bar 25 μm . (D) The percentage of B_{ZP} and B_{Ctrl} with at least one sperm bound ($B_{ZP}SB\%$) (left panel) and the average number of sperm per bead (S/B) (right panel) were recorded after coincubation period. A higher mean number of sperm per bead was observed in B_{ZP2} compared with B_{Ctrl} , B_{ZP3} and B_{ZP4} ($P < 0.01$). Different letters (a, b, c) show significant differences between groups ($P < 0.05$).

sperm bound ($B_{ZP}SB\%$) and as the mean number of sperm bound to each B_{ZP} (S/B_{ZP}) after 2 h of sperm-bead incubation. We found that for each group of glycoprotein-conjugated beads (B_{ZP2} , B_{ZP3} , B_{ZP4}) more than 64.0% of beads had bound at least one sperm (Fig. 1D, left panel). However, B_{ZP2} had a significantly higher percentage of S/B_{ZP} (8.8 ± 0.6 , $n = 289$) compared to the control B_{Ctrl} (4.9 ± 0.3 , $n = 270$), B_{ZP3} (6.7 ± 0.6 , $n = 253$) and B_{ZP4} (3.9 ± 0.3 , $n = 297$) groups ($P < 0.01$; Fig. 1D, right panel). Therefore, B_{ZP2} decoyed the highest number of sperm cells.

CB_{ZP} model supports the sperm binding following a similar pattern than B_{ZP} model

Cumulus ZP-bead complexes were generated and co-incubated for 2 h with double centrifuged porcine sperm (Fig. 2A). The complex formed by cumulus cell membranes and the carbohydrate sepharose (CB_{ZP}) was confirmed by field emission scanning electron microscopy (Fig. 2B) where membrane projections were clearly observed in cumulus cells on the bead's surface, and sperm interacting with the proteinaceous surface of the bead. Moreover, the surface of the cumulus cells attached to the B_{ZP} is rugose and vesicles-like structures can be observed in some cells. Observation of CB_{ZP} under confocal microscopy after staining with rhodamine-phalloidin showed that attached cumulus cells emit projections penetrating the bead's surface (Fig. 2C).

As mentioned previously, the percentage of CB_{ZP} with at least one sperm bound ($CB_{ZP}SB\%$) and the total sperm bound per B_{ZP} (TS/CB_{ZP}) were assessed. Regarding $CB_{ZP}SB\%$, more than 84.0% of CB_{ZP} in all models showed at least one sperm bound (Fig. 2D, left panel), with $B_{ZP}SB\%$ significantly higher for CB_{ZP2} model (96.6 ± 1.4 , $n = 176$, $P < 0.001$) compared to the other two models, CB_{ZP3} and CB_{ZP4} . Additionally, CB_{ZP2} was also the model with a higher TS/CB_{ZP} (Fig. 2D, right panel), being 13.9 ± 0.7 ($n = 173$), 6.5 ± 0.5 ($n = 168$) and 10.1 ± 0.6 ($n = 158$) for CB_{ZP2} , CB_{ZP3} and CB_{ZP4} , respectively ($P < 0.001$). Showing that, same as before, CB_{ZP2} decoys the highest number of boar sperm.

ZP2 is involved in sperm binding and ZP3 and ZP4 in acrosome reaction

Acrosome integrity was assessed in the sperm cells bound to B_{ZP} and in sperm that remained unbound in the well after co-incubation with B_{ZP} . Acrosome status just before and 3 min after sperm preparation was $4.7 \pm 0.9\%$ ($n = 600$) and $29.3 \pm 1.9\%$ ($n = 600$), respectively. The percentage of reacted sperm bound to B_{ZP} increased over time in all groups (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article). After 0.5 h co-incubation, the B_{ZP2} model showed a higher rate of reacted bound sperm

($77.5 \pm 3.8\%$, $n = 154$) than B_{ZP3} (69.8 ± 3.8 , $n = 158$) and B_{ZP4} (65.0 ± 3.4 , $n = 155$) ($P < 0.05$) (Fig. 3A). Differences were also observed in the unbound sperm assessed at 0.5 h co-incubation. The lowest percentage of acrosome reacted sperm was found in the group incubated with B_{ZP2} (45.2 ± 2.0 , $n = 600$) compared to B_{ZP3} and B_{ZP4} groups (57.7 ± 2.0 , $n = 600$; and 52.8 ± 2.0 , $n = 600$, respectively) ($P < 0.05$) (Fig. 3B). Additionally, acrosomal shrouds were observed on the B_{ZP} surface incubated with porcine sperm and its number increased over time in all models. At 2 h incubation, B_{ZP2} group showed the lowest number of acrosomal shrouds per bead, 2.7 ± 0.1 ($n = 153$), compared to B_{ZP3} and B_{ZP4} (3.8 ± 0.2 , $n = 156$; and 3.5 ± 0.1 , $n = 153$, respectively) ($P < 0.01$) (Fig. 3C). Altogether the data show that the highest percentage of bound reacted sperm is found in the B_{ZP2} model, while the percentage of unbound acrosome reacted sperm is the lowest in this group.

Insemination of partially denuded COCs in presence of B_{ZP2} increases the final efficiency of IVF in porcine species

To assess whether the presence of B_{ZP2} did or did not have an effect on in vitro fertilisation (IVF) in porcine species three experimental groups were proposed: COCs alone, COCs together with B_{ZP2} (B_{ZP2} group), and COCs together with B_{Ctrl} (B_{Ctrl} group) (Fig. 4A). After 24 h co-incubation, the B_{ZP2} model presented a higher percentage of beads with at least one sperm bound ($B_{ZP}SB\%$) (Fig. 4B panel left) and a higher number of sperm bound per bead (S/B_{ZP}) than B_{Ctrl} (Fig. 4B panel right).

Fertilisation rate of eggs exposed to B_{ZP2} were assessed (Table 1). Penetration rate was similar, with all experimental groups being around 75.0%. The monospermy rate was higher when COCs were simultaneously inseminated with B_{ZP2} ($43.9 \pm 3.3\%$, $n = 303$) than with B_{Ctrl} ($33.1 \pm 3.0\%$, $n = 315$) and COCs inseminated in absence of beads ($34.2 \pm 2.9\%$, $n = 350$) ($P < 0.05$). Both the total number of spermatozoa and the number of decondensed spermatozoa per oocyte were lower in the COCs inseminated with B_{ZP2} (2.3 ± 0.1 and 0.7 ± 0.1 , respectively) than B_{Ctrl} and COCs alone ($P < 0.001$). The final output of the IVF system was higher when COCs were inseminated in presence of B_{ZP2} ($P < 0.05$). No differences were found in the male pronucleus formation rate (%) and the number of male pronuclei per oocyte, demonstrating that the presence of B_{ZP} did not have a harmful effect on fertilising spermatozoa.

Discussion

The ZP is a relevant egg coat but the role of each ZP protein among mammalian species is still controversial given that there are multiple proposed models to explain the sperm-ZP binding, such as the glycan-release model

Figure 2 Sperm-CB_{ZP} sperm binding assay. (A) Schematic representation of sperm-CB_{ZP} sperm binding assay. Attachment of cumulus cells to B_{ZP} (upper panel) and coincubation (2 h) of CB_{ZP} with double centrifuged porcine sperm (lower panel). (B) Images from FESEM of an in vitro matured oocyte (left) and a CB_{ZP} (middle) and CB_{ZP} with a sperm bound to beads' surface and another sperm attached to a cumulus cell (right). Vesicle-like structures (*) and cell projections can be observed (▲). (C) Images taken by confocal microscopy of an in vitro matured oocyte and CB_{ZP} stained with rhodamine-phalloidin. Attached cumulus cells emit projections with ramifications penetrating on the bead's surface. (D) The percentage of CB_{ZP} with at least one sperm bound (CB_{ZP}SB%) (left) and the average number of total sperm per bead (TS/CB_{ZP}) (right) were recorded after coincubation period. A higher mean number of total sperm per bead was observed in CB_{ZP2} compared with CB_{ZP3} and CB_{ZP4} ($P < 0.001$). Different letters (a, b, c) show significant differences between groups ($P < 0.05$).

(Bleil & Wassarman 1980, Vazquez *et al.* 1989) and the ZP2 cleavage model in mice (Burkart *et al.* 2012, Avella *et al.* 2014, 2016). Recently, we proposed and validated a 3D model that mimics the spherical shape of the oocyte as a new tool to study the role of

single proteins involved in gamete interaction, which is easily transferable and scalable among mammals. Specifically, porcine recombinant ZP glycoproteins (ZP2, ZP3 and ZP4) were incubated with magnetic sepharose beads (B_{ZP}) and the correct conjugation was

Figure 3 Acrosome reaction assessment and acrosomal shrouds detection. (A) Percentage of acrosome reacted sperm bound to B_{ZP} at 0.5 h. B_{ZP2} showed a higher percentage of acrosome reacted sperm bound to beads. (B) Percentage of acrosome reacted sperm unbound to B_{ZP} at 0.5 h. being the sperm coincubated with B_{ZP2} the ones presenting the lowest percentage of acrosome reaction. (C) Acrosomal shrouds (*) on the surface of B_{ZP}, which were fixed (0.5% glutaraldehyde) and stained with 4 µg/mL PNA-FITC (fluorescein isothiocyanate-conjugated peanut agglutinin) and 0.01 mM bisbenzimidazole. Sperm with reacted acrosome appear stained with PNA-FITC (arrow) whereas intact acrosome is not stained. Images were taken in an epifluorescence microscope. Sperm was also stained with propidium iodide to facilitate the visualisation of the acrosomal shrouds. Number of acrosomal shrouds were recorded per B_{ZP} over time. At 2 h coincubation, B_{ZP2} group presented a lower number of acrosomal shrouds per bead. Different letters (a, b, c) show significant differences between groups ($P < 0.05$).

confirmed by SDS-PAGE and Western blot (Hamze *et al.* 2019). In this present study, the sperm-ZP binding assays using a validated 3D model showed that beads conjugated to ZP2 (B_{ZP2}) have the highest ability to bind sperm. Our results indicate that ZP2 appears to have a key role in sperm-ZP binding in pigs. Indeed,

it has been shown that N-terminal domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans (Baibakov *et al.* 2012, Avella *et al.* 2014) and following fertilisation, ovastacin, an oocyte-specific protein is released from cortical granules and is responsible for the postfertilisation cleavage of ZP2 (Burkart *et al.* 2012). The ZP2 cleavage site was defined biochemically (LA↓DE) (Gahlay *et al.* 2010) and the diacidic (shaded) proteolytic cleavage site (arrow) in the N-terminal region of ZP2 is conserved in human, mice, bonnet monkey, cow, pig, dog, cat, rabbit, hamster, and rat (Burkart *et al.* 2012). Therefore, the presence of the cleavage sequence in porcine ZP2 together with the observed results suggest that, as in humans and mice, in porcine, ZP2 would be responsible for recognition between gametes. Our results differ from previous studies where porcine glycoprotein ZP4 has been proposed as responsible for the sperm-binding activity by competitive inhibition binding assay using native and recombinant ZP4 and ZP3 (Yonezawa *et al.* 2012). In these studies, however, ZP2 was not included, thus its role could not be elucidated.

Most knowledge regarding molecular recognition between spermatozoa and oocyte is derived from in vitro studies in which the cumulus cells have been removed from the oocyte and studies suggest a likely role of cumulus cells and their matrix on further activity of spermatozoa (Jin *et al.* 2011). In this current study, cumulus cells and sperm bind to the models (CB_{ZP}) allowing for the study of different ZP glycoproteins over a spherical 3D surface offering a new tool to reconstruct artificial cumulus-oocyte complexes. First, the nature of this interaction was demonstrated by means of FESEM and confocal microscopy, which revealed that cumulus cells bind to the rugose surface of the bead. The cells emit cytoplasmic prolongations, vesicle-like structures, and appear to penetrate the bead's surface. In the native oocyte, cumulus cells emit transzonal projections (Del Collado *et al.* 2017) and extracellular vesicles (de Ávila *et al.* 2020) in order to communicate and cooperate metabolically with the oocyte. The current observations might reflect an attempt of cumulus cells to perform the aforementioned functions. Secondly, the proposed models serve as a starting point to explore the role of isolated proteins even the surface markers of oocytes and the 3D model proposed in the current study are different what could potentially impact the cellular interaction between ZP and sperm. In the future, the models might be scaled to produce more complex surfaces by the combination of several ZP recombinant proteins, cumulus cells, and oviductal secretions. This new 3D model might offer a new device to study physiological characteristics of cellular communication. The generation of models with bound native cumulus cells allows the sperm to interact first with the cells inducing the necessary potential changes on its surface before contacting the ZP glycoprotein.

Figure 4 Porcine in vitro fertilisation in presence of B_{ZP2} and B_{Ctrl} . (A) Schematic representation of porcine in vitro fertilisation with B_{ZP2} model. (B) Percentage of B_{ZP2} and B_{Ctrl} with at least one sperm bound ($B_{ZP2SB\%}$) (left) and the mean number of sperm per bead (S/B) (right) were recorded after coincubation period. A higher mean number of sperm per bead was observed in the B_{ZP2} model compared to B_{Ctrl} . Different letters (a, b) show significant differences ($P < 0.05$). (C) Inseminated porcine oocytes assessed at 18–20 h post insemination. From left to right: unfertilised egg showing a metaphase plate (m), a polar body (*) and attached cumulus cell (cc); monospermic zygote showing two pronuclei (▲) and two polar bodies (*); polyspermic zygote with three pronuclei (▲); fertilised egg with a decondensed sperm head (spz) and a pronucleus (▲).

Therefore, the addition of the cumulus cells to the B_{ZP} might have some advantages since a more physiological microenvironment is being reproduced and, to some extent, the cells might exert a chemoattractant effect on the sperm (Tanghe *et al.* 2002). When applying the sperm-cumulus-beads complex assay, again, ZP2 shows the higher number of total sperm bound confirming that porcine sperm bind preferentially to ZP2 compared to ZP3 and ZP4.

In the oocyte (ZP)–sperm interaction, the acrosome reaction is a crucial event necessary for sperm penetration through the ZP. Different studies suggest that ZP3 is the glycoprotein inducing the acrosome reaction in mice sperm (Vazquez *et al.* 1989, Gupta & Bhandari 2011), whereas in humans it is ZP1 (Ganguly *et al.* 2010), ZP3 (Bray *et al.* 2002) and ZP4 (Chiu *et al.* 2008). Moreover, only acrosome reacted sperm to bind to human ZP2 (Gupta 2018). Accordingly, results presented in this current study showed a higher percentage of bound reacted sperm in the B_{ZP2} group whereas the percentage of unbound reacted sperm is significantly higher in

the groups incubated with B_{ZP3} and B_{ZP4} . Additionally, the presence of acrosomal content (phenomenon known as acrosomal shrouds) deposited over the beads surface was observed as it has been described in the native porcine ZP after in vitro insemination of empty ZPs and ZP-enclosed oocytes (Romar *et al.* 2012). In the current 3D model, the observation of these ‘sperm prints’ indicating sperm contacts and acrosomal content deposition on the bead’s surface is time-dependent, increasing over time and being higher for ZP3 and ZP4 groups. It is worthwhile to note that previous studies in pigs employing the same culture medium for insemination (IVF-TALP) and coincubation times (0, 0.5 and 2 h) showed a similar pattern of acrosomal content deposition on oocyte’s ZP (Romar *et al.* 2012) indicating that the 3D model reproduced this feature of native ZP and suggests that ZP3 and ZP4 glycoproteins are involved in triggering the acrosome reaction in boar spermatozoa.

In porcine species, the in vitro production of viable embryos is achievable, but not yet in a commercial

Table 1 Fertilisation results of in vitro matured porcine complexes (COCs) inseminated together with ZP2-coated-beads (B_{ZP2}), control beads (B_{Ctrl}) or without beads (COCs group).

Group	n	Penetration (%)	Monospermy* (%)	Output** (%)	MPNf* (%)	Mean number spermatozoa/oocyte*	Number decondensed spermatozoa/oocyte*	Number MPN/oocyte*
COCs	350	76.0 ± 2.3	34.2 ± 2.9 a	26.0 ± 2.3 a	99.2 ± 0.5	2.9 ± 0.1 a	1.1 ± 0.1 a	1.7 ± 0.11
COCs+ B_{ZP2C}	303	75.2 ± 2.5	43.9 ± 3.3 b	33.0 ± 2.7 b	100	2.3 ± 0.1 b	0.7 ± 0.08 b	1.5 ± 0.18
COCs+ B_{Ctrl}	315	75.9 ± 2.4	33.1 ± 3.0 a	25.1 ± 2.4 a	98.3 ± 0.8	2.9 ± 0.1 a	1.3 ± 0.1 a	1.6 ± 0.1
P value		0.973	0.029	0.049	0.132	<0.001	<0.001	0.103

Results were assessed 18 h postinsemination. MPN, male pronuclear formation rate; MPN/oocyte, number of male pronucleus in each penetrated oocyte). Data from six replicates.

*From penetrated oocytes. Different letter in the same column indicates significant differences ($P < 0.05$). **From total number of inseminated oocytes.

scale. This is due to the low efficiency of the IVF system (Romar *et al.* 2019), with high rates of polyspermy being the main reason leading to non-viable embryos (Grupen 2014). The 3D model proposed in this study, besides being a valuable tool to study proteins involved in gamete interaction and avoiding the likely interactions among them, has many potential applications. A study carried out in mice found that the addition of beads conjugated to a ZP2 peptide decoy sperm, reduce drastically the penetration rate when IVF is performed and beads can even be used as a non-hormonal reversible contraceptive method (Avella *et al.* 2016). Here, the ZP2 model (B_{ZP2}) was added to the insemination well where porcine gametes were coincubated without reducing penetrability and increasing monospermy and final output. In this new IVF system, B_{ZP2} showed an attractive effect on sperm thus preventing its interaction with oocytes and reducing polyspermy levels. The presence of inert beads in the insemination well did not affect the mechanisms of block to polyspermy of native oocytes. In porcine IVF systems, a reduced sperm concentration is necessary to avoid high polyspermy levels, but it cannot be extremely low because then penetration rate dramatically decreases. The use of B_{ZP2} model during gametes coculture would allow the use of moderate sperm concentrations with high monospermy rates thus being a cheap and reliable tool to increase the efficiency of pig IVF system without modifying the composition of IVF medium. It is noteworthy to mention that addition of beads did not affect male pronuclear formation and the ratio B_{ZP2} :oocyte in this assay was 1:1. The potential effect of higher ratios should be explored in future experiments.

In conclusion, the 3D model proposed to allow for the study of single ZP glycoproteins involved in gamete interaction through sperm binding and its role on triggering acrosome reaction. Here we show that, ZP3 and ZP4 proteins mainly induce acrosome reaction whereas ZP2 is involved in sperm-ZP binding in porcine species. The ability of the model to decoy sperm might be a useful tool to develop new protocols of porcine IVF improving the final outcome of this technique in pigs.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0123>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J G H, M J M and R R performed the experiments. M J M and R R conceived, planned and supervised the experiments. J G H, M J M and R R wrote the original draft, reviewed and edited the manuscript. M J M and R R obtained funding. All authors participated in final editing providing critical feedback and helped shape the research, analysis and manuscript.

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