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Development, validation and *in vitro* applications of novel 3D models to study gamete interaction in mammals

Desarrollo, validación y aplicaciones *in vitro* de nuevos modelos 3D para el estudio de la interacción entre gametos de mamíferos

Dª. Julieta Gabriela Hamze Araujo

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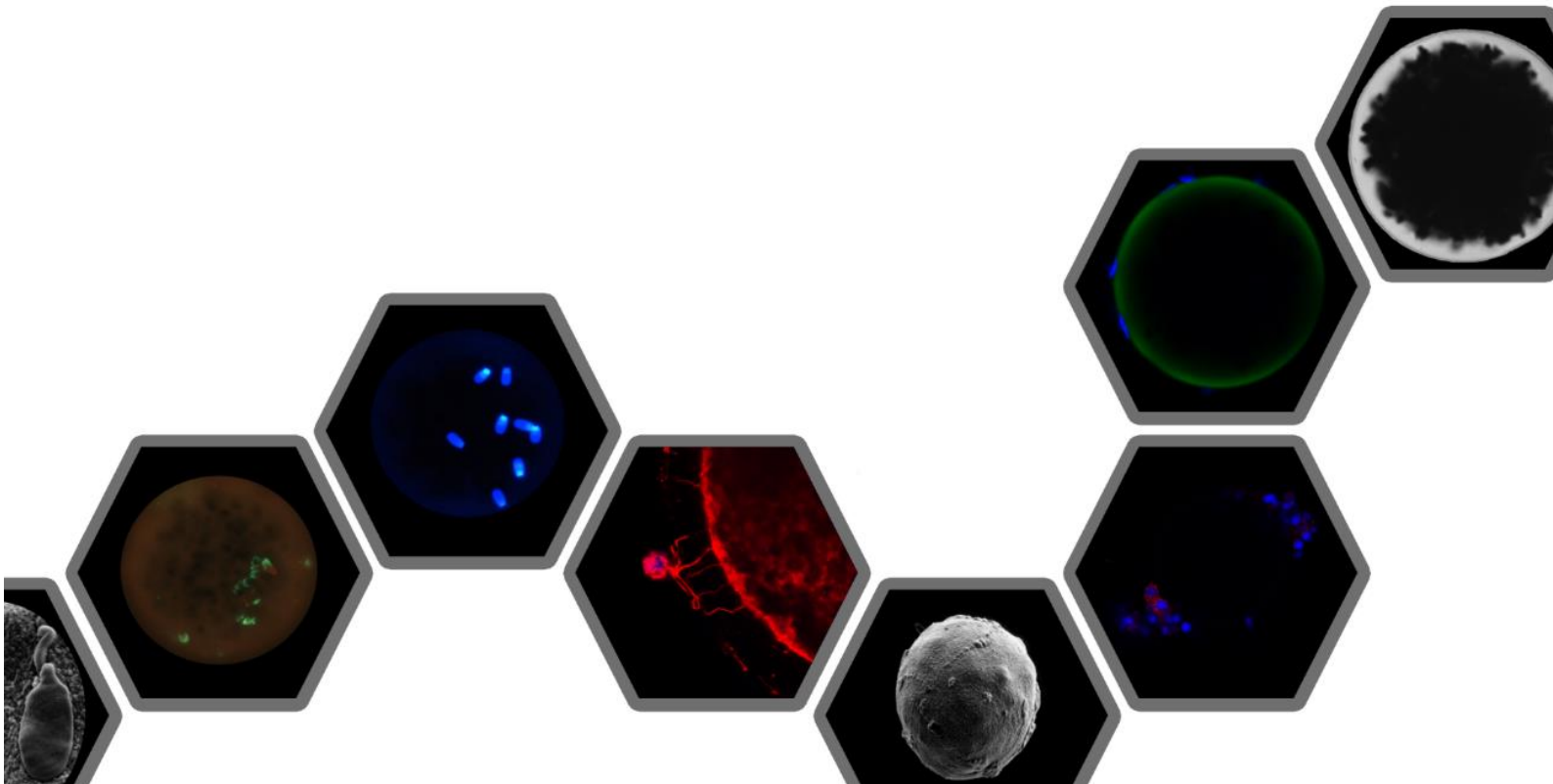

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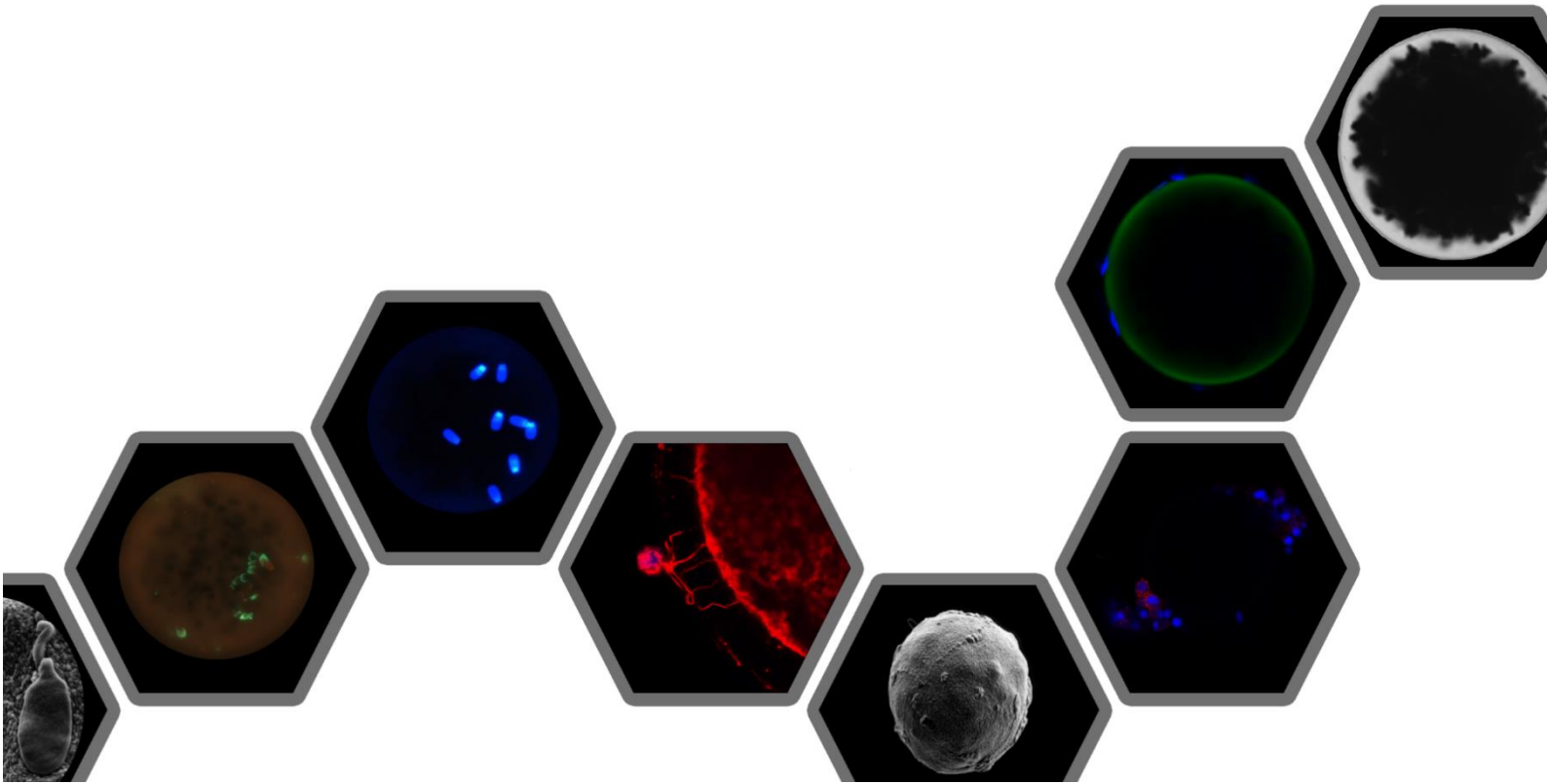
SUMMARY/RESUMEN



Summary

The binding and fusion of gametes, the oocyte and the sperm, to generate a new and unique individual is defined as fertilization and for it to be successful, a series of steps need to happen in an orchestrated manner during a reduced window of time. Just several hundred of mature and capacitated sperm cells reach the fertilization site and finally only one will cross through all egg vestments in order to fuse with the oolemma. Despite gamete recognition is a widely studied process, not all the molecular mechanisms are known in detail and especially in livestock species such as porcine and bovine, the knowledge is scarce. One of the egg's vestments, the zona pellucida (ZP), an extracellular matrix composed by three or four glycoproteins depending on the species is involved in several mechanisms since oogenesis in the ovary until embryo implantation in the uterus. ZP is also involved in fertilization process by participating in gamete recognition and blocking of the polyspermy. A series of studies using genetic modified mice lines showed that ZP2 glycoprotein contains a sperm-binding domain in its N-terminus, which is cleaved after fertilization by a protease released during cortical granule exocytosis. In addition, many studies described that the ZP is involved in acrosome reaction, being ZP3 glycoprotein the candidate as an acrosome exocytosis inducer in mice sperm; and ZP1, ZP3 and ZP4 in humans. Furthermore, the ZP provides protection to the oocytes and embryos during development through the reproductive tract prior implantation. Once the spermatozoon has crossed through the *cumulus oophorus* cells and the ZP, it will bind and fuse with the oolemma. Most of the specific molecules involved in these events as well as the underlying mechanisms of the whole process, are still unknown. In fact, to date only a few molecules have been demonstrated

to be essential during this process. Three of them described in the mammalian oocyte (CD9, CD81 and JUNO) and another three in the sperm (IZUMO, TMEM95 and PtdSer). Moreover, the recent discovery of JUNO in the egg as the binding partner of IZUMO in the sperm determines these proteins as the first cell surface receptor pair essential for gamete recognition in any organism. In order to study and discover other specific molecules involved in gamete recognition, binding and fusion it would be necessary to have appropriate tools where the role of individual proteins could be study.

Research in the field of reproductive biology is a challenging task due to the transient nature of the gamete interaction event and the existence of several limitations such as gamete retrieval and its use for research purposes since there are bioethical implications and, in some species, implies the slaughter or surgical intervention of the animal. Additionally, the availability of gametes of endangered species is scarce. Therefore, new reproducible and scalable strategies to carry on studies in the reproductive biology field should be developed to elucidate the molecular mechanisms of gamete recognition, to improve the assisted reproductive technologies and to design new non-hormonal contraceptives. Ideally, these strategies should be easily transferable between species.

The main objective of this Doctoral Thesis was to develop and validate a new *in vitro* tool to study gamete interaction and to predict the fertility in seminal samples. The three-dimensional (3D) model extensively studied during this Thesis is based in the use of magnetic sepharose beads coated with recombinant proteins that are known to be involved in gamete interaction. This 3D model mimics the shape of the oocyte, is scalable, reproducible and easily transferable between species. Some of the possible applications of the model will be developed and validated during the different chapters.

In the first chapter (Chapter 1), the model based on magnetic sepharose beads (B) coated with single recombinant porcine ZP glycoproteins (B_{ZP}) that mimic the 3D oocyte's shape was generated. In order to obtain the models, porcine recombinant ZP2 (C and N-terminus), ZP3 and ZP4 glycoproteins were expressed with peptide tags to allow their identification and conjugation to magnetic sepharose beads. Beads coated with individual zona pellucida glycoproteins (B_{ZP2}, B_{ZP3} and B_{ZP4}) were used to study: i) their ability to support adhesion of porcine *cumulus oophorus* cells; ii) their potential to bind spermatozoa; iii) their ability to induce sperm acrosome exocytosis; and iv) whether these interactions are affected by sperm preparation method. The obtained results validated the functionality of the model. Moreover, the models were characterized by protein SDS-PAGE, immunoblot and imaging with confocal and electron microscopy. From a structural point of view, the B_{ZP} closely mimic native cumulus-oocyte complexes by maintaining a spherical shape, support cumulus cell adhesion and provide a glycoprotein-specific, variegated surface analogous to the native ZP. From a biological point of view, the B_{ZP} support sperm binding, recapitulates induction of acrosome exocytosis and acrosomal shrouds can be observed on the B_{ZP} surface. Furthermore, the sperm binding was different depending on the capacitation method used. From these observations, the conclusion of the first chapter is that the recombinant zona glycoprotein coated beads are stable through the time, maintain its support cumulus cells adhesion, sperm biding and provide a valuable tool to explore the molecular basis of gamete recognition in pigs. This straightforward 3D model would be easily transferable to a wide range of mammals.

In the second chapter (Chapter 2), the application of the model to study the role of single proteins in gamete interaction was explored. The objective of this chapter was to

employ the generated 3D model in porcine species to better understand the specific roles of ZP2, ZP3 and ZP4 glycoproteins in sperm interaction with ZP, induction of sperm acrosome reaction and degree of cumulus cells adhesion. The specific objectives of this chapter were: i) to investigate the direct role of each ZP coated-beads to decoy sperm; ii) to validate binding ability of sperm to ZP coated-beads surrounded by cumulus cells; iii) to analyze the acrosomal status of sperm cells bound to ZP coated-beads; and iv) to study the likely modulating effect of adding ZP coated-beads to the porcine *in vitro* fertilization (IVF) system. To achieve these objectives, ZP coated-beads were incubated with fresh ejaculated boar spermatozoa and with isolated cumulus cells obtained from *in vitro* matured cumulus-oocyte complexes (COCs). Sperm binding, acrosomal shrouds on bead's surface and acrosome integrity (by means of PNA-FITC lectin staining) of sperm bound to the beads and unbound sperm (in the fertilization dish) were studied. Moreover, fertilization results were assessed after insemination of *in vitro* matured COCs in presence of B_{ZP2} or B_{Ctrl}. Results at 2 h incubation showed that over 60 % of beads had at least one sperm bound, being B_{ZP2} model the one with the highest number of sperm bound per bead (8.82 ± 0.63, N=289). However, B_{ZP3} and B_{ZP4} models presented a higher number of acrosomal shrouds (3.77 ± 0.17 N=156; and 3.48 ± 0.15 N=153 respectively) than B_{ZP2} (2.67 ± 0.14 N=153). Additionally, the percentage of acrosome reacted sperm bound to the beads was higher for B_{ZP2} than B_{ZP3} and B_{ZP4} whereas the percentage of unbound acrosome reacted sperm was higher for B_{ZP3} and B_{ZP4} models than $B_{ZP2.}$ IVF output and monospermy rate increased when porcine COCs were inseminated in presence of $B_{ZP2.}$ Cumulus cells attached to the surface of B_{ZP} acquired a roundish shape emitting membrane projections and vesicle-like structures through the sepharose coat. The conclusion of this second chapter is that ZP3 and ZP4 glycoproteins

mainly induce acrosome reaction whereas ZP2 is involved in sperm-ZP binding in porcine species. Besides, B_{ZP2} model might be a useful tool to improve the final IVF efficiency in pigs and a first step to generate artificial COCs to be used in the laboratory.

After studying the role of the single porcine ZP glycoproteins successfully in Chapter 2, using the 3D model developed and validated in Chapter 1, in the last chapter (Chapter 3), the 3D model was tested in bovine species. This time, a protein involved in sperm-egg binding at a plasmatic membrane level was selected. Therefore, a new model based on magnetic sepharose beads coated with recombinant bovine JUNO protein (B_{JUNO}) was developed and validated. It is relevant to mention that variation in fertility exists between bulls when artificial insemination (AI) is performed, despite seminal samples pass the sperm quality control checks. Thus, the development of *in vitro* tests to predict bull fertility is important for selecting high fertility bulls prior to their use under commercial conditions. Here, a sperm-binding assay using the B_{JUNO} model was developed. In order to validate the B_{JUNO} model it was study: i) the ability of recombinant JUNO protein to be conjugated to the sepharose beads and its over time stability; ii) the potential of B_{JUNO} to bind bull sperm; iii) the behavior of the model when sperm from different origin is used (epididymal vs. fresh ejaculated) and; iv) the behavior of the model when it is coincubated with frozen-thawed semen from bulls with different fertilizing capacity. Results showed that B_{JUNO} is stable through time and bull sperm bound specifically to B_{JUNO} suggesting that JUNO-IZUMO1 interaction is conserved in cattle. Moreover, B_{JUNO} model responded differently between epididymal and ejaculated sperm being higher the number of sperm bound per bead when the beads were incubated with fresh ejaculated sperm instead of corpus or cauda epididymal sperm. Lastly, the number of sperm cells bound to B_{JUNO} was lower when inseminated

with frozen-thawed sperm from low fertility bulls vs. high fertility. The conclusion of this Chapter is that the findings here described document an innovative and valid sperm-binding assay to predict mammalian fertility by responding in a different manner between ejaculates from different fertilizing capacity.

In summary, it has been developed and validated a novel 3D model based on magnetic sepharose beads coated with recombinant proteins involved in gamete interaction that mimics the oocyte's shape. This model can be successfully used in different species and can be a useful tool to study the gamete interaction in depth, the sperm binding based on the used capacitation method, the induction of the acrosome reaction, the improvement of IVF protocols and it might be implemented as a semen fertility predictor in the future. It is relevant to mention that the models and methods described in this Thesis are scalable and reproducible, being a potential tool to be transferred to the industry.

Resumen

La unión y fusión de los gametos, los ovocitos y los espermatozoides, para generar un individuo nuevo y único se define como fecundación y para que sea exitosa, una serie de pasos deben ocurrir de manera orquestada durante un período reducido de tiempo. Únicamente varios cientos de espermatozoides maduros y capacitados alcanzan el sitio de fecundación y, finalmente, solo uno atravesará todas las envolturas del ovocito para fusionarse con el oolema. A pesar de que el reconocimiento entre gametos es un proceso ampliamente estudiado, no todos los mecanismos moleculares son conocidos en detalle y especialmente en especies de interés ganadero como el porcino y bovino, el conocimiento es escaso. Una de las envolturas del ovocito, la zona pelúcida (ZP), una matriz extracelular compuesta por tres o cuatro glicoproteínas dependiendo de la especie, está involucrada en varios mecanismos desde la ovogénesis en el ovario hasta la implantación del embrión en el útero. La ZP también participa en el proceso de fecundación al estar implicada en el reconocimiento entre gametos y el bloqueo de la polispermia. Una serie de estudios donde se utilizaron líneas genéticas modificadas de ratones mostraron que la glicoproteína ZP2 contiene un dominio de unión de espermatozoides en su extremo N-terminal. Este dominio se escinde tras la fecundación gracias a la acción de una proteasa liberada durante la reacción cortical. Además, muchos estudios han descrito la implicación de la ZP en la inducción de la reacción acrosómica, siendo la glicoproteína ZP3 la candidata en los espermatozoides de ratón; y ZP1, ZP3 y ZP4 en humanos. Además, la zona pelúcida proporciona protección a los ovocitos y embriones durante el desarrollo a través del tracto reproductivo antes de la implantación. Una vez que el espermatozoide cruce el *cumulus oophorus* y la ZP, se unirá

y fusionará con el oolema. La mayoría de las moléculas específicas involucradas en estos eventos, así como los mecanismos subyacentes de todo el proceso, aún se desconocen. De hecho, hasta la fecha solo se ha demostrado que unas pocas moléculas son esenciales en este proceso. Tres de ellas descritas en el ovocito de mamíferos (CD9, CD81 y JUNO) y otras tres en el espermatozoide (IZUMO, TMEM95 y PtdSer). Además, el reciente descubrimiento de JUNO en el ovocito como ligando de IZUMO en el espermatozoide es el primer par de receptores de la superficie celular esencial para el reconocimiento de gametos descrito en cualquier organismo. Para estudiar y descubrir otras moléculas específicas involucradas en el reconocimiento, la unión y la fusión de los gametos, sería necesario contar con las herramientas adecuadas para poder estudiar el papel de las proteínas de manera individual.

La investigación en el campo de la biología reproductiva es una tarea desafiante debido a la naturaleza transitoria de la interacción entre los gametos y la existencia de varias limitaciones, como, por ejemplo, la recuperación de los gametos y su uso con fines en investigación, ya que existen implicaciones bioéticas y en algunas especies, implica el sacrificio o la intervención quirúrgica del animal. Además, la disponibilidad de gametos de especies en peligro de extinción es escasa. Por lo tanto, se deben desarrollar nuevas estrategias que sean reproducibles y escalables en el campo de la biología reproductiva para dilucidar los mecanismos moleculares del reconocimiento de gametos, mejorar las tecnologías de reproducción asistida y diseñar nuevos anticonceptivos no hormonales. Idealmente, estas estrategias deberían ser fácilmente transferibles entre especies.

El objetivo principal de esta Tesis Doctoral fue desarrollar y validar una nueva herramienta *in vitro* para estudiar la interacción entre los gametos y predecir la fertilidad

en muestras seminales. El modelo tridimensional (3D) ampliamente estudiado durante esta Tesis se basa en el uso de microesferas magnéticas de sefarosa recubiertas con proteínas recombinantes que se sabe que están involucradas en la interacción entre los gametos. Este modelo 3D imita la forma del ovocito, es escalable, reproducible y fácilmente transferible entre especies. Algunas de las posibles aplicaciones del modelo se desarrollarán y validarán durante los diferentes capítulos.

En el primer capítulo (Capítulo 1), se generó el modelo basado en microesferas magnéticas de sefarosa (B) recubiertas con glicoproteínas recombinantes de la ZP porcina (B_{ZP}) que imitan la forma tridimensional del ovocito. Para obtener los modelos, las glicoproteínas recombinantes porcinas ZP2 (C y N-terminal), ZP3 y ZP4 se expresaron con etiquetas peptídicas para permitir la correcta identificación y conjugación con microesferas magnéticas de sefarosa. Las microesferas recubiertas con glicoproteínas individuales de la zona pelúcida (B_{ZP2}, B_{ZP3} y B_{ZP4}) se utilizaron para estudiar: i) su capacidad para soportar la adhesión de células del *cumulus oophorus* porcino; ii) su potencial para unir espermatozoides; iii) su capacidad para inducir la reacción acrosómica de los espermatozoides; y iv) si estas interacciones se ven afectadas por el método de capacitación espermática. Los resultados obtenidos validaron la funcionalidad del modelo. Además, los modelos se caracterizaron por electroforesis (SDS-PAGE), inmunotransferencia y captación de imágenes utilizando microscopía confocal y microscopía electrónica de barrido. Desde un punto de vista estructural, el modelo B_{ZP} imita estrechamente los complejos de cúmulos y ovocitos nativos al mantener una forma esférica, respaldar la adhesión de las células del *cumulus oophorus* y presentar una superficie recubierta glicoproteica análoga a la de la ZP nativa. Desde un punto de vista biológico, el modelo B_{ZP} admite la unión de los espermatozoides,

recapitula la inducción de la reacción acrosómica y se pueden observar restos acrosomales en la superficie de las microesferas B_{ZP}. Además, la unión de los espermatozoides fue diferente dependiendo del método de capacitación utilizado. A partir de estas observaciones, la conclusión del primer capítulo es que las microesferas recubiertas con las glicoproteínas recombinantes de la zona pelúcida porcina son estables a lo largo del tiempo, que permiten la adhesión de células del cúmulo a su superficie, así como la unión de espermatozoides, proporcionando una herramienta valiosa para explorar la base molecular del reconocimiento de gametos en cerdos. Este sencillo modelo 3D sería fácilmente transferible a una amplia gama de mamíferos.

En el segundo capítulo (Capítulo 2), se exploró la potencial aplicación del modelo en el estudio del rol de proteínas individuales en la interacción entre gametos. El objetivo de este capítulo es emplear el modelo 3D generado en el Capítulo 1 en la especie porcina para comprender mejor los roles específicos de las glicoproteínas ZP2, ZP3 y ZP4 en la interacción de los espermatozoides con la ZP, la inducción de la reacción acrosómica de los espermatozoides y el grado de adhesión de las células del cúmulo. Los objetivos específicos de este capítulo son: i) investigar la capacidad las microesferas conjugadas alas diferentes proteínas recombinantes de la ZP de unir espermatozoides; ii) validar la capacidad de unión de los espermatozoides a los modelos B_{ZP} rodeados por células del cúmulo (CB_{ZP}); iii) analizar la reacción acrosómica de los espermatozoides unidos a las microesferas conjugadas; y iv) estudiar el posible efecto modulador de añadir B_{ZP} al sistema de fecundación *in vitro* porcina (FIV). Para lograr estos objetivos, las microesferas conjugadas a las glicoproteínas recombinantes de la ZP se incubaron con espermatozoides porcinos eyaculados frescos y con células del *cumulus oophorus* aisladas de complejos de cúmulos-ovocitos (COC) maduros *in vitro*. Se evaluó la unión

de los espermatozoides, la presencia de restos acrosomales en la superficie del modelo B_{ZP} y la integridad del acrosoma (mediante tinción con lectina PNA-FITC) de los espermatozoides unidos a las microesferas y de los espermatozoides no unidos (en la placa de fecundación). Además, los resultados de la fecundación *in vitro* tras la inseminación de COC madurados *in vitro* en presencia de B_{ZP2} o B_{Ctrl} fueron evaluados. Los resultados a las 2 h de incubación mostraron que más del 60 % de las microesferas tenían al menos un espermatozoide unido, siendo el modelo B_{ZP2} el que tenía el mayor número de espermatozoides unidos por microesfera (8.82 ± 0.63, N = 289). Sin embargo, los modelos B_{ZP3} y B_{ZP4} presentaron un mayor número de restos acrosomales (3.77 ± 0.17 N = 156; y 3.48 ± 0.15 N = 153 respectivamente) que B_{ZP2} (2.67 ± 0.14 N = 153). Además, el porcentaje de espermatozoides reaccionados unido a las microesferas fue mayor para B_{ZP2} que B_{ZP3} y B_{ZP4}, mientras que el porcentaje de espermatozoides reaccionados no unidos fue mayor para los modelos B_{ZP3} y B_{ZP4} que B_{ZP2}. El rendimiento de la FIV y la tasa de monospermia aumentaron cuando los COC porcinos se inseminaron en presencia de B_{ZP2}. Las células del cúmulo unidas a la superficie de B_{ZP} presentaban una forma redondeada que emitía proyecciones de membrana y estructuras en forma de vesículas. La conclusión de este segundo capítulo es que las glicoproteínas ZP3 y ZP4 están implicadas principalmente la inducción de la reacción acrosómica, mientras que ZP2 está involucrada en la unión de espermatozoide-ZP en la especie porcina. Además, el modelo B_{ZP2} podría ser una herramienta útil para mejorar el rendimiento final de la FIV en cerdos y un primer paso para generar COC artificiales que se utilizarán en el laboratorio.

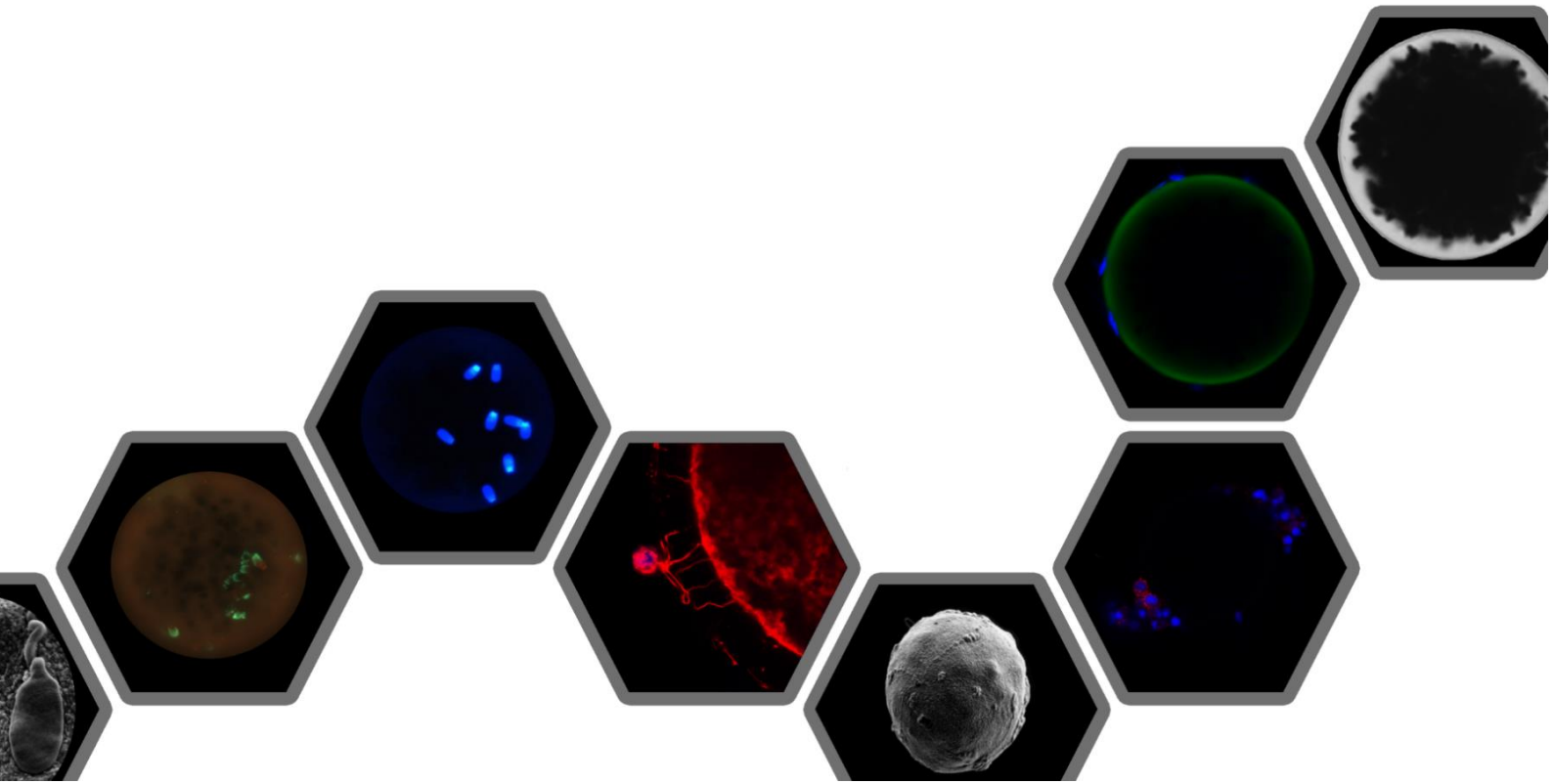
Después de estudiar el papel de las glicoproteínas de la ZP porcina de manera individual con éxito en el Capítulo 2, utilizando el modelo 3D desarrollado y validado en el Capítulo

1, en el último capítulo (Capítulo 3), el modelo 3D se probó en la especie bovina. Esta vez, se seleccionó una proteína implicada en la unión de espermatozoides a nivel de membrana plasmática. Para ello, se desarrolló y validó un nuevo modelo basado en microesferas magnéticas de sefarosa recubiertas con la proteína recombinante JUNO bovina (B_{JUNO}). Es relevante mencionar que existe una variación en la fertilidad entre toros cuando se realiza inseminación artificial (IA), a pesar de que las muestras seminales pasan los controles de calidad pertinentes. Por lo tanto, el desarrollo de pruebas *in vitro* para predecir la fertilidad de los toros es importante para seleccionar toros de alta fertilidad previo a su uso en condiciones comerciales. En este capítulo, se desarrolló un ensayo de unión de espermatozoides utilizando el modelo B_{JUNO}. Para validar el modelo B_{JUNO} se estudió: i) la capacidad de la proteína JUNO recombinante para conjugarse con las microesferas de sefarosa y su estabilidad en el tiempo; ii) el potencial de B_{JUNO} para unir espermatoziodes de toro; iii) el comportamiento del modelo al utilizar espermatozoides de diferente origen (epididimario versus fresco eyaculado) y; iv) el comportamiento del modelo cuando se incuba con semen congelado-descongelado de toros con diferente capacidad de fecundación. Los resultados mostraron que B_{JUNO} es estable en el tiempo y los espermatozoides de toro se unen específicamente a B_{JUNO}, lo que sugiere que la interacción JUNO-IZUMO1 está conservada en esta especie. Además, el modelo B_{JUNO} respondió de manera diferente al incubarlo con espermatozoides epididimarios y eyaculados, siendo mayor el número de espermatozoides unidos por microesfera cuando las éstas se incubaron con espermatozoides frescos eyaculados en lugar de espermatozoides epididimarios del cuerpo o la cola. Por último, el número de espermatozoides unidos a B_{JUNO} fue menor cuando se incubó con espermatozoides congelados-descongelados de toros de baja

fertilidad versus alta fertilidad. La conclusión de este capítulo es que los hallazgos aquí descritos documentan un ensayo de unión de espermatozoides innovador y válido para predecir la fertilidad de los mismos, respondiendo de manera diferente entre eyaculados con diferentes capacidades de fecundación.

En resumen, durante esta Tesis Doctoral, se ha desarrollado y validado un nuevo modelo 3D que imita la forma del ovocito. Éste está basado en microesferas magnéticas de sefarosa recubiertas por proteínas recombinantes involucradas en la interacción entre los gametos a nivel de la zona pelúcida y la membrana plasmática. Este modelo se puede utilizar con éxito en diferentes especies de mamíferos y puede ser una herramienta útil para estudiar en profundidad la interacción entre el ovocito y el espermatozoide, la unión de los espermatozoides según el método de capacitación utilizado, la inducción de la reacción acrosómica, la mejora de los protocolos de fecundación *in vitro* y, además, podría implementarse como predictor de fertilidad del semen en el futuro. Es relevante mencionar que los modelos y métodos descritos en esta Tesis son escalables y reproducibles, siendo una potencial herramienta para ser transferida a la industria.

LITERATURE REVIEW



Literature review

1. Fertilization

The union of the two gametes, the oocyte and the sperm, to generate a new and unique individual is defined as fertilization (Wassarman 1999). This process ensures the survival of a species through the genetic recombination events that occur during fertilization. To be successful, a series of steps need to happen in an orchestrated manner during a reduced window of time (Bianchi and Wright 2016).

Under *in vivo* conditions, the sperm deposited in the female genital tract must reach the fertilization site fully matured in order to be able to fertilize the egg, which will be ovulated surrounded by the zona pellucida (ZP) and the expanded *cumulus oophorus,* composed by cumulus cells embedded in a hyaluronan matrix as can be observed in Figure 1. Once the mature sperm reach the fertilization site, located in the oviductal ampulla in mammals, they have to i) penetrate the *cumulus oophorus*, ii) bind and penetrate the ZP and iii) fuse with the oolemma (oocyte plasma membrane) in order to generate a zygote (Cuasnicú et al. 2018). Once the membrane fusion has occurred, the oocyte suffers the cortical reaction and the ZP is then modified in order to avoid the polyspermy (Wassarman 1999).

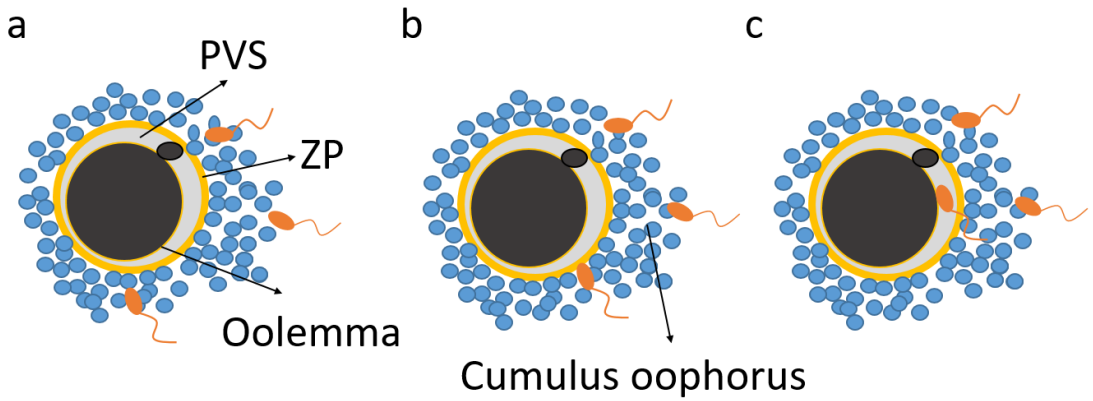


Figure 1. *Schematic representation of the gamete recognition and fusion. a) Sperm penetrating the cumulus oophorus. b) Spermatozoon binding and penetrating the ZP. c) Spermatozoon membrane fusing the oolemma. PVS: Perivitelline space.*

Gamete recognition and fertilization is a widely studied process but not all mechanisms involved have been elucidated (Bianchi et al. 2014). To understand in depth all molecules and pathways involved in this process is essential to fully understand infertility and be able to develop more effective assisted reproductive techniques (ARTs) and new non-hormonal contraceptive methods.

1.1. Spermatozoa: Capacitation and acrosome reaction

Upon leaving the testis, sperm are immature and immotile. In order to fuse and fertilize the egg, they should overcome a maturation process. The first step occurs, during their path through the epididymis where they acquire the ability to interact with the oocyte and become motile (Nixon and Bromfield 2018). The second step occurs through the female tract where they undergo a process defined as “capacitation” (Austin 1952), which grants the ability to fertilize the egg. The few sperm that reach the egg are highly selected and have suffered a series of changes by means of: i) a change in their

movement pattern, they are being hyperactivated, ii) a remodeling of their surface, facilitating the expression of the receptors and iii) the activation of the machinery necessary for the acrosome reaction (AR) (Aitken and Nixon 2013).

The acrosome is a sperm vesicle located in the apical region of the head (Wassarman 1999). AR is essential for fertilization (La Spina et al. 2016); during this process of exocytosis, the outer acrosomal membrane fuses with the plasmatic membrane of the sperm at multiple points resulting in vesicle formation and liberation of the acrosomal content as can be observed in Figure 2 (Georgadaki et al. 2016).

In mice and bull sperm it is described that the AR has to occur for some receptors as IZUMO1 to be relocated to the equatorial segment of the sperm head, which allows the oocyte-sperm fusion (Sosnik et al. 2009; Fukuda et al. 2016). In the case of the mouse spermatozoa, some studies suggest that most fertilizing sperm suffered the AR before reaching the cumulus-oocyte complexes (COCs) (Jin et al. 2011) beginning in the upper isthmus region of the oviduct (La Spina et al. 2016).

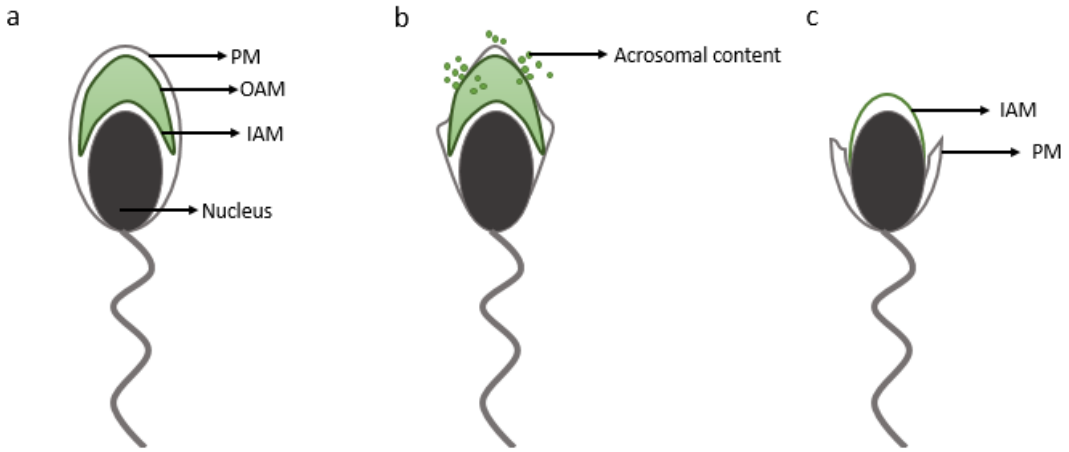


Figure 2. *Schematic representation of the acrosome reaction (AR). a) Non-acrosome reacted sperm. PM: plasmatic membrane, OAM: outer acrosomal membrane, IAM: Inner acrosomal membrane. b) Sperm undergoing AR and release of the acrosomal content. c) Acrosome reacted sperm.*

1.2. Cumulus-oocyte complexes (COCs): maturation and ovulation

Mature oocytes surrounded by the ZP and the cumulus oophorus are released from pre-ovulatory follicles through a process called ovulation. Ovulation is triggered by a surge of luteinizing hormone (LH), which occurs during the estrous cycle stimulated by gonadotropins (McGee and Hsueh 2000). In each cycle, primordial follicles are recruited to mature and grow through primary and secondary follicles before an antral cavity is formed. The classification of the follicles is based on the i) size of the oocyte, ii) size of the follicle and number of follicular cells surrounding the oocyte, and iii) the morphology of the follicle (Pedersen and Peters 1968). During folliculogenesis, the follicle and the oocyte increase their volume 300-fold approximately (Griffin et al. 2006). A schematic representation of this process can be observed in Figure 3.

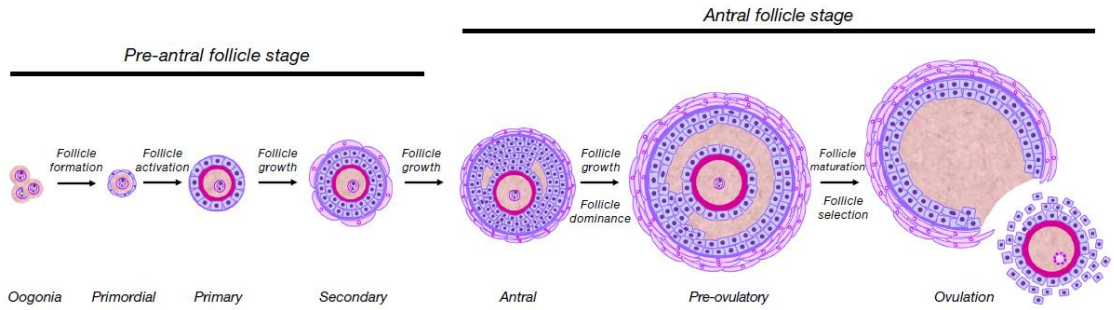


Figure 3. *Schematic representation of the folliculogenesis. Taken from* Woodruff et al. 2018.

In all pre-antral follicles, the nucleus of oocytes are arrested in the prophase I of the first meiotic division (Shuhaibar et al. 2015). The meiosis is reassumed due to the LH peak and in the moment of the ovulation, the oocyte is at the metaphase II stage, competently mature at a nuclear and cytoplasmic level, ready to be fertilized (Woodruff et al. 2018). An exception are COCs from bitch which are ovulated at prophase I stage, and oocyte meiosis resumption takes place in the oviduct (Tsutsui 1989).

1.2.1. Zona Pellucida (ZP)

The ZP is a glycoproteic extracellular matrix that surrounds mammalian oocytes and preimplantational embryos. The non-growing oocytes found in the primordial follicles do not have yet a ZP. It is not clear whether ZP proteins are synthesized in the granulosa cells, in the oocytes or both. However several studies evidence that ZP glycoproteins are secreted by the oocyte and appear in the growing follicles (Lefièvre et al. 2004; Familiari et al. 2006; Gupta 2018). During folliculogenesis, the ZP increases its width from 1.6 µm

to 6.2 µm in mice (Wassarman and Litscher 2018). The ZP thickness (1-27 nm) and the its protein content (1-30 ng) differs between species (Gupta et al. 2012). In porcine species, ZP thickness is 16 µm and contains 30-35 ng protein (Nakano et al. 1996).

The human and mice ZP semblance in scanning electron microscopy images is that both show different sized porous network formed by interconnected filaments. The outer surface pores are larger than the ones in the inner surface as it can be observed in the Figure 4 (Familiari et al. 1992). These differences in the ultrastructure have been also observed by transmission electron microscopy in porcine species, where a different network organization is found in the outer and inner ZP (Funahashi et al. 2001). Moreover, morphological changes in ZP are observed when comparing *in vitro* matured and ovulated porcine oocytes before and after fertilization (Funahashi, Ekwall, and Rodriguez-Martinez 2000).

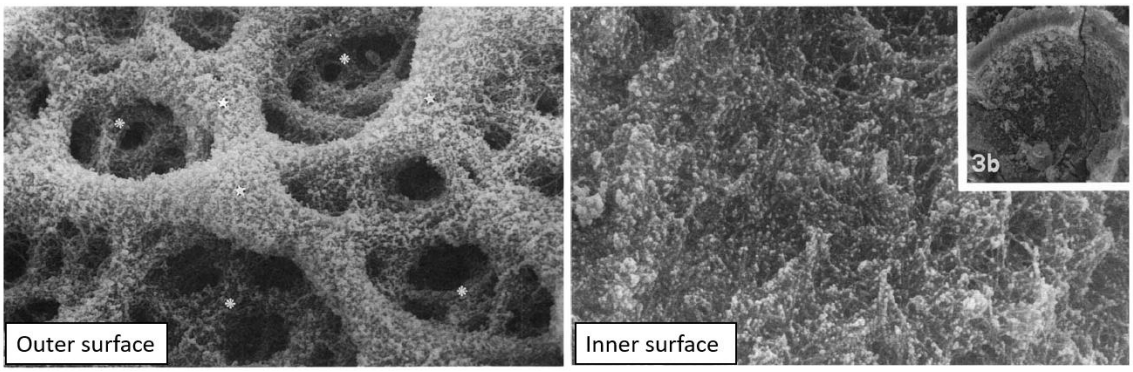


Figure 4. *Scanning electron microscopy images. Outer and inner surface of the ZP of a mature oocyte. Taken from* Familiari et al. 1992.

1.2.1.1. ZP composition

The mammalian ZP is composed by three or four glycoproteins depending on the species. In cows (Noguchi et al. 1994), pigs (Hedrick and Wardrip 1987) and dogs (Goudet et al. 2008), the ZP is comprised by ZP2, ZP3 and ZP4, while mice have ZP1 instead of ZP4 (Bleil and Wassarman 1980b), since *Zp4* has been described as a pseudogene in this species (Goudet et al. 2008). Other mammals such as, rat (Hoodbhoy et al. 2005), hamster (Izquierdo-Rico et al. 2009), bonnet monkey (Ganguly et al. 2008), cat (Moros-Nicolas et al. 2018a) and human (Lefievre et al. 2004), have all the four proteins (ZP1, ZP2, ZP3 and ZP4).

In marsupials, two different composition models were recently proposed. The first one for South American species with four proteins: ZP1, ZP2 and two ZP3 copies , and the second model with six proteins in Australian species: ZP1, ZP2, three ZP3copies,and ZP4 (Moros-Nicolas et al. 2018b).

It is important to mention that ZP2 and ZP3 glycoproteins are present in all mammalian species studied to date. *Zp1* and *Zp4* are paralogs, they share a higher sequence similarity and it is suggested that they have evolved from a common ancestor (Goudet et al. 2008).

1.2.1.2. Structure, secretion and processing of ZP glycoproteins

In mice, there is a single copy of each zona gene in the genome and the transcripts of these genes are accumulated during oogenesis. Once translated, ZP1, ZP2 and ZP3 are secreted in order to form the extracellular matrix (Bleil and Wassarman 1980a; Shimizu, Tsuji, and Dean 1983). All the zona proteins contain a “ZP domain” in the C-terminus,

formed by approximately 260 amino acids (aa), including eight conserved cysteine residues (Meczekalski et al. 2015). The ZP domain is found in many secreted proteins with different biological functions indicating that this conserved module is involved in the polymerization into filaments of extracellular proteins. ZP domain is described in other proteins that are not in the oocyte such as Tamm-Horsfall protein (THP), glycoprotein-2 (GP-2), α- and β-tectorins and transforming growth factor (TGF)-β among others (Jovine et al. 2002).

Furthermore, all the ZP glycoproteins share a signal peptide in the N-terminus, which conducts the protein precursors to the secretory pathway through the plasmatic membrane. This signal peptide is removed by the enzyme signal peptidase of the oocyte at some stage of the protein biosynthesis or during the assembly into the ZP matrix to obtain the mature protein (Bokhove and Jovine 2018; Gupta 2018).

The Trefoil domain (TD) (42 aa) has been described in human ZP1 and ZP4 (Gupta 2018) despite its function within these proteins has not been elucidated yet. Some authors suggest that TD could be important in the ZP assembly or in maintaining the ZP structure during the proteolytic action by acrosomal or cortical granules enzymes that occurs at fertilization (McLeskey et al. 1998). TD domain is associated to proteins that are thought to be growth factors contributing to mucosal defense mechanisms, mainly in the gastrointestinal tract (Gupta 2018).

The four ZP glycoproteins have the consensus furin cleavage site (CFCS) shortly after the ZP domain. Studies with recombinant human ZP3 suggest that this cleavage is essential for ZP proteins secretion and matrix assembly (McLeskey and Saling 2002).

Finally, all ZP proteins contain a hydrophobic transmembrane-like domain (TMD) and a short cytoplasmic tail. This hydrophilic cytoplasmic tail, prevents premature interactions between ZP2 and ZP3 proteins in order to assure the correct secretion and assembly of the ZP matrix in mice (Jimenez-Movilla and Dean 2011). A schematic representation of the ZP glycoproteins and their domains can be observed in Figure 5.

The basic structure of animal egg coat is conserved over millions of years of evolution, finding ZP homologous proteins in the vitelline envelope of non-mammalian vertebrates (Monne and Jovine 2011).

Figure 5. *Schematic representation of ZP glycoprotein. Signal peptide (pink), Trefoil domain (TD, red), ZP domain (blue), processing region (green) and transmembrane-like domain (TMD, orange). ZP glycoproteins also have short cytoplasmic tail. Various domains are not drawn to scale.*

1.2.1.3. Functions of ZP

The ZP matrix is involved in several mechanisms and its role is essential from the oogenesis until the preimplantational embryo. During oogenesis, the ZP is important in the establishment of cellular communications, it stabilizes the intercellular connections as gap junctions between the follicular cells and the oocyte, ensuring the proper

nurturing of the oocytes through the transfer of nutrients, metabolites and other molecules from the external environment (Wassarman and Litscher 2018).

ZP also regulates fertilization in oocytes participating in gamete recognition and blocking the polyspermy after sperm penetration. In some species such as bovine and porcine, the block of the polyspermy begins before fertilization by the ZP hardening (Coy et al. 2008). It also provides protection to the oocytes and embryos during development through the reproductive tract prior implantation in the uterus (Wassarman and Litscher 2018). Recently, it has been described that ZP4 confers structural properties to the ZP being essential for early embryo development (Lamas-Toranzo et al. 2019)

Several mutations have been reported in human ZP glycoproteins which produce infertility (Zhou et al. 2019), corroborating the importance of ZP in fertilization process.

2. Molecular basis of gamete interaction

It is known that ZP has a key role in gamete interaction. The use of models developed during this doctoral thesis will help elucidate the molecular basis of this interaction. Nowadays, two different models have been described to explain how the sperm-ZP binding occurs, being the N-terminal of ZP2 model the one that is acquiring more strength in the last years. In this section, these models will be reviewed.

2.1. Sperm-ZP binding models

2.1.1. Glycan-release model

A competitive sperm-binding assay with ovulated eggs using soluble ZP proteins suggested that ZP3 protein is the one responsible for mouse sperm binding to ZP, considering this protein as the sperm receptor in the oocyte. The authors hypothesized that once fertilization occurs, ZP3 is modified and the sperm is not able to bind again (Figure 6a) (Bleil and Wassarman 1980c). Later studies proposed that O-glycans attached to Ser^{332} and Ser^{334} residues of ZP3 were the responsible for sperm-ZP binding. After fertilization, as a result of cortical reaction, a glycosidase is exocytosed removing these glycans thus preventing sperm binding to zygotes and embryos (Florman et al. 1984; Chen et al. 1998). The origin of these carbohydrate ligands has remained controversial. In mice, the α-1, 3 galactose in ZP (Bleil and Wassarman 1988) and β-1,4- galactosyl-transferase in sperm (Miller et al. 1992) were proposed to be involved in sperm-ZP binding, meanwhile in humans it is believed that the carbohydrate ligand is the sialyl-$Lewis^X$, which is the most abundant in human ZP (Pang et al. 2011). Nonetheless, mice lacking α-1, 3 galactose (Thall et al. 1995) or β-1,4- galactosyl-transferase in the sperm (Lu and Shur 1997) were fertile. Mutant mice lacking O-glycans inZP3, presumably involved in sperm-ZP binding, were also fertile (Gahlay et al. 2010).

It remains unclear why the first biochemical assays supported the ZP3 glycan-release model and the recent genetic-based assays disagree with this hypothesis. One possible explanation may be that soluble ZP proteins isolated and re-natured do not act in the same manner than the insoluble ZP (Avella et al. 2013). This discrepancy has caused new models to emerge in order to explain sperm-ZP interaction and binding in mammals.

2.1.2. ZP2 cleavage model

In a tentative to explain the role of each ZP protein in the sperm-ZP interaction and binding, mouse lines lacking ZP1, ZP2 or ZP3 were stablished. $ZP1^{-/-}$ mice remained fertile although their fertilizing capacity was decreased (Rankin et al. 1999). In the case of $ZP2^{-/-}$ (Rankin et al. 2001) or $ZP3^{-/-}$ (Rankin et al. 1996) mice lines, no ZP was observed surrounding ovulated eggs and animals were sterile. These studies suggested that ZP2 or ZP3 were the proteins involved in sperm binding.

A series of studies using genetic modified mice lines (Rankin et al. 2003; Baibakov et al. 2012; Avella et al. 2014) in which mouse ZP1, ZP2 and ZP3 were replaced by human ZP proteins were performed to further investigate the roles of ZP2 and ZP3. The results showed that human sperm bound to the mouse oocytes with human ZP2, but not to the oocytes with human ZP1 and ZP3. Using recombinant technology, the N-terminus of ZP2 ($ZP2^{51-149}$ sperm-binding domain) was proposed as the gamete recognition site (Figure 6b).

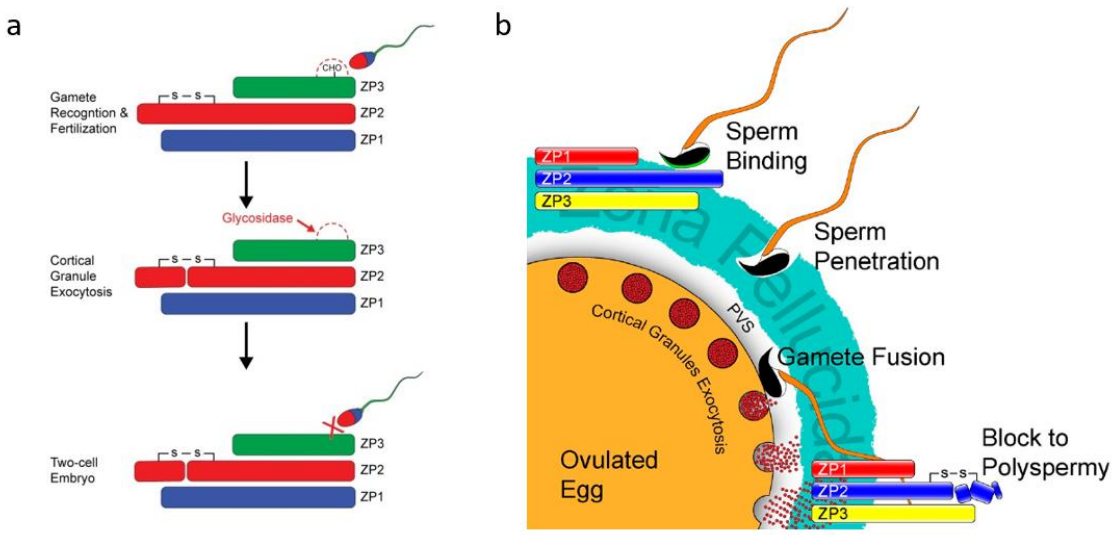


Figure 6. *Schematic representation of the sperm-ZP binding models. a) Glycan-release model. Taken from Avella et al. 2013. b) ZP2 cleavage model. Taken from Avella et al. 2014.*

After fertilization, the N-terminus of ZP2 is cleaved preventing polyspermy. When the endogenous ZP2 is replaced by a ZP2 protein that cannot be cleaved ($ZP2^{mt}$), the sperm bound to the embryos despite fertilization and cortical granule exocytosis having already occurred (Gahlay et al. 2010). Ovastacin, a metalloendoprotease member of the astacin family and present in the mice cortical granules content before fertilization, was proposed by Burkart et al. (2012) as the responsible for ZP2 cleavage. Moreover, recombinant ovastacin was proven to be effective in cleaving the protein in the native ZP. Genetic ablation of the gene encoding this protein showed that females could not cleave the ZP2 after fertilization, allowing the sperm bind to the embryos (Burkart et al. 2012).

The ZP2 cleavage model arises from the observations explained above. In this model, sperm recognize and attach to the $ZP2^{51-149}$ sperm-binding domain located in the N-terminus of the ZP2 prior to ZP penetration and gamete fusion. After fertilization, the cortical granules content is exocytosed and ovastacin is released, thus cleaving ZP2 protein and preventing polyspermy (Avella et al. 2013).

2.2. Receptors involved in fertilization

Fertilization is the process where egg and sperm recognize, bind and fuse with each other to generate a new and unique organism (Bianchi et al. 2014). Despite the years invested in research in the reproduction field, most of the specific molecules involved in fertilization and the underlying mechanisms of this process are still unknown. To date, only few proteins have been demonstrated to be essential during this process. Three of them described in the mammalian oocyte (CD9, CD81 and JUNO), and three in the sperm (IZUMO, TMEM95 and PtdSer).

2.2.1. Oocyte

2.2.1.1. CD9 and CD81

The first molecule described as essential for fertilization was CD9 (Le Naour et al. 2000; Miyado et al. 2000). This cell surface protein belongs to the tetraspanin superfamily and participates in cell migration, cell adhesion, proliferation, differentiation and signal transduction. The tetraspanins are associated with several molecules situated in the cell surface such as β1 integrins (Le Naour et al. 2000). CD9 could be implicated in the assembly or the stabilization of these adhesion molecules (Kaji et al. 2000).

To study the role of CD9 in fertilization, CD9 deficient mice were generated ($CD9^{-/-}$). Meanwhile the heterozygous mice ($CD9^{+/-}$) displayed the same phenotype as the wild type (WT), the $CD9^{-/-}$ females showed a severely reduced fertility. The ovaries and the ovulation rate were normal, but mating experiments demonstrated that litter size from CD9 deficient females lower than the rates of WT and heterozygous mice.

Therefore, *in vitro* fertilization (IVF) experiments were performed in order to elucidate in which step of fertilization the failure occurred. The ZP was penetrated by WT sperm, but they were not able to fuse with the oolemma and form pronuclei. IVF using $CD9^{-/-}$ ZP-free oocytes was then performed, observing the same pattern as in conventional IVF. Fusion rates of 98% for $CD9^{+/+}$ ZP-free oocytes and 4% for $CD9^{-/-}$ ZP-free oocytes were obtained when evaluated 1 h after insemination. When intracytoplasmic sperm injection (ICSI) was accomplished, similar implantation and embryo development rates were obtained in WT and $CD9^{-/-}$ mice. All these results confirmed the implication of CD9 in sperm-egg interaction (Miyado et al. 2000).

Some studies suggested that CD9 might have a structural role in the oocyte. This protein is enriched on the oocyte microvillar membrane generating fusion competent sites for fertilization (Jegou et al. 2011) and it is important for a normal shape and distribution of the membrane microvilli (Runge et al. 2007).

The oocytes express more tetraspanins in addition to CD9. For instance CD81 has been described in mouse and human oocytes (Takahashi et al. 2001), and CD151 is also present in human eggs (Ziyyat et al. 2006). CD81 deficient female mice ($CD81^{-/-}$) presented a 40% reduction in fertility and IVF assays suggested that this phenotype is due to a failure in sperm-egg fusion. CD9 and CD81 might play a complementary role in

gamete fusion because double knockout animals ($CD9^{-/-}$ $CD81^{-/-}$) were completely infertile (Rubinstein et al. 2006; Ohnami et al. 2012).

Presence of CD9 and CD81 has been recently described also in the sperm head, over the acrosome and in the plasma membrane respectively, suggesting once more, the critical role of these proteins in sperm-egg fusion (Frolikova et al. 2018).

2.2.1.2 JUNO

In 2014, a glycosylphosphatidylinositol (GPI)-anchored protein was identified in the oocyte as essential for fertilization (Bianchi et al. 2014). This protein encoded by the *Folr4* gene was known as Folate receptor 4 because of its putative function in folate uptake. Its expression on $CD4^{+}CD25^{+}$ regulatory T-lymphocytes in mice, its use in anti-tumor therapy and its function as a mediator of responses to dietary folate was widely studied, but its presence and function in the oocytes was unknown until 2014. In this study, it was demonstrated that Folr4 was unable to bind folate but it was essential for fertilization. Therefore, they renamed this protein to “Juno”, in honor to the fertility and marriage Roman goddess.

JUNO was discovered to be involved in gamete recognition when a screening to find the binding partner of a sperm protein described in 2005, IZUMO1 (Inoue et al. 2005), was being carried out. It took nine years to find the IZUMO1 partner in the oocyte, probably because of the transient nature of this interaction, and to date it is the only pair of proteins described that interact when the sperm meets the egg.

Direct interaction between JUNO (in the egg) and IZUMO1 (in the sperm) was showed using the AVEXIS assay (Bushell et al. 2008), designed to identify low affinity interactions

between extracellular proteins. This assay also helped to confirm whether this interaction was conserved within mammals due to the existence of *Izumo1* and *Juno* orthologues in all sequenced mammalian genomes.

The essential role of JUNO in the interaction between the sperm membrane and the oolemma was assessed by IVF assays in mice (Bianchi et al. 2014). Firstly, when IVF was performed pre-incubating for 10 minutes zona-free eggs with an anti- JUNO antibody. The results showed no fertilization. Secondly, female JUNO deficient mice ($Juno^{-/-}$) were generated, being infertile. No differences were observed in the oocyte's morphology and in the number of ovulated oocytes when compared to the wild-type, but oocytes from $Juno^{-/-}$ mice were not fertilized after natural mating or IVF. Moreover, ZP-free-$Juno^{-/-}$ oocytes were also tested in IVF, obtaining the same result. All these experiments confirmed the involvement of JUNO in sperm-egg binding. A schematic representation of this process can be observed in Figure 7.

Another interesting observation from Bianchi and Wright's study (2014) was that JUNO seems to be involved in the block of polyspermy occurring after fertilization by disappearing from the oolemma within 40 min post sperm penetration (Bianchi et al. 2014).

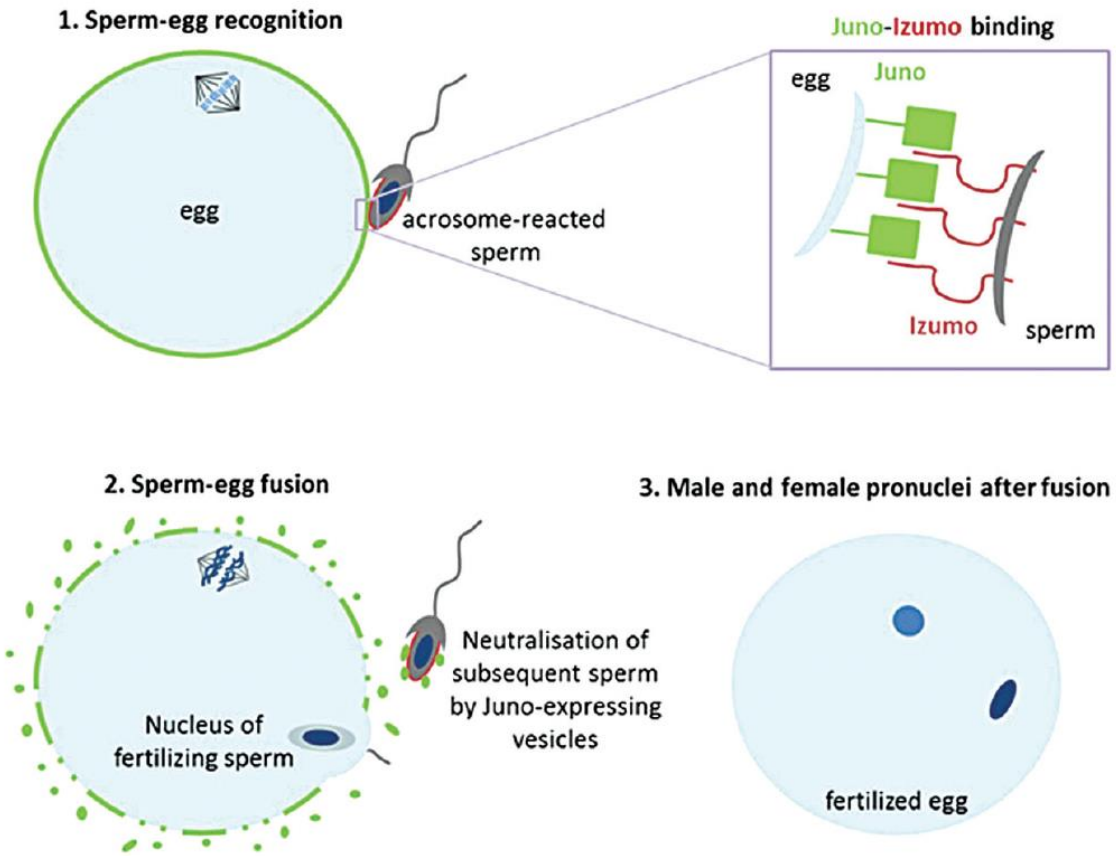


Figure 7. *Schematic representation of the fertilization where IZUMO1-JUNO interaction takes place. Taken from Bianchi and Wright 2014.*

Two years later of JUNO's identification as the IZUMO1 binding partner, several research groups revealed the tertiary structure of JUNO and JUNO–IZUMO1 complex. Knowing these structures allowed the identification of the amino acids involved in this recognition, suggesting that W148, H157 and R160 of IZUMO1, and W62 and L81 of JUNO were specifically the responsible for sperm-oocyte plasma membrane interaction (Aydin et al. 2016; Ohto et al. 2016; Han et al. 2016; Kato et al. 2016; Inoue 2017).

In a tentative to know the precise time when JUNO starts to be expressed during oocyte maturation, experiments in mice were performed. The results showed that JUNO expression began when the oocytes gained the ability to fuse with the sperm (13-22 µm). Researchers found that JUNO was expressed at the germinal vesicle (GV) stage, at prophase I, and its expression continued at similar levels until metaphase II (MII) stage (Suzuki et al. 2017).

A recent study confirmed that JUNO is involved in human gamete recognition by the interaction with human IZUMO1 and that its expression progressively increases during oocyte maturation, starting at GV stage and significantly increasing at metaphase I (MI) and MII stages (Jean et al. 2019).

2.2.1.3. BOUNCER

In 2018, a (GPI)-anchored protein was identified as crucial for fertilization in zebrafish. This protein is a member of the Ly6/urokinase-type plasminogen activator receptor (Ly6/uPAR) superfamily. To study the function of this protein, a *bouncer* knockout (*bncr*$^{-/-}$) zebrafish was established. I*n vivo* mating showed problems in obtaining embryos. Furthermore, egg activation in *bncr*$^{-/-}$ oocytes was normal and IVF results showed no penetration when using these eggs. Moreover, ICSI was performed and the embryo development was restored. The authors concluded that BOUNCER protein which is localized in the egg membrane and in vesicles within the egg, is essential for sperm-egg binding in zebrafish (Herberg et al. 2018). Sequence analyses showed that the sperm protein SPACA4 is the closest homolog in mammalian species. Additionally, *in vitro* assays suggested that SPACA4 is involved in gamete interaction, but it function in vivo is still unknown (Shetty et al. 2003).

2.2.2. Sperm

2.2.2.1 IZUMO

In 2005, an immunoglobulin superfamily protein was identified in the sperm as essential for gamete interaction and fusion (Inoue et al. 2005). Authors of the study renamed the protein "IZUMO" as a Japanese marriage shrine. IZUMO was only detectable in acrosome reacted sperm, suggesting that its function might be related with gamete fusion. To demonstrate IZUMO's function, deficient IZUMO mice were generated (Izumo$^{-/-}$) showing a sterile phenotype although their mating behavior was normal. Then, IVF experiments showed fertilization failure, the sperm was able to penetrate the ZP but remained in the perivitelline space. IVF using ZP-free eggs confirmed that spermatozoon were not able to fuse with oocytes despite bounding to the plasma membrane. ICSI was performed to clarify whether the defect was produced by a fusion failure or extended to later developmental stages. The embryos developed correctly, confirming once more the role of IZUMO in gamete fusion (Inoue et al. 2005). In addition to the immunoglobulin-like domain, IZUMO1 presented a conserved glycosylation site, which has been proven to prevent its degradation, but it is unnecessary to its function (Inoue et al. 2008).

As described earlier, sperm must suffer the acrosome reaction in order to be able to fertilize the egg. During this exocytosis event, IZUMO1 is relocated from the acrosomal membrane to the equatorial segment of the sperm surface (Satouh et al. 2012). Tssk6, a kinase of the Tssk (Testis Specific Kinase) family seems to be involved in this relocation of IZUMO1. Male Tssk6 deficient mice are sterile, confirming the importance of the correct relocation of IZUMO1 after acrosome reaction (Sosnik et al. 2009).

Nonetheless, JUNO can bind to monomeric IZUMO1 in the sperm. Once this contact has occurred, IZUMO1 N-terminal bends into the interior of the molecule forming a dimer. Dimeric IZUMO1 loses the ability to interact with JUNO. The authors of this study hypothesized that dimeric IZUMO1 interacts with a putative oocyte receptor, which leads to the fusion of the membranes (Inoue et al. 2015).

The discovery of the proteins involved in gamete recognition and fusion is essential to the development of new contraceptive methods. Some researchers have investigated the use of IZUMO1 as a vaccine to induce contraception in female mice (Xue et al. 2016). It has been described the presence of anti-IZUMO1 antibodies in infertile female patients (Clark et al. 2013; Naz 2014) supporting the importance of the study of these proteins as well as its putative application as contraceptives.

2.2.2.2. Transmembrane protein 95 (TMEM95)

A genome-wide association study, after analyzing the results of 15.3 million artificial inseminations (AI) in a population of Fleckvieh bulls detected a nonsense mutation in the gene encoding the transmembrane protein 95 (TMEM95). Forty homozygous bulls for this mutation exhibited poor fertility rates after mating and only 1.7% out of a total of 35.671 AI performed resulted in successful pregnancies (Pausch et al. 2014). Despite the sperm concentration, morphology, viability and motility of these ejaculates were normal, the parameters assessed after IVF showed a fertilization failure. Blastocyst rate at day 8 was lower in the mt/mt group than in the wt/mt or wt/wt groups. These results along with the reduce ability of mt/mt sperm for penetrating ZP-free oocytes but the normal development rates when ICSI was performed suggested that this protein is involved in gamete recognition in cattle (Fernandez-Fuertes et al. 2017).

2.2.2.3. Phosphatidylserine (PtdSer)

A lipid on cell membranes named phosphatidylserine (PtdSer) has been considered a marker of dead or non-viable sperm until now. It has been recently demonstrated that PtdSer might be essential for successful fertilization (Rival et al. 2019). Although a genetic ablation of the gene encoding this molecule is not possible because of its implication in normal cellular functions, this recent study masking or competitively interfering with sperm PtdSer and combinatorial targeting multiple PtdSer receptors on oocytes suggest a role of these molecules on sperm entry into oocytes. The authors also convey that PtdSer and its receptors in the eggs are functional players that may work together with IZUMO1 and JUNO to promote sperm-oocyte fusion during fertilization.

2.3. Gamete recognition in porcine and bovine species

During this doctoral thesis new strategies have been developed to study gamete interaction in porcine and bovine species, therefore, a deepen review about how the egg and the sperm recognize and interact in these species is made in this section.

Porcine and bovine species' ZP are composed by 3 glycoproteins (ZP2, ZP3 and ZP4) (Hedrick and Wardrip 1987; Noguchi et al. 1994). As occurred in the murine model, some studies tried to elucidate the role of these proteins in sperm-ZP interaction, suggesting that the heterocomplex ZP3/ZP4 is the responsible of the sperm binding activity of ZP in these species. Specifically, the carbohydrate structures in ZP4 in pigs (Yonezawa et al. 2012) and the N-glycosylation in the flexible hinge region of ZP3 in cows (Suzuki et al. 2015) have been proposed as the regions involved in sperm binding.

Regarding to CD9, its expression in the oocyte plasma membrane and its role in sperm-oocyte binding and fusion have been described in porcine (Li et al. 2004) and bovine species (Zhou et al. 2009). A recent study suggests that CD9 would be important in transzonal projections (TZP). The researchers showed different expression patterns of CD9 and CD81 in porcine and bovine ZP, hypothesizing a new species-specific role of these proteins associated with intercellular communication and cell adhesion during fertilization (Jankovicova et al. 2019).

As for IZUMO1, it has been also described both in porcine (Tanihara et al. 2014) and bovine spermatozoa (Kim 2015). Tahinara et al. (2014) reported that porcine ZP induces the AR and this process results in IZUMO1 exposure in boar spermatozoa in order to complete fertilization. As it occurs in mice, this IZUMO1 translocation after AR has also been described in bull spermatozoa species (Fukuda et al. 2016).

3. Systems to study gamete interaction in mammals

Research in the field of reproductive biology has several limitations. For instance, gamete retrieval and its use for research purposes have bioethical implications and is not allowed nor possible in all species. The use of human gametes or embryos in research is forbidden in most countries and the projects must overcome strict evaluations by ethical committees. Moreover, gamete or embryo studies in some other species imply the sacrifice or surgical intervention. The European directive 2010/63/UE (https://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF) refers to the principle of Replacement, Reduction and Refinement (3R) by which the use of animals for research purposes should be substituted when possible, using the

minimum number of animals, minimizing the pain and the angst as well as improving the well-being of the animals used. Furthermore, in endangered species such as the white rhinoceros, the availability of gametes is scarce (Hildebrandt et al. 2018).

Specifically, the study of gametes interaction is specially challenging due to its transient nature. Therefore, new strategies as the ones proposed in this Doctoral Thesis should be developed to study this dynamic process in depth in order to overcome these limitations. Moreover, the models here proposed could be very valuable because they are easily transferable between species, scalable and reproducible. The tools currently available for the study of this interaction can be divided into *in vivo* or *in vitro* models. In the next sections the most important models will be explained.

3.1. *In vivo* models

In the last 30 years, a revolution took place in the genetic engineering field, we are now able to generate gene-edited animals, allowing the study of target genes as well as the phenotype and health status of the mutant animals. These genes can be disrupted, losing its function, by stopping its expression, generating a truncated protein or suffering the deletion of the entire gene. Most of the experiments using transgenic animals are performed in mice (Le Naour et al. 2000; Inoue et al. 2005; Avella et al. 2014; Bianchi et al. 2014). This fact has provided new insights into mouse and human fertilization, but it is not always easy to transfer the obtained results to other species.

Since 2013, an outbreak discovery has revolutionized the scientific community, a new tool to edit the genome in a precise and efficient manner, considerably reducing the time and effort to obtain a transgenic organism or cell line. This tool is based in the CRISPR-Cas system (Clustered Regularly Interspaced Short Palindromic Repeats and

CRISPR-associated proteins (Cas)) (Jinek et al. 2012; Gasiunas et al. 2012). Since its description, several research groups are working not only in mice, but in other species such as pigs (Hai et al. 2014; Whitworth et al. 2014) to acquire knowledge in pathogenesis, treatment and prevention of a great number of diseases. With no doubt, CRISPR-Cas9 technique will allow the finding of new molecules involved in fertilization and will help to elucidate the molecular basis underlying this process.

3.2. *In vitro* models

To study the contribution of a single protein potentially involved in the steps of gamete recognition or fusion, biochemically-based *in vitro* models can be used. These models are usually based on the blocking potential of the sperm-egg interaction.

Sperm-ZP recognition has been studied by means of solubilized ZP, purified native ZP proteins and recombinant proteins. For instance, researchers showed that heat-solubilized ZP (Bhandari et al. 2010) and recombinant ZP glycoproteins (Caballero-Campo et al. 2006) induce the AR in capacitated human sperm. Native ZP glycoproteins purified by immunoaffinity columns were used to described the biological activity of each human ZP protein (Chiu et al. 2008).

Another widely used approach to study gamete interaction by blocking the protein of interest is the use of antibodies against specific epitopes. This technique has been employed to block sperm binding using antibodies anti-ZP proteins (Aitken and Richardson 1981) and to discover essential proteins involved in gamete recognition and fusion, such as IZUMO1 (Inoue et al. 2005).

3.2.1. Three-dimensional (3D) models

Nowadays, three-dimensional (3D) models are highly valuable tools to study the different steps of reproductive events, from ovarian follicular growth to gamete interaction and early embryo-maternal communication. Some advantages of these systems are the recreation *in vitro* of the *in vivo* microenvironment, keeping the levels of cell differentiation and tissue organization as well as the intercellular communication.

3D models have been developed to study the role of different molecules such as hormones or growth factors on follicle development and oocyte maturation (Brito et al. 2014). Efforts are being made to mimic the oviduct structure *in vitro* in order to recreate the environment were fertilization and early embryo-maternal communication occurs (Ferraz et al. 2017; Chen et al. 2017). Another applications of the 3D systems are the 3D microfluidic devices designed to select sperm before insemination (Matsuura et al. 2013), 3D scaffolds to restore the ovarian activity in mice (Laronda et al. 2017) or microfluidics platforms for phenocopying the menstrual cycle and pregnancy-like endocrine loops (Xiao et al. 2017).

Furthermore, a 3D model based on the ZP2 cleavage model of gamete recognition by terms of mouse ZP2 peptides attached to agarose beads has shown its ability to decoy sperm and prevent fertilization *in vitro* and *in vivo* (Avella et al. 2016).

4. Summary and perspective

Fertilization is a complex process and despite the years invested in elucidating the molecular basis; the available information is scarce. To date, it is known that a capacitated sperm and a mature oocyte should meet at the fertilization site in the oviduct during a short window of time to obtain a successful fertilization and therefore, a new and unique individual. Once the sperm meets the egg, it should overcome the *cumulus oophorus*, bind to the N-terminal of ZP2 to overpass the ZP and bind to JUNO in the oolemma to finally, fuse its exposed plasma membrane and enter the oocyte.

To fully understand this process, new strategies need to be created and more studies need to be done in other species other than mice. This knowledge will allow the development of new ARTs and the improvement of the existent ones. Moreover, to assess all the molecules involved in gamete recognition and fusion will allow the development of new non-hormonal contraceptives.

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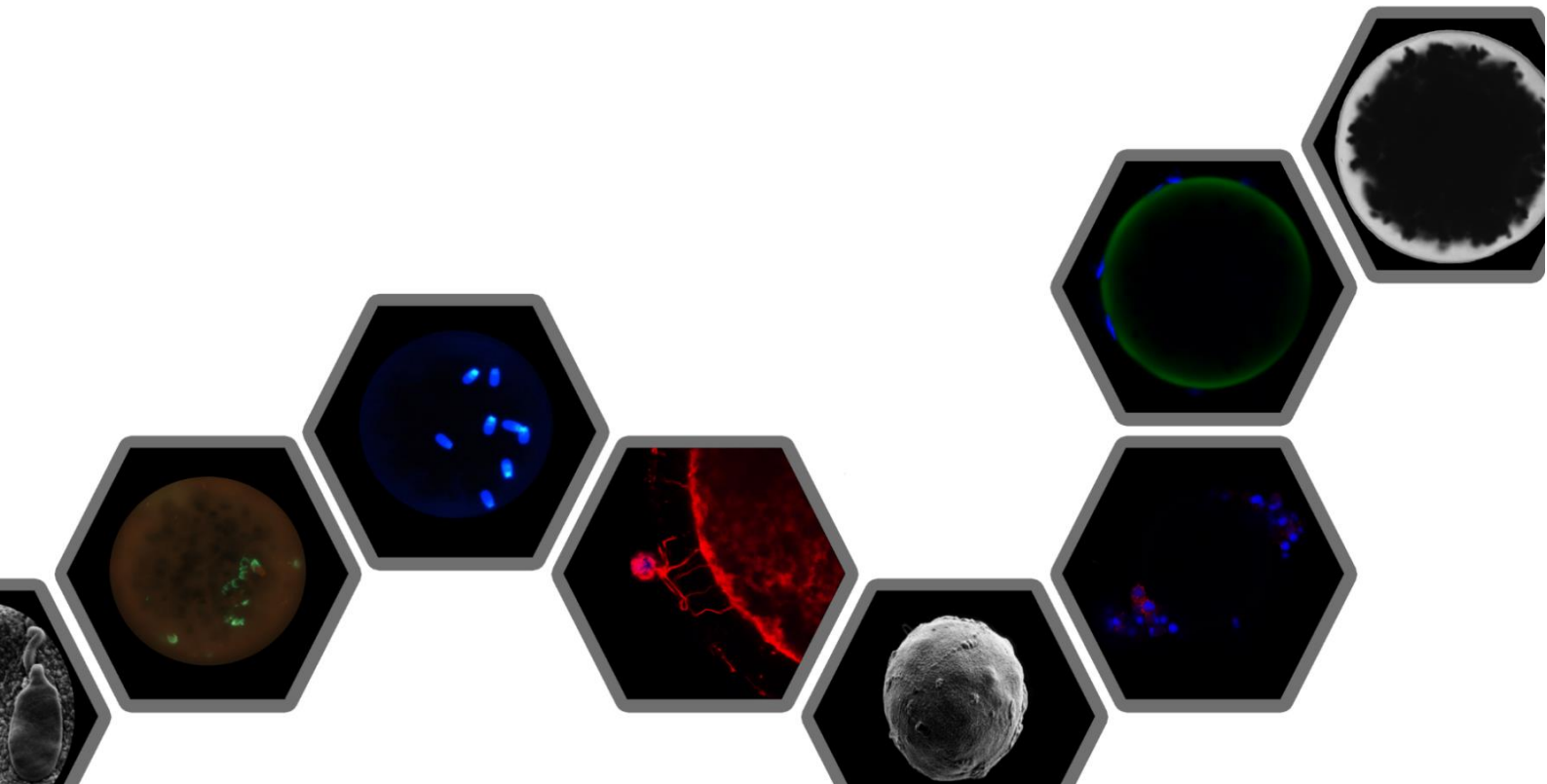
OBJECTIVES/OBJETIVOS



Hypothesis and Objectives

HYPOTHESIS

The main hypothesis of this Doctoral Thesis is that it is possible to generate three-dimensional models that mimic the shape and vestments (cellular and protein) of the female gamete, being used for studies of gamete interaction in different species and with interesting applications in assisted reproduction techniques (ART). For this, we hypothesize that to develop these models, magnetic sepharose beads could be conjugated to recombinant ZP glycoproteins and with cumulus cells adhered to its surface, as well as proteins from the oolemma such as JUNO. This model would be stable over time, allow the binding of sperm using different capacitation protocols and sperm from different sources, facilitating the study of proteins of interest in the fertilization process and could be applied to increase the output in *in vitro* fertilization and to predict the fertilizing capacity of a seminal sample.

OBJECTIVES

The main objective of this thesis is on one hand to develop and validate a new *in vitro* tool to study mammalian gamete interaction and, on the other hand, to look its likely applications in Assisted Reproduction Techniques (ART). In order to achieve this main objective, in chapter 1 the three-dimensional (3D) model consisting of sepharose beads coated with recombinant zona pellucida (ZP) glycoproteins is generated, described and validated in porcine species. In chapter 2, the specific role of each ZP glycoprotein (specifically ZP2, ZP3 and ZP4) is studied using the 3D model developed in chapter 1. Moreover, the role of B_{ZP2} model in increasing porcine *in vitro* fertilization (IVF) efficiency

is assessed. In chapter 3, the model is translated to bovine species using JUNO, a protein essential for gamete recognition. B_{JUNO} model is characterized and validated as a method to study sperm samples' fertilizing capacity.

The following specific objectives were stablished to accomplish the main objective:

CHAPTER 1

Objective 1. To generate, describe and validate the 3D model based on magnetic sepharose beads (B) coated with single porcine recombinant ZP glycoproteins (B_{ZP}).

Objective 1.1. Design and expression of ZP2 (C and N-terminus), ZP3 and ZP4 glycoproteins in mammalian cells.

Objective 1.2. Conjugation of the recombinant ZP glycoproteins to the magnetic sepharose beads and the over time stability of the conjugation.

Objective 1.3. Description and validation of the model by studying:

1.3.1. Its ability to support adhesion of porcine *cumulus oophorus* cells obtained from *in vitro* matured cumulus-oocyte complexes.

1.3.2. Its potential to bind porcine spermatozoa.

1.3.3. Its ability to induce acrosome exocytosis.

1.3.4. Its response to sperm binding when different capacitation protocols are used.

CHAPTER 2

Objective 2. To study the specific role of each porcine ZP glycoprotein using the 3D model developed in chapter 1 and its potential application in porcine IVF system.

Objective 2.1. To evaluate the ability of the three models (B_{ZP2}, B_{ZP3} y B_{ZP4}) to decoy sperm.

Objective 2.2. To assess the sperm acrosome reaction and the ability of the three models to trigger this event.

Objective 2.3. To observe whether the cumulus cells adhere and interact with the three models.

Objective 2.4. To analyze the effect of B_{ZP2} model on porcine IVF efficiency.

CHAPTER 3

Objective 3. To develop and validate the model in bovine species and to study its potential application as a fertility predictor *in vitro*.

Objective 3.1. To design and express recombinant bovine JUNO (rbJUNO) glycoprotein in mammalian cells.

Objective 3.2. To conjugate rbJUNO to magnetic sepharose beads (B_{JUNO}) and to study the over time stability of the model.

Objective 3.3. To validate B_{JUNO} by its ability to bind bull spermatozoa.

Objective 3.4. To analyze the ability of epididymal and ejaculated sperm to interact with the B_{JUNO} model.

Objective 3.5. To study the response of the model when incubated with frozen-thawed ejaculated semen from bulls with different fertilizing capacity.

Hipótesis y objetivos

HIPÓTESIS

La hipótesis principal de este trabajo es que se pueden generar modelos tridimensionales que imiten la forma y las envolturas (celular y proteica) del gameto femenino, siendo empleado para estudios de interacción entre gametos en distintas especies y con aplicaciones interesantes en distintas técnicas de reproducción asistida (TRA). Para ello, hipotetizamos que es posible utilizar como soporte microesferas conjugadas a proteínas recombinantes de la zona pelúcida y con células del cumulus adheridas a su superficie, así como proteínas del oolema como JUNO. Este modelo sería estable en el tiempo, permitiría la unión de espermatozoides capacitados de distinta manera y de distinto origen, facilitaría el estudio de proteínas de interés en el proceso de fecundación y podría ser aplicado para aumentar la eficiencia de la fecundación *in vitro* y predecir la capacidad fecundante de una muestra seminal.

OBJETIVOS

El objetivo principal de esta tesis es el desarrollo y la validación de una nueva herramienta *in vitro* que permita, por un lado, ahondar en el estudio de la interacción entre gametos y, por otro, buscar posibles aplicaciones del modelo en técnicas de reproducción asistida. Para alcanzar este objetivo, en el Capítulo 1, el modelo tridimensional (3D) es generado, descrito y validado en la especie porcina. En el Capítulo 2, el rol específico de cada glicoproteína de la ZP (específicamente ZP2, ZP3 y ZP4) es estudiado usando el modelo 3D desarrollado en el capítulo 1. Además, se evalúa el papel del modelo B_{ZP2} en el aumento de la eficiencia de la fecundación *in vitro* porcina. En el

Capítulo 3, el modelo es trasladado a la especie bovina utilizando JUNO, una proteína esencial para el reconocimiento entre gametos. El modelo BJUNO se caracteriza y valida como un método para estudiar la capacidad fecundante de muestras seminales.

Para alcanzar el objetivo principal se establecieron los siguientes objetivos específicos:

CAPÍTULO 1

Objetivo 1. Generación, descripción y validación del modelo 3D basado en la conjugación de las proteínas recombinantes de la zona pelúcida (ZP) porcina de manera individual a microesferas magnéticas de sefarosa (B_{ZP}).

Objetivo 1.1. Diseño y expresión de las glicoproteínas ZP2 (C y N-terminal), ZP3 y ZP4 en células de mamífero.

Objetivo 1.2. Conjugación de las proteínas recombinantes de la ZP a las microesferas magnéticas de sefarosa y estudio de la estabilidad de la unión.

Objetivo 1.3. Descripción y validación del modelo estudiando:

1.3.1. Su capacidad de adhesión de células del *cumulus oophorus* maduradas *in vitro*.

1.3.2. Su potencial de unir espermatozoides porcinos.

1.3.3. Su competencia para inducir la reacción acrosómica.

1.3.4. Si estas interacciones se ven afectadas según el protocolo utilizado para capacitar los espermatozoides.

CAPÍTULO 2

Objetivo 2. Estudiar el rol específico de cada glicoproteína de la ZP en la especie porcina utilizando el modelo desarrollado en el capítulo 1 y su potencial aplicación en el sistema de fecundación *in vitro* (FIV) en cerdo.

Objetivo 2.1. Evaluación de la habilidad de los tres modelos (B_{ZP2}, B_{ZP3} y B_{ZP4}) para unir espermatozoides.

Objetivo 2.2. Evaluación de la reacción acrosómica y la habilidad de los tres modelos de inducirla.

Objetivo 2.3. Descripción de la interacción de las células del *cumulus oophorus* con los tres modelos.

Objetivo 2.4. Estudio del efecto del modelo B_{ZP2} en la eficiencia del sistema de FIV porcina.

CAPÍTULO 3

Objetivo 3. Desarrollar y validar el modelo en la especie bovina y estudiar su aplicación potencial como predictor de la fertilidad *in vitro*.

Objetivo 3.1. Diseño y expresión de la glicoproteína recombinante bovina JUNO (rbJUNO) en células de mamífero.

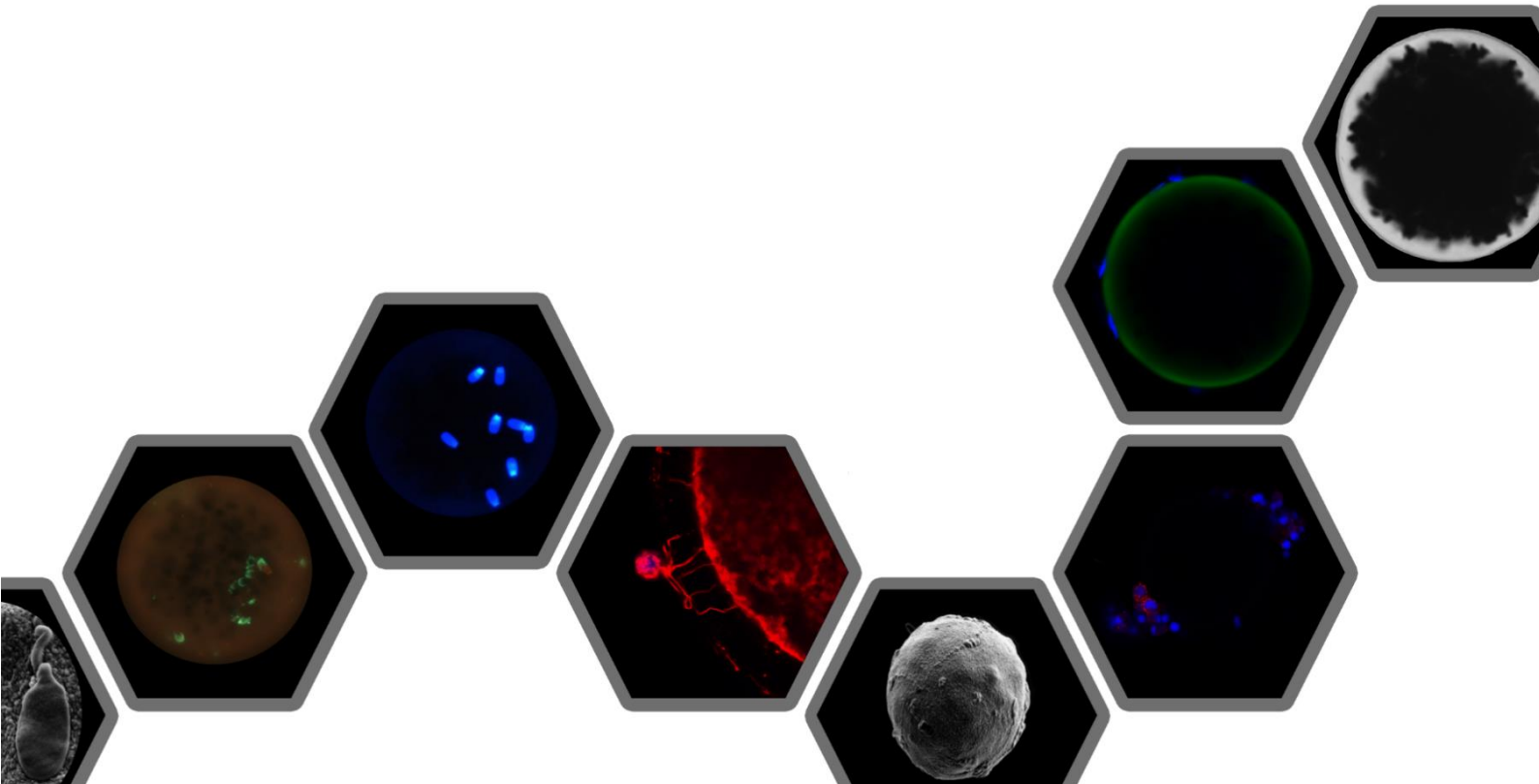
Objetivo 3.2. Conjugación de rbJUNO a las microesferas magnéticas de sefarosa (B_{JUNO}) y estudio de la estabilidad de la unión.

Objetivo 3.3. Evaluación del potencial de B_{JUNO} para unir espermatozoides bovinos.

Objetivo 3.4. Análisis del comportamiento del modelo empleando espermatozoides de distinto origen (epididimarios vs. eyaculados).

Objetivo 3.5. Descripción del comportamiento del modelo cuando se incuba con semen eyaculado congelado-descongelado procedente de toros con diferente capacidad fecundante.

CHAPTERS



CAPÍTULO 1

Los espermatozoides de mamíferos y las células del cúmulo se unen a un modelo 3D basado en microesferas conjugadas a proteínas recombinantes de la zona pelúcida

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RESUMEN

El ovocito es una célula esférica encapsulada por la zona pelúcida (ZP) que forma una matriz filamentosa compuesta por distintas glicoproteínas que median el reconocimiento entre gametos durante la fecundación. Los estudios sobre los mecanismos moleculares de la unión entre el ovocito y el espermatozoide están limitados en muchas especies de mamíferos por la escasez de ovocitos, por las preocupaciones éticas que surgen por la manipulación de este tipo de células y por el alto coste que supone la producción de animales genéticamente modificados. Para abordar estas limitaciones, hemos reproducido un modelo tridimensional (3D) que imita la forma del ovocito, basado en microesferas magnéticas de sefarosa conjugadas a glicoproteínas recombinantes de la ZP (B_{ZP}) y células del cúmulo. Se obtuvieron tres preparaciones diferentes, compuestas por ZP2 (C y N-terminal; B_{ZP2}), ZP3 (B_{ZP3}) o ZP4 (B_{ZP4}) y los modelos fueron caracterizados por SDS-PAGE, inmunotransferencia e imágenes obtenidas mediante microscopía confocal y electrónica. La funcionalidad del modelo fue validada por la adhesión de células del cúmulo, la habilidad de las microesferas recubiertas de glicoproteínas de unir espermatozoides e inducir la reacción acrosómica. Por lo tanto, nuestros hallazgos documentan que las microesferas conjugadas a las proteínas recombinantes de la ZP proporcionan una herramienta 3D novedosa para investigar el papel de proteínas específicas en la interacción entre

gametos, convirtiéndose en una herramienta relevante como método para predecir la funcionalidad espermática, una vez transferida a la industria.

1. Introducción

La reproducción sexual es utilizada por diferentes organismos e implica la fusión de dos células haploides altamente especializadas, el ovocito y el espermatozoide. Para asegurar la fecundación, los espermatozoides primero deben cruzar las células del cúmulo que rodean el ovocito y luego, contactar, reconocer y penetrar la capa extracelular del ovocito (zona pelúcida, ZP) para fusionarse con la membrana plasmática. El reconocimiento inicial de espermatozoides por la ZP es un paso esencial para la fecundación y la eliminación bioquímica de la ZP o su ausencia en ratones editados genéticamente causa infertilidad (Bronson and McLaren 1970; Rankin et al. 1996; Rankin et al. 2001). A pesar de la crucial importancia de la fecundación, nuestro conocimiento sobre la base molecular de esta interacción es limitado en humanos y ratones (Gupta et al. 2012; Avella, Xiong, and Dean 2013), y prácticamente inexistente en otras especies de mamíferos. Las limitaciones al obtener y manipular gametos suponen un gran desafío para obtener resultados concluyentes al estudiar moléculas implicadas en la unión de los espermatozoides y la ZP. Por ejemplo, en algunas especies de mamíferos no es factible obtener espermatozoides capacitados y ovocitos maduros debido a preocupaciones éticas (humanos) o a un número restringido de gametos (especies en peligro de extinción). También existen desafíos técnicos en el análisis y evaluación de las interacciones espermatozoides-ovocito debido a que es un proceso dinámico que involucra múltiples macromoléculas. En los últimos años, los sistemas tridimensionales (3D) se han convertido en una herramienta valiosa para estudiar y mejorar nuestra comprensión de las diferentes etapas de la reproducción. (Brito et al. 2014; Ferraz et al. 2017). Un sistema 3D que recree los ovocitos en su forma esférica y

las características bioquímicas de la ZP sería una herramienta útil para investigar problemas no resueltos relacionados con la fecundación *in vitro*. (Raquel Romar, Funahashi, and Coy 2016).

La ZP de mamíferos está compuesta por tres o cuatro glicoproteínas designadas como ZP1, ZP2, ZP3 y ZP4. En ratones, la ZP contiene ZP1, ZP2 y ZP3. ZP4 está codificada por un pseudogén que no expresa la proteína (Goudet et al. 2008). Aunque la ZP en la especie porcina (Hedrick and Wardrip 1987), bovina (Noguchi et al. 1994) y perros (Goudet et al. 2008) está también compuesta por 3 proteinas, la ZP de estas especies no contienen ZP1 (en su lugar, contienen ZP4), siendo un pseudogén en estas especies (Goudet et al. 2008). Las ZP de otras especies de mamíferos incluyendo ratas (Hoodbhoy et al. 2005), hámster (Izquierdo-Rico et al. 2009), macaco coronado (Ganguly, Sharma, and Gupta 2008) y humanos (Lefievre et al. 2004) contienen las cuatro glicoproteínas. Dependiendo de la especie de mamífero, cada glicoproteína de la ZP se ha propuesto como ligando para el espermatozoide (Bleil and Wassarman 1980; Ganguly et al. 2010; Yonezawa et al. 2012; Suzuki et al. 2015; Avella, Baibakov, and Dean 2014). Por ejemplo, en ratones y humanos, los espermatozoides se unen al extremo N-terminal de la ZP2 (Avella, Baibakov, and Dean 2014; Baibakov et al. 2012) mientras en la especie porcina o bovina, ZP3 y/o ZP4 se han visto implicadas en la interacción ovocito-espermatozoide (Yonezawa et al. 2012; Suzuki et al. 2015). Esto sugiere que el papel individual de las glicoproteínas de la ZP durante la fecundación puede diferir entre los mamíferos y debe investigarse de forma independiente en lugar de extrapolar los hallazgos de una especie a otra. Dichas investigaciones se verían facilitadas por modelos *in vitro* que incorporen glicoproteínas recombinantes de la ZP de orden específico para la validación de las

interacciones espermatozoide-ZP en diferentes mamíferos. La contribución de las proteínas individuales de la ZP al reconocimiento entre gametos se ha estudiado bioquímicamente basándose en el bloqueo de posibles interacciones espermatozoides-ZP con ZP solubilizada (Bhandari et al. 2010; Franken et al. 1996; Tesarik et al. 1993), proteínas nativas purificadas de la ZP (Chiu et al. 2008) y proteínas recombinantes de la ZP (Caballero-Campo et al. 2006; van Duin et al. 1994; Yonezawa et al. 2005). Además, los anticuerpos dirigidos contra epítopos específicos, se han utilizado para evaluar la candidatura de proteínas de la ZP de manera individual en el reconocimiento entre gametos (Aitken and Richardson 1981). En los últimos años, la facilidad para establecer ratones editados genéticamente, ha abierto la posibilidad de estudiar el papel de las glicoproteínas de la ZP *in vivo*, lo que ha proporcionado nuevas ideas sobre la fecundación de ratones y humanos. (Avella, Baibakov, and Dean 2014). Basado en el modelo de escisión de la ZP2 de reconocimiento entre gametos, se ha demostrado que microesferas de agarosa conjugadas al extremo N-terminal de la ZP2 pueden unir espermatozoides y prevenir la fecundación *in vitro* e *in vivo* (Baibakov et al. 2012). Estas mismas microesferas conjugadas al péptido de la ZP2 se pueden usar para seleccionar espermatozoides humanos que parecen tener una capacidad mejorada para unirse y penetrar la ZP en la que la ZP2 humana reemplaza a la ZP2 de ratón endógena y puede resultar útil si se utilizan en las técnicas de reproducción asistida (Aitken and Richardson 1981). Aunque el uso de animales transgénicos dobles y triples puede servir como modelos para el estudio del reconocimiento entre gametos, el establecimiento de tales animales requiere mucho tiempo, es costoso y se limita principalmente a ratones, que pueden no ser el mejor modelo para otros mamíferos. Por lo tanto, existe un considerable interés en desarrollar nuevas estrategias para decodificar el

reconocimiento ovocito-espermatozoide (Okabe 2013; Chiu et al. 2014; Wright and Bianchi 2016), lo que avanzará nuestra conocimiento sobre fecundación *in vitro* y mejorará la reproducción humana asistida y la producción ganadera. En este estudio, se propone y valida un nuevo modelo *in vitro*. El modelo se basa en microesferas magnéticas de sefarosa conjugadas a glicoproteínas recombinantes de la ZP (B_{ZP}) que imitan la forma del ovocito. Las glicoproteínas recombinantes porcinas ZP2 (C y N-terminal), ZP3 y ZP4 se expresaron con etiquetas peptídicas para permitir su identificación y conjugación a microesferas magnéticas de sefarosa. Se estudiaron microesferas con glicoproteínas de la ZP de manera individual: 1) por su capacidad para soportar la adhesión de células del cúmulo porcinas maduras *in vitro*; 2) su potencial para unir espermatozoides; 3) su capacidad para inducir la reacción acrosómica; y 4) determinar si estas interacciones se vieron afectadas por el protocolo utilizado para la capacitación de los espermatozoides. En resumen, este sistema recrea un entorno 3D de ovocitos ovulados que es escalable y ofrecerá información sobre los mecanismos moleculares del reconocimiento entre gametos en mamíferos.

2. Materiales y métodos

A menos que se indique lo contrario, todos los productos químicos se compraron a Sigma-Aldrich Química (Madrid, España). El estudio se realizó en estricta conformidad con las recomendaciones de los Principios Rectores para el Cuidado y Uso de Animales (Publicación DHEW, NIH, 80–23). El protocolo fue aprobado por el Comité Ético para la Experimentación con Animales de la Universidad de Murcia, España (Código del proyecto: AGL2015-70159-P y AGL2015–66341 - R).

2.1. Diseño y expresión de las glicoproteínas recombinantes de la zona pelúcida (ZP) porcina.

Los plásmidos de expresión (pcDNA3.1 [+]) se diseñaron y construyeron para codificar glicoproteínas de la ZP porcina (ZP2: UniProt P42099; ZP3: UniProt P42098; y ZP4: UniProt Q07287) (GeneArt, Life Technology, Thermo Fisher Scientific). Se agregó una etiqueta de histidina (6x) al extremo N de la ZP2N (38-43 aa), ZP3 (41-46 aa) y ZP4 (22-27 aa), y al extremo C de ZP2C (644-649 aa). Además, se agregó una etiqueta FLAG a ZP2C (38-45 aa) y ZP2N (642-649 aa); se agregó una etiqueta HA a ZP3 (313-321 aa); y una etiqueta V5 a ZP4 (469-482 aa). Después de la verificación por secuencia de ADN, los plásmidos de expresión ZP2C, ZP2N, ZP3 y ZP4 se amplificaron usando Library Efficiency DH5α Competent cells (Thermo Fisher Scientific) y se purificaron con el kit GenEluted Plasmid. Se cultivaron células de ovario de hámster chino (células CHO, ECACC, The European Collection of Authenticated Cell Cultures) (37 ° C, 5% de CO2 y 95% de humedad) durante 48-72 h 80-90% de confluencia utilizando medio F-12 (Biowest, Nuaillé, Francia) suplementado con suero bovino fetal al 10% (Biowest, Nuaillé, Francia) y penicilina-estreptomicina 100 U/ml (GibcoBRL-Life Technologies, Gaithersburg, EE. UU.). Las transfecciones transitorias se realizaron con X-tremeGene HP (Roche Applied Science, Indianápolis, EE. UU.) De acuerdo con el protocolo del fabricante. Para cada transfección, se agregaron 4 µL de reactivo de transfección X-tremeGene HP a un volumen final de 200 µL de medio de suero reducido Opti-MEM (Gibco-Invitrogen) predisuelto con 2 µg de plásmido e incubado durante 15 minutos a temperatura ambiente (RT). El complejo se diluyó mediante la adición de 2 ml de Opti-MEM y se superpuso en células en crecimiento (37 °C, 5% de CO2 y 95% de humedad). El medio que contenía las glicoproteínas secretadas se recogió después de 48 h, se

centrifugó (3.939 xg, 5 min, 4 ºC) para eliminar los restos celulares y se concentró en Vivaspin Turbo 4 de 10.000 Da (Sartorius), obteniendo un volumen final de 200-300 µL de glicoproteínas concentradas en tampón fosfato de sodio 20 mM, pH 7,4.

2.2. Conjugación de las glicoproteínas recombinantes a las microesferas magnéticas

Las microesferas magnéticas de sefarosa (His Mag Sepharose Excel GE Healthcare) se manejaron con una gradilla magnética (MagRack 6, GE Healthcare). Las microesferas magnéticas de sefarosa se resuspendieron homogéneamente y se pipetearon 10 µL de de microesferas en suspensión en un tubo de microcentrífuga que contenía 500 µL de tampón de fosfato de sodio 20 mM, pH 7,4. Las microesferas se lavaron con 500 µL de tampón de lavado (fosfato de sodio 20 mM, NaCl 0,5 M, imidazol 10 mM, pH 7,4) y finalmente con 500 µL de tampón de unión (fosfato de sodio 20 mM, NaCl 0,5 M, Tween-20 al 0,1% pH 7,4). Las glicoproteínas recombinantes de la ZP concentradas (ZP2C, ZP2N, ZP3 y ZP4) se incubaron con microesferas magnéticas lavadas (1: 1 v / v) durante la noche a 4 ºC con agitación orbital. Después de la incubación, las microesferas recubiertas con glicoproteínas (B_{ZP2C}, B_{ZP2N}, B_{ZP3} and B_{ZP4}) se lavaron dos veces con tampón de fosfato de sodio 20 mM (pH 7,4) para eliminar las proteínas no conjugadas, se resuspendieron en tampón de fosfato de sodio 20 mM y se almacenaron a 4 ºC hasta su uso. Como control, las perlas se incubaron con medios de células CHO no transfectadas procesadas como si contuvieran proteínas recombinantes (B_{Ctrl}). Las glicoproteínas recombinantes de la ZP se eluyeron agregando 100 µL de tampón de elución (fosfato de sodio 20 mM, NaCl 0,5 M, imidazol 500 mM, pH 7,4) y se incubaron a 4 ºC y 750 g en un termo-mezclador durante 1 h. Las concentraciones de glicoproteína ZP2C, ZP3 y ZP4 se estimaron por colorimetría de Bradford en el Servicio de Biología

Molecular (SACE, Universidad de Murcia, España). ZP2C, ZP3 y ZP4 se separaron por SDS-PAGE y las bandas correspondientes a cada glicoproteína se aislaron y procesaron para análisis proteómico en el Servicio de Biología Molecular (SACE, Universidad de Murcia, España). La identidad de la proteína se determinó mediante espectrometría de masas, realizada mediante el uso de un sistema HPLC/MS, en el que un HPLC Agilent Serie 1100 (Agilent Technologies, Santa Clara, CA, EE. UU.) está conectado a un espectrómetro de masas Agilent Ion Trap XCT Plus (Agilent Technologies, Santa Clara, CA, EE. UU.) Utilizando un electrospray (ESI). El procesamiento de datos se realizó mediante el programa de análisis de datos para LC/MSD Trap Versión 3.3 (Bruker Daltonik, GmbH, Alemania) y Spectrum Mill MS Proteomics Workbench (Rev A.03.02.060B, Agilent Technologies, Santa Clara, California, EE. UU.).

2.3. Western blot

El medio de cultivo celular que contiene glicoproteínas concentradas se separó por SDS-PAGE y se transfirió a membranas de PVDF que se probaron con anticuerpos: anti-Flag para ZP2 (1: 1000 v / v en TBST 1x, 1% BSA); anti-ZP3 amablemente donado por el Dr. Hedrick (Berger et al. 1989), para ZP3 (1:2000 v/v in TBST 1x, 1% BSA); y V5 Epitope Tag Antibody para ZP4 (1:2000 v/v in TBST 1x, 1% BSA)(Thermo Fisher Scientific) antes de la visualización por quimioluminiscencia (Pierce ECL-Plus, Thermo Fisher Scientific).

B_{ZP} fueron analizados por SDS-PAGE para verificar que las glicoproteínas recombinantes se unieran con éxito a las microesferas de sefarosa. Para estudiar la estabilidad de la conjugación, después del almacenamiento (4 °C, tampón de fosfato de sodio 20 mM, pH 7,4) durante 0, 24, 48, 72 y 144 h, las B_{ZP} se lavaron dos veces con tampón de fosfato de sodio 20 mM, pH 7,4, se resuspendieron en tampón de fosfato de sodio 20 mM, pH 7,4 y se solubilizaron en condiciones reductoras en tampón de muestra SDS 4X (Millipore,

EE. UU.). Después de 10 minutos a 100 ° C, el sobrenadante se separó por SDS-PAGE, se transfirió a membranas de PVDF y se probaron con los anticuerpos mencionados anteriormente.

2.4. Microscopía confocal

Tras la conjugación, las B_{ZP} se incubaron (1 h, RT) con anticuerpos diluidos en PBS libre de calcio y magnesio suplementado con BSA al 1%. Las diluciones de anticuerpos fueron: anti-FLAG para ZP2 (1: 200 v/v); anti-ZP3 para ZP3 (1: 200 v/v); y V5 Epitope Tag Antibody para ZP4 (1: 200 v / v). Las B_{ZP} luego se incubaron (1 h, RT) con anticuerpos secundarios (conjugado anti-conejo IgG-FITC (1: 100 v/v) para ZP2 y ZP4; Alexa Fluor 488 Donkey IgG anti-cabra (1: 200 v/v) para ZP3), lavado en PBS y fijado con paraformaldehído al 2% (Electron Microscopy Sciences, Hatfield, Filadelfia, EE. UU.). Las B_{ZP} teñidas se colocaron en un portaobjetos con cámara (cavidad de 25 µl) con Gene Frame (Advanced Biotechnologies, Leatherhead, Reino Unido) y se cubrieron con un cubreobjetos. Las muestras se analizaron con un microscopio confocal Leica TCS SP8 y el análisis de imagen se realizó con el software de análisis de software LAS X Core (Leica Microsystems, España). FITC y Alexa Fluor 488 se excitaron a 488 nm.

2.5. Incubación de microesferas conjugadas a proteínas de la ZP con células del cúmulo aisladas y maduradas *in vitro*

Para recrear un modelo 3D *in vitro* que se asemeja a un complejo cúmulo-ovocito, los modelos B_{ZP2C}, B_{ZP3} y B_{ZP4} se incubaron con células de cúmulo obtenidas de complejos cúmulo-ovocitos porcinos madurados *in vitro* (COCs) (Romar, Coy, and Rath 2012). COCs se aislaron de ovarios obtenidos de animales de 6-7 meses sacrificados en un matadero. Después de la maduración *in vitro*, los COC se decumularon en PBS-1% BSA mediante pipeteo. Se descartaron los ovocitos y las células del cúmulo se centrifugaron dos veces

(1.200 × g, 10 min) en medio TALP modificado (Rath et al. 1999). Las B_{ZP} (50–55) se incubaron (24 h, 38,5 °C, 5% de CO2, 20% de O2 y humedad saturada) con 2.500 células del cúmulo aisladas/B_{ZP} (Campos et al. 2001) en 500 µl TALP. La adhesión de las células del cúmulo se confirmó microscópicamente observando la formación de aglomerados de células del cúmulo alrededor de las microesferas recubiertas (CB_{ZP}). Las CB_{ZP} se pipetearon entonces suavemente tres veces en PBS suplementado con PVA al 0.1% para eliminar las células del cúmulo ligeramente adheridas, se fijaron con glutaraldehído al 0.5% (v/v), se tiñeron con Hoechst 33342 0.01 mM, se montaron en cubreobjetos y se observaron con un microscopio de epifluorescencia a 40X. Se realizaron tres replicados doble ciego para determinar el número de células de cúmulo que permanecen unidas a la superficie de las microesferas.

2.6. Preparación de espermatozoides

Los espermatozoides eyaculados de verracos que demostraron ser fértiles (Landrace × Largewhite, 12–24 meses de edad; CEFU, SA, Pliego, Murcia, España) del Centro de Inseminación Artificial (AIC) se diluyeron (1: 5 v/v) en Beltsville Thawing Solution (BTS). Para validar la unión de los espermatozoides a las B_{ZP}, se prepararon muestras heteroespérmicas ya sea por doble centrifugación (Matas et al. 2010) o swim-up (Canovas et al. 2017). Para la doble centrifugación, se centrifugaron muestras de espermatozoides diluidas (300 × g, 3 min), se recogió el sobrenadante y se centrifugó nuevamente (800 × g, 5 min). Se desechó el sobrenadante y se resuspendió el sedimento de espermatozoides en medio TALP modificado (Rath et al. 1999) suplementado con piruvato de sodio 1 mM, BSA al 0,6% y gentamicina 50 µg/ml. Para el protocolo swim-up, se colocó 1 ml de muestra de semen diluido debajo de 1 ml de medio swim-up de espermatozoides NaturARTs PIG (EmbryoCloud, Murcia, España) en el fondo de un tubo

cónico. Después de 20 minutos a 37 ° C (con el tubo en un ángulo de 45 °), se obtuvieron 800 µl de la parte superior del tubo. Las concentraciones finales de espermatozoides se ajustaron a 200,000 espermatozoides/ml con medio TALP modificado suplementado.

2.7. Microscopía electrónica de barrido

Las B_{ZP} se incubaron conjuntamente (2 h) con espermatozoides doblemente centrifugados (glutaraldehído al 1% en PBS (v/v), 30 min), se lavaron tres veces en agua de biología molecular y se transfirieron a un cubreobjetos. Las muestras fueron procesadas por la Unidad General de Microscopía (Universidad de Alicante, España). Finalmente, las perlas se observaron bajo un microscopio electrónico JEOL JSM 840 SEM (Jeol Limited, Londres, Reino Unido). Se registraron la forma y las características de la superficie de las B_{ZP}, así como la integridad del acrosoma de los espermatozoides unidos.

2.8. Ensayo de unión de B_{ZP}-espermatozoides

Grupos de B_{ZP} (50–55) conjugadas con ZP2C, ZP3, ZP4 se lavaron dos veces en medio TALP y se incubaron conjuntamente (38,5 °C, 5% de CO2, 20% de O2 y humedad saturada) con espermatozoides doblemente centrifugados (200,000 espermatozoides/ml) en 500 µl de medio. Además, B_{ZP2C} se inseminó con espermatozoides preparados por swim-up (200,000 espermatozoides/ml) para validar B_{ZP2C} como un modelo adecuado para el reconocimiento de espermatozoides-ZP independientemente del método de preparación de los espermatozoides. Para evaluar el número de espermatozoides unidos y la integridad de sus acrosomas después de 0,5, 1, 2 y 20 h de incubación, se lavó una alícuota de B_{ZP2C} tres veces en PBS, suplementado con PVA al 0,1%, se fijaron con glutaraldehído al 0,5% en PBS (v/v), y se tiñeron con 0,01 mM Hoechst 33342, 4 µg/ml de aglutinina de cacahuete conjugada con isotiocianato de fluoresceína (PNA-FITC) y 20 µg / ml de yoduro de propidio (PI). Las B_{ZP} se montaron en

portaobjetos, y el número de espermatozoides unidos y el estado del acrosoma (espermatozoides reaccionados en verde), se registraron bajo una microscopía de epifluorescencia (Leica, DMLS, Barcelona, España). Se registró el porcentaje de microesferas con al menos un espermatozoide unido ($B_{ZP}SB$) y el número medio de espermatozoides por microesfera (S/B_{ZP}) y se evaluó la integridad del acrosoma. Los espermatozoides con acrosoma dañado y contenido acrosómico sobre la superficie de las B_{ZP} se observaron en verde. Se realizaron tres replicados.

2.9. Análisis estadístico

Los datos se presentan como media ± SEM y todos los porcentajes se modelaron de acuerdo con el modelo binomial de variables y la transformación de arcoseno para lograr una distribución normal. El porcentaje de microesferas con al menos un espermatozoide unido, el número de espermatozoides por microesfera, las microesferas con al menos una célula de cúmulo unida y el número medio de células de cúmulo por microesfera se analizaron mediante ANOVA unidireccional. Cuando ANOVA reveló un efecto significativo, los valores se compararon mediante la prueba de Tukey. Un valor $P < 0.05$ se consideró estadísticamente significativo. El análisis se realizó utilizando Systat v13.1 (Systat Software, Inc San José, CA, EE. UU.).

3. Resultados

3.1. Las proteínas recombinantes de la ZP son secretadas y se unen de manera estable y uniforme a las microesferas.

Los plásmidos de expresión que codifican las proteínas porcinas ZP2C, ZP2N, ZP3 y ZP4 (Figura 1.1) se expresaron en células de ovario de hámster chino (CHO) y las glicoproteínas secretadas se aislaron con éxito.

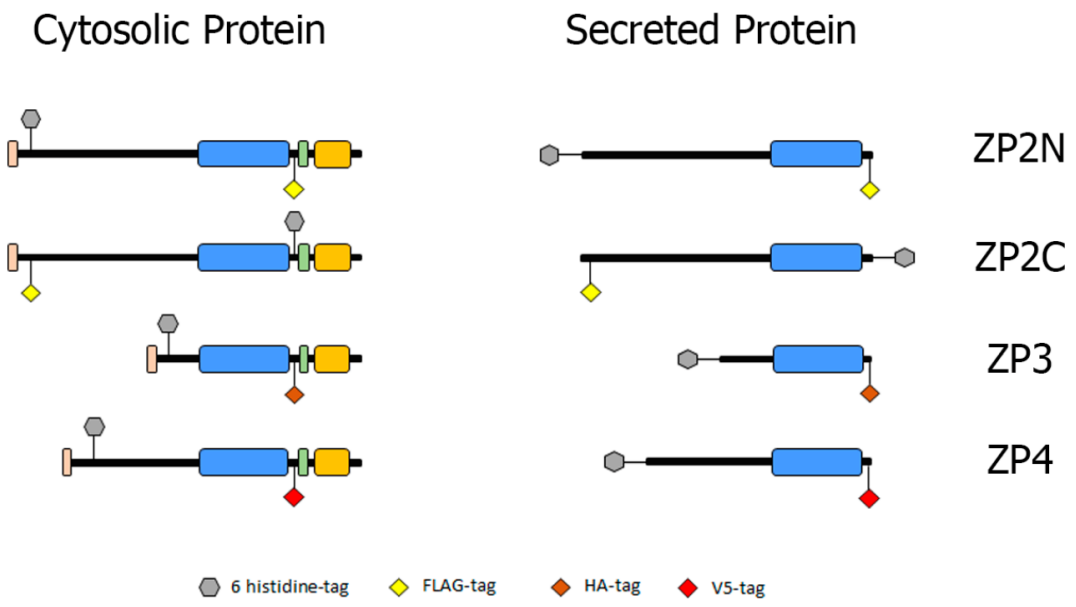

Figura 1.1. *Diseño de proteínas recombinantes de la ZP porcina. Representación esquemática de las glicoproteínas recombinantes de la ZP porcina ZP2N, ZP2C, ZP3 y ZP4. Péptido de señal (rosa), dominio ZP (azul), región de procesamiento (verde) y dominio transmembrana (naranja).*

Cada glicoproteína tuvo el peso molecular esperado (Hedrick and Wardrip 1987). Las glicoproteínas ZP2C y ZP2N mostraron un peso molecular de 100 kDa, ZP3 alcanzó 55 kDa y ZP4 fue de 65 kDa en membranas incubadas con anticuerpos anti-Flag (ZP2C y ZP2N), anti-ZP3 (ZP3) y anti-V5 (ZP4) (Figura 1.2).

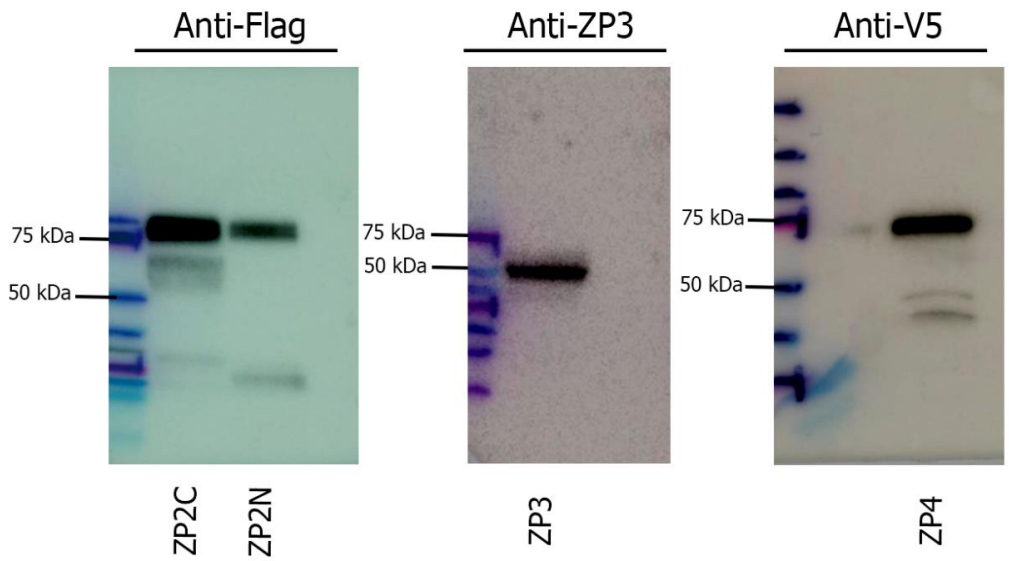

Figura 1.2. *Expresión de proteínas recombinantes de la ZP porcina. Las proteínas se expresaron en células CHO, se separaron por SDS-PAGE y se analizaron por transferencia a membranas de PDVF. Las proteínas ZP se probaron con anticuerpos anti-Flag para ZP2N, ZP2C, anti-ZP3 para ZP3 y el anticuerpo V5 Epitope Tag para ZP4. Marcadores de peso molecular, izquierda.*

Después de la incubación de microesferas con el secretado de células CHO transfectadas, las glicoproteínas recombinantes se conjugaron con éxito a las microesferas (Figura 1.3). La electroforesis y las transferencias confirmaron sus pesos moleculares esperados (100 kDa para B_{ZP2}, 55 kDa para B_{ZP3} y 65 kDa para B_{ZP4}) tanto en el medio secretado antes de la conjugación (línea 1) como después de la elución de las proteínas de las microesferas (línea 2). Las proteínas no se detectaron en el medio tras conjugar (línea 3) confirmando que toda la glicoproteína secretada en el medio CHO se unió con éxito a las microesferas.

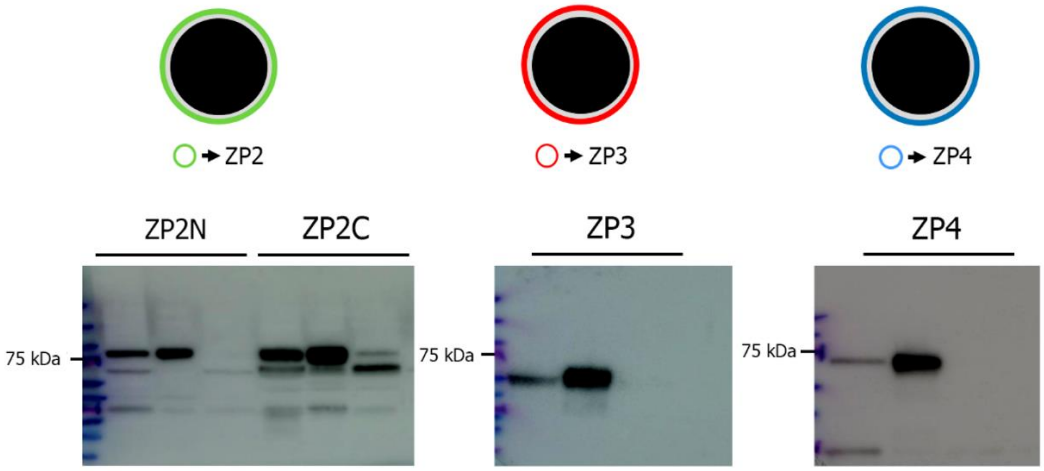

Figura 1.3. *Conjugación de proteínas recombinantes de la ZP a microesferas magnéticas de sefarosa. Representación esquemática de las microesferas recubiertas de proteínas recombinantes de la ZP (B_{ZP2}, B_{ZP3} y B_{ZP4}) (superior). SDS-PAGE y Western blot de proteínas ZP2C, ZP3 y ZP4 conjugadas con microesferas magnéticas (inferior). Medio con proteínas secretadas antes de la conjugación (carril 1), en la fracción eluida (carril 2) y medios después de la conjugación (carril 3). El anticuerpo anti-Flag se usó para ZP2, anti-ZP3 para ZP3 y el anticuerpo V5 Epitope Tag para ZP4.*

La electroforesis y las transferencias documentaron que la adherencia de la glicoproteína recombinante a las microesferas de sefarosa era estable después del almacenamiento durante 0, 24, 48, 72 y 144 h (Figura 1.4).

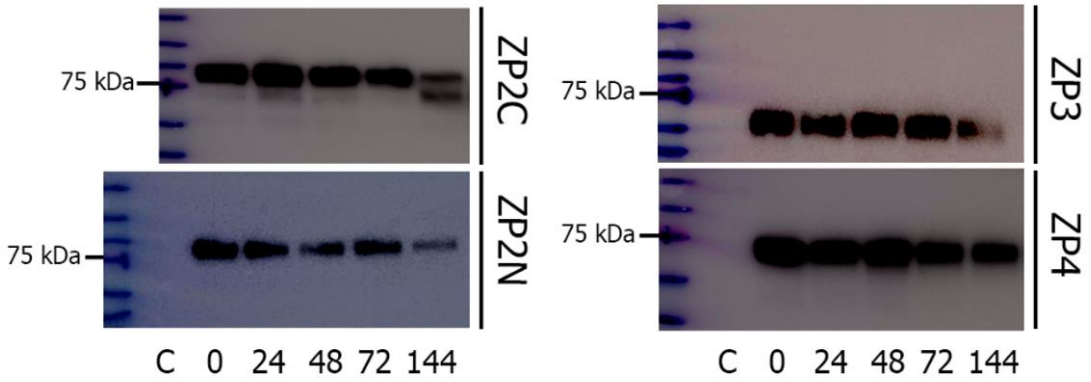

Figura 1.4. *SDS-PAGE y western blot de las proteínas ZP2N, ZP2C, ZP3 y ZP4 conjugadas con microesferas magnéticas tras de la conjugación y almacenamiento durante 0, 24, 48, 72 y 144 h. Calle C, microesferas incubadas con medio secretado de células CHO sin transfectar.*

Las glicoproteínas eluidas se analizaron por espectrometría de masas para confirmar sus identidades. Todos los péptidos validados por espectrometría de masas tenían una intensidad (SPI) superior al 70% y una puntuación superior a 5 (Appendix I). Las concentraciones de proteína tras eluir fueron 0.157, 0.125 y 0.172 µg/µl para ZP2C, ZP3 y ZP4, respectivamente. Se confirmó el recubrimiento y distribución de las glicoproteínas recombinantes de la ZP uniforme en la superficie de las microesferas mediante microscopía confocal inmunofluorescente y no se observó señal en las microesferas incubadas con medio de células CHO sin transfectar (B_{Ctrl}) (Figura 1.5). Una vez que se caracterizaron tanto ZP2C como ZP2N, en los ensayos posteriores se utilizó ZP2C debido a una mayor abundancia de ZP2C en relación con ZP2N.

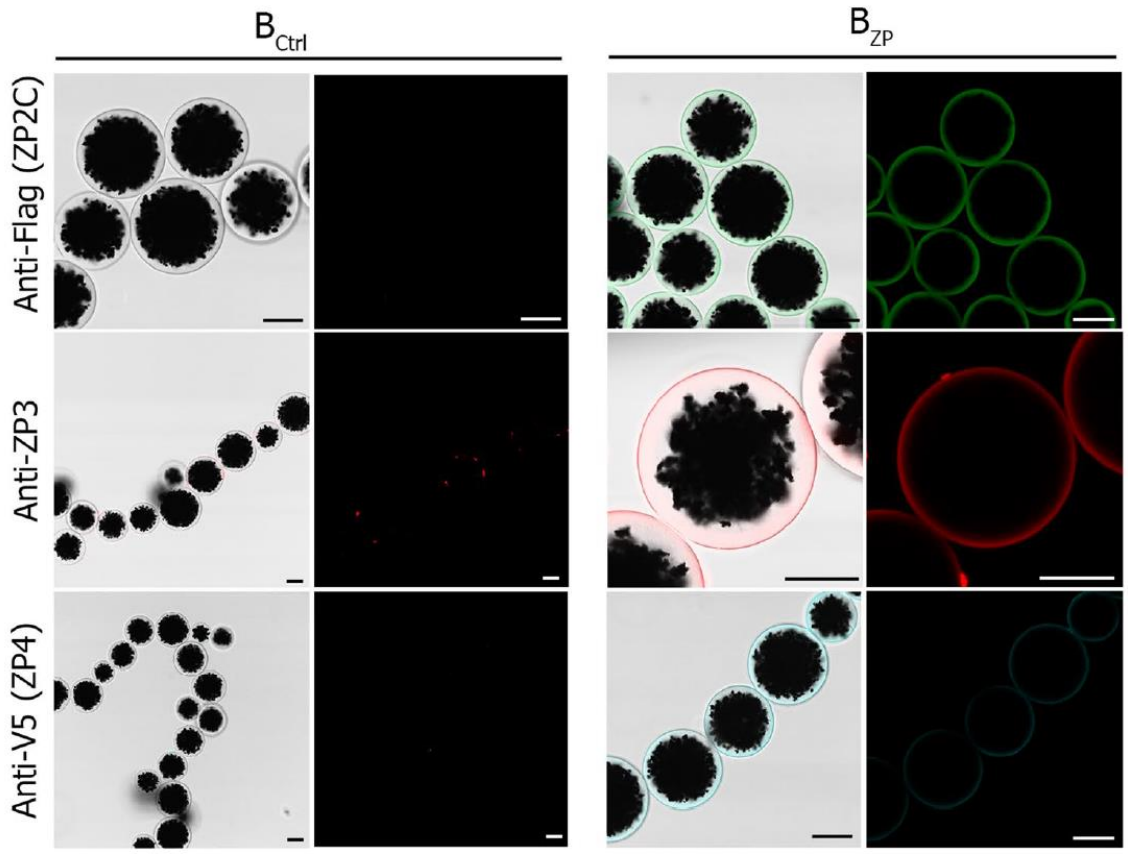

Figura 1.5. *Imágenes de microscopía confocal de microesferas sin proteína recombinante (B_{Ctrl}) (izquierda) y microesferas conjugadas (B_{ZP}) (derecha) que muestran un recubrimiento uniforme de microesferas con proteínas ZP. Escala, 40 µm.*

3.2. Las microesferas recubiertas con las proteínas de la ZP tienen una forma similar a un ovocito 3D que admite la unión de los espermatozoides.

Las glicoproteínas recombinantes de la ZP conjugadas a microesferas magnéticas de sefarosa (B_{ZP}) forman esferas 3D (~ 120 µm) que se aproximan al tamaño de los ovocitos nativos (Figura 1.6a). La microscopía electrónica de barrido proporciona imágenes de la superficie de las microesferas mostrando una superficie rugosa que se aproxima a la ZP de los ovocitos nativos (Figura 1.6a). La forma esférica de las B_{ZP} es una ventaja sobre las superficies planas porque permite el análisis del contacto y la unión de los

espermatozoides en tres dimensiones cuando se obtienen imágenes mediante microscopía electrónica estereoscópica, óptica y de barrido (Figura 1.6b, c). Además, al unirse, la energía cinética de los espermatozoides resultó en la deformación de la superficie proteica de la B_{ZP} (Figura 1.6b). Los ensayos de unión de espermatozoides-B_{ZP} se realizaron y analizaron para evaluar la actividad espermática. La capacidad de los espermatozoides porcinos eyaculados para unirse a B_{ZP2}, B_{ZP3} y B_{ZP4} varió entre las proteínas de la ZP a lo largo del tiempo (Figura 1.6c). El número de B_{ZP} con al menos un espermatozoide unido (B_{ZP}SB) aumentó con el tiempo y después de 60 minutos, todas las preparaciones tenían más del 85% de las microesferas con al menos un espermatozoide unido.

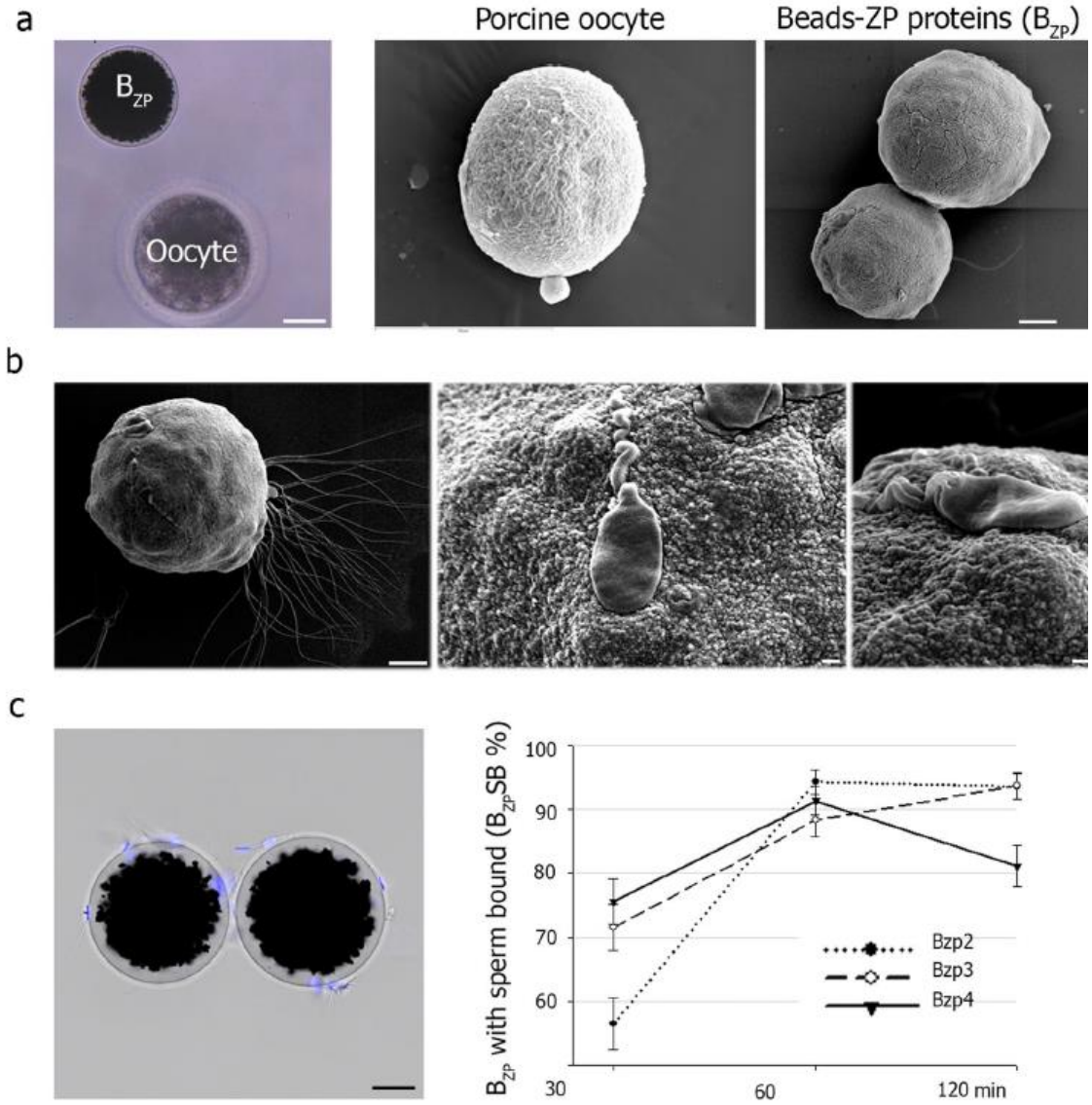

Figura 1.6. *Caracterización de la unión de espermatozoides-B_{ZP}. (a) Microesfera magnética conjugada con proteínas de la ZP (B_{ZP}) y ovocito porcino denudado nativo observado por estereomicroscopía. Escala, 30 µm (izquierda). Ovocito porcino denudado y microesferas conjugadas con proteínas ZP (B_{ZP}) observadas bajo microscopio electrónico de barrido. La estructura típica de la ZP en forma de esponja se observa en el ovocito (centro) y la superficie de las microesferas de proteína-sefarosa (derecha). Escala, 10 µm. (b) Microscopía electrónica de barrido de microesferas conjugadas a las glicoproteínas recombinantes de la ZP (B_{ZP})*

después de la incubación con espermatozoides porcinos. Escala, 10 µm (izquierda). Mayor aumento de la unión de los espermatozoides que documenta pequeñas deformaciones después de la interacción con la superficie de una B_{ZP} (centro, derecha). Escala, 1 µm. (c) Microesferas recubiertas de ZP (B_{ZP}) con espermatozoides porcinos unidos después de la incubación con 200.000 espermatozoides/ml y observadas bajo estereomicroscopio (el ADN de espermatozoides en azul tras teñirse con bisbencimida; Escala, 30 µm) (izquierda). Después de la incubación durante 30, 60 y 120 minutos, se fijaron las B_{ZP} (glutaraldehído al 0,5%), se tiñeron (bisbencimida 0,01 mM) y se evaluaron con un microscopio de epifluorescencia (derecha). Se registró el porcentaje de B_{ZP} con al menos un espermatozoide unido (B_{ZP}SB) en cada momento.

3.3. Las microesferas conjugadas a las proteínas recombinantes de la ZP recapitulan el tamaño y la forma de los complejos cúmulo-ovocitos (COC) porcinos nativos.

Después de 24 h de incubación, las células del cúmulo maduradas *in vitro* se adhirieron a la superficie de las B_{ZP}. Esto recreó el tamaño y la forma de los complejos cúmulo-ovocitos porcinos nativos (COC) en los que las células del cúmulo y las glicoproteínas de la ZP forman dos capas externas al ovocito (Figura 1.7).

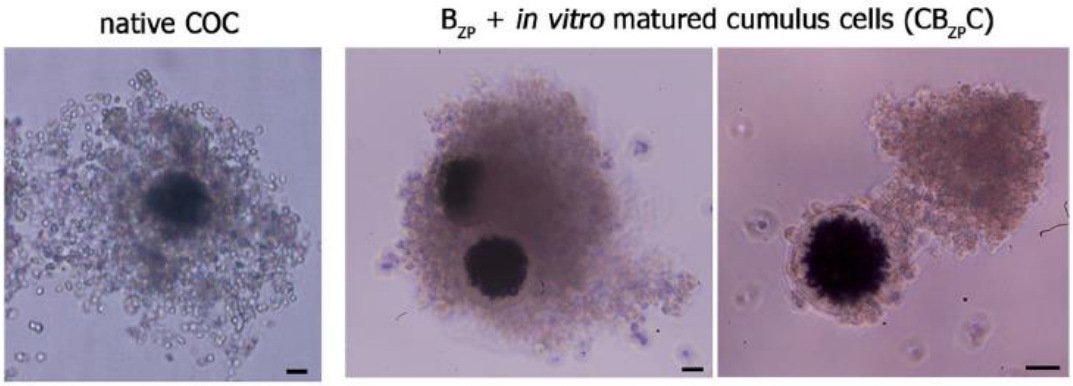

Figura 1.7. *Imitando el complejo cúmulo-ovocito (CB_{ZP}). Complejo cúmulo-ovocito nativo (COC) expandido después de la maduración in vitro. Escala, 30 µm (izquierda). Microesferas recubiertas con las glicoproteínas recombinantes de la ZP después de 24 h de incubación con células de cúmulo aisladas obtenidas de COC madurados in vitro, complejo de microesferas recubiertas de células del cúmulo-ZP (CB_{ZP}) Escala, 30 µm. Las células se adhieren a la superficie de las esferas, asemejándose a un COC nativo con dos capas externas, células del cúmulo y proteínas ZP (centro, derecha).*

El complejo formado entre las células del cúmulo y las B_{ZP} (CBZP) persistió después de lavar mecánicamente (Figura 1.8) y no se observaron diferencias en la adherencia entre las tres glicoproteínas recombinantes de la ZP. En todos los casos, más del 95% de B_{ZP} tenía al menos una célula del cúmulo unida después del lavado (datos no mostrados). Curiosamente, las microesferas utilizadas como control (sin proteínas de la ZP) en su superficie (microesferas incubadas con medio secretado por células CHO; B_{Ctrl}) mostraron un número menor de células del cúmulo por microesfera (5.99 ± 0.45, n = 201) que B_{ZP3} (8.92 ± 0.52, n = 212), B_{ZP4} (10.01 ± 0.49, n = 199) (P <0.001) y B_{ZP2} (8.79 ±

0.54, n = 195). No se encontraron diferencias entre las tres glicoproteínas recombinantes de la ZP (Figura 1.8).

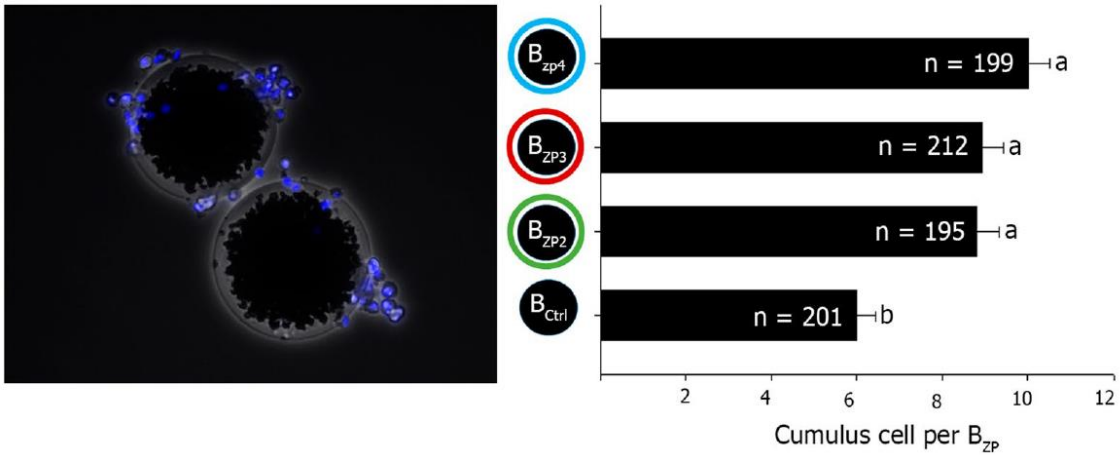

Figura 1.8. *Cuantificación del número de células adheridas a las microesferas recubiertas de ZP (B_{ZP}) teñidas con bisbencimida 0,01 mM y lavadas mecánicamente (media ± SEM). No se observó diferencia en el número de células adheridas entre las tres proteínas de la ZP unidas a las microesferas de sefarosa (B_{ZP2}, B_{ZP3}, B_{ZP4}). Sin embargo, las microesferas control sin proteínas de la ZP tenían menos células unidas. Diferentes letras (a, b) indican diferencias significativas entre grupos (P <0.001).*

3.4. Integridad acrosomal de los espermatozoides unidos a microesferas conjugadas con glicoproteínas recombinantes de la ZP

El estado acrosomal de los espermatozoides unidos a las microesferas se evaluó mediante tinción con PNA-FITC y se confirmó a nivel ultraestructural mediante microscopía electrónica de barrido. El estado del acrosoma se registró para el 93% de

los espermatozoides unidos a los 30 minutos, el 85% a los 60 minutos y el 79% a los 120 minutos (Figura 1.9).

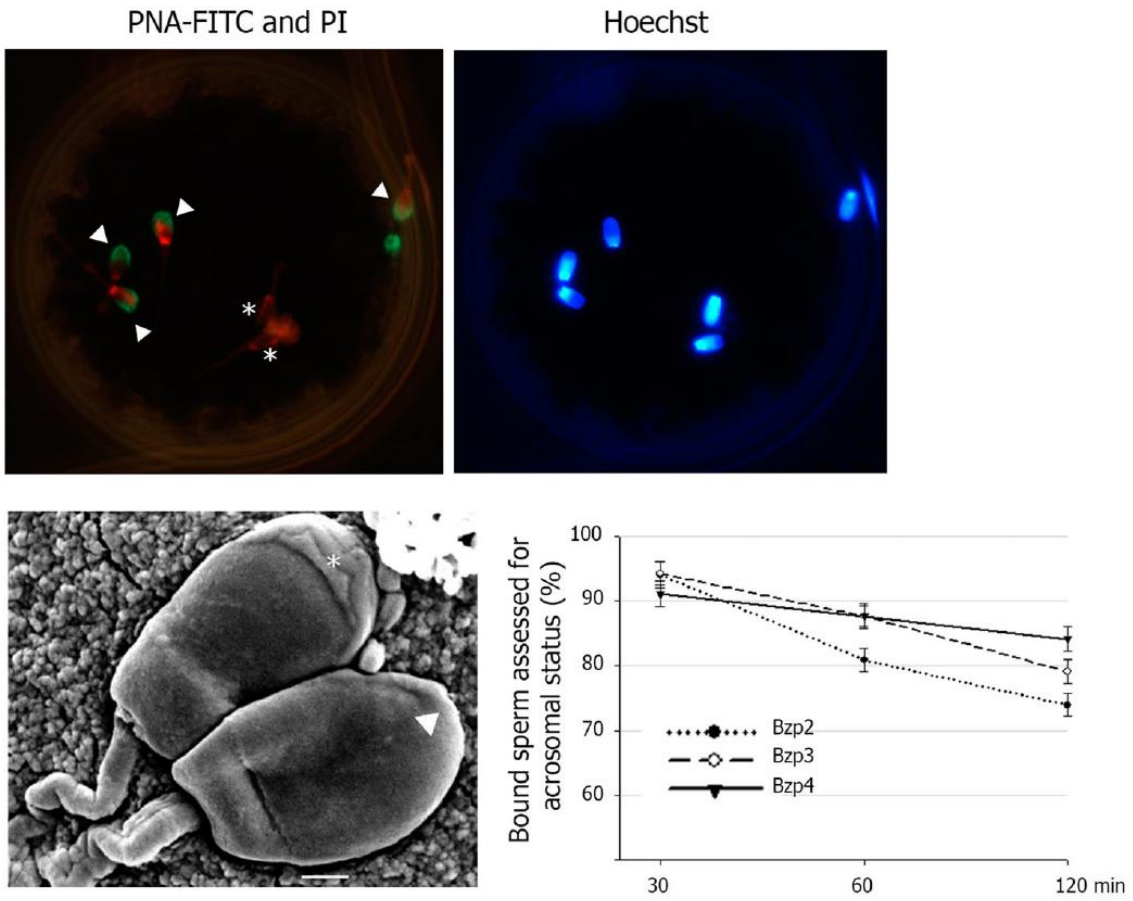

Figura 1.9. *Estado del acrosoma de los espermatozoides unidos a las microesferas (B_{ZP}). B_{ZP} co-incubadas con espermatozoides se fijaron (glutaraldehído al 0,5%) y se tiñeron con 4 µg/ml de PNA-FITC (aglutinina de cacahuete conjugada con isotiocianato de fluoresceína), 20 µg/ml de IP (yoduro de propidio) y bisbenzimida 0,01 mM. Se observaron espermatozoides con acrosoma reaccionado (Δ) y sin reaccionar (*) en la superficie de las microesferas. Imágenes tomadas por microscopía de epifluorescencia (arriba) y microscopía electrónica de barrido (abajo a la izquierda). Escala, 1 µm Porcentaje de espermatozoides unidos a las B_{ZP} evaluado para el estado del acrosoma a lo largo del tiempo de incubación (abajo a la derecha).*

Se observó exocitosis en la superficie de los tres modelos (B_{ZP2}, B_{ZP3}, B_{ZP4}) y la exocitosis aumentó con el tiempo. Cuarenta y cinco por ciento de las B_{ZP} mostraron restos acrosomales en su superficie después de 30 minutos de incubación, 70% de las B_{ZP} a los 60 minutos y más del 80% de las B_{ZP} a los 120 minutos (Figura 1.10).

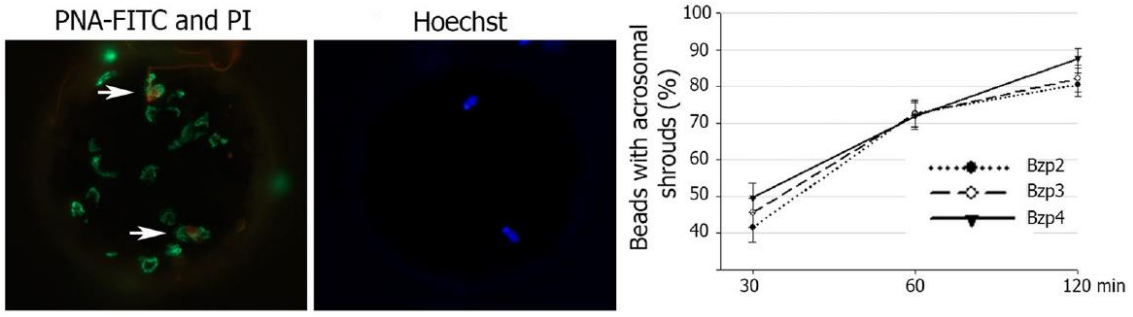

Figura 1.10. *Restos acrosomales en la superficie de las B_{ZP}. Las microesferas se fijaron y se tiñeron como en (Figura 1.9). Se observaron restos acrosomales (verde) y cabezas de espermatozoides (flecha) mediante microscopía de epifluorescencia (izquierda, centro). El número de B_{ZP} con restos acrosomales en la superficie aumentó con el tiempo (P <0.05) (derecha).*

3.5. Unión de espermatozoides a las microesferas conjugadas a las glicoproteínas recombinantes de la ZP según el método de capacitación.

Las B_{ZP2C} se incubaron durante 0,5, 1, 2 y 20 h con espermatozoides capacitados por centrifugación doble o swim-up y el número de microesferas con al menos un espermatozoide unido (B_{ZP2}SB) y el número de espermatozoides unidos por microesfera (S/B_{ZP2}) se evaluó (Figura 11). Con cualquiera de los dos métodos de capacitación, el B_{ZP2}SB aumentó de manera constante durante las primeras 2 h de incubación y luego disminuyó después de 20 h debido al desprendimiento de los espermatozoides de las

microesferas (Figura 11 panel izquierdo). El perfil temporal de S/B_{ZP2} fue similar a $B_{ZP2}SB$. No se encontraron diferencias en el número de S/B_{ZP2} entre los protocolos de capacitación de espermatozoides a las 0,5 h, pero se observó un mayor S/B_{ZP2} tras la capacitación con doble centrifugación a 1 h (13.21 ± 1.11 n = 245 vs. 10.04 ± 0.68 n = 253, P = 0.015), 2 h (40.68 ± 1.93 n = 278 vs. 17.28 ± 0.98 n = 268, P <0.001) y 20 h (27.02 ± 2.14 n = 204 vs. 10.42 ± 0.74 n = 234, P <0.001) (Figura 11 panel derecho).

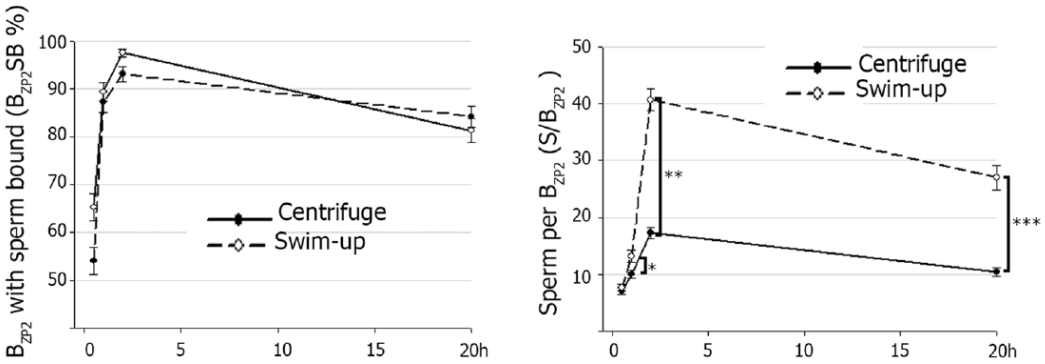

Figura 1.11. *Unión de los espermatozoides a las microesferas (B_{ZP2}) tras la capacitación utilizando dos métodos diferentes. Se incubaron las B_{ZP2C} durante 0,5, 1, 2 y 20 h con 200.000 espermatozoides porcinos/ml capacitados por doble centrifugación o swim-up. Después de la incubación, las B_{ZP2C} se fijaron (glutaraldehído al 0,5%), se tiñeron (bisbencimida 0,01 mM) y se evaluaron con un microscopio de epifluorescencia para calcular el porcentaje de B_{ZP2} con al menos un espermatozoide unido ($B_{ZP2}SB$) (izquierda) y evaluar el número de espermatozoides unido por microesfera (S/B_{ZP2}) (derecha). Se observó un mayor número de espermatozoides unidos a B_{ZP2} después de la capacitación con doble centrifugación a 1 h (* P = 0.015), 2 h (** P <0.001) y 20 h (*** P <0.001).*

4. Discusión

La base molecular del reconocimiento entre gametos en mamíferos sigue sin estar clara. En ratones editados genéticamente, IZUMO (Inoue et al. 2005), JUNO (Bianchi et al. 2014), CD9 (Miyado et al. 2000) y ZP2 (Avella, Baibakov, and Dean 2014) son necesarios para el reconocimiento entre espermatozoide y ovocito. Sin embargo, estas proteínas no se han probado en otros mamíferos, o los resultados han sido controvertidos debido a las características específicas de cada taxón durante la fecundación. Un impedimento para obtener resultados inequívocos ha sido la ausencia de un ensayo consistente para el reconocimiento entre gametos. Por lo tanto, hemos investigado la utilización de las microesferas de sefarosa conjugadas a las glicoproteínas recombinantes de la ZP (B_{ZP}) como un modelo 3D de interacción entre gametos de mamíferos que imita la forma esférica de los ovocitos, se traslada fácilmente entre mamíferos y es escalable. Las microesferas magnéticas de sefarosa se conjugan con glicoproteínas recombinantes de la ZP a las que se unen células del cúmulo y los espermatozoides, permitiendo evaluar el estado del acrosoma en condiciones experimentales variables. Esta tecnología representa un primer paso necesario para validar el papel de las proteínas individuales del ovocito en el reconocimiento de espermatozoides. Como ejemplo, en este estudio hemos probado las tres glicoproteínas de la ZP, potencialmente involucradas en la interacción entre los gametos porcinos. Las proteínas de la ZP se procesan, eliminando el péptido señal en su extremo N-terminal y se liberan del dominio transmembrana del extremo C-terminal para que un ectodominio pueda participar en la zona pelúcida extracelular que rodea los ovocitos ovulados (Boja et al. 2003; Jovine et al. 2004; Jimenez-Movilla and Dean 2011). Por lo tanto, se debe tener cuidado al diseñar las proteínas recombinantes para asegurar que la secuencia de interés esté presente en las

glicoproteínas recombinante de la ZP. Debido a la glicosilación y los enlaces disulfuro múltiples, es preferible el uso de células de mamífero heterólogas para la producción de las proteínas recombinantes, para asegurar que éstas son biológicamente activas, aunque los patrones de glicosilación difieran de las proteínas endógenas. Las glicoproteínas diseñadas se secretaron con éxito, se detectaron mediante anticuerpos comerciales y se conjugaron a microesferas magnéticas de sefarosa, proporcionando un recubrimiento homogéneo sobre toda la superficie. La conjugación fue estable, permitiendo un amplio intervalo de tiempo para realizar los experimentos deseados. Las B_{ZP} inertes fueron seleccionadas debido a su tamaño, superficie abigarrada y facilidad de manejo. La naturaleza porosa de la ZP nativa y su superficie rugosa pueden ser importantes para las respuestas fisiológicas cuando los espermatozoides se unen a su superficie (Baibakov et al. 2007). Aunque las proteínas recombinantes de la ZP no forman matrices de manera individual, al conjugarlas a microesferas, se obtiene una superficie rugosa (Ghaeidamini et al. 2018) con suficiente rigidez que responde a la presión mecánica del contacto espermatozoide-ZP. Según los modelos actuales, la energía cinética de los espermatozoides se convierte en deformación elástica de la ZP, la cual es necesaria para una penetración exitosa. (Gefen 2010). La textura sefarosa-glicoproteína permite esta deformación que imita, hasta cierto punto, las propiedades mecánicas de la ZP. Además, se han descrito dos patrones de unión de espermatozoides cuando se observan mediante SEM y técnicas de microscopía electrónica de transmisión (TEM). El patrón más común, una unión plana y tangencial de la cabeza del espermatozoide a la superficie de la ZP, que luego es seguida por la intrusión en la zona precisamente en esta posición horizontal. (Sathananthan et al. 1982) y una unión del espermatozoide vertical, penetrando la ZP por la punta de la cabeza. Este último patrón

se encuentra en los ovocitos donde son visibles cantidades de espermatozoides unidos en forma de racimo (Familiari et al. 2008). Ambos patrones de unión se observan en los modelos B_{ZP}. El espermatozoide unido a la superficie de la microesfera que muestra unión apical se observa en la Figura 1.6b (imagen izquierda) y el espermatozoide aplanado en la superficie de la microesfera se observa en la Figura 1.6b (imágenes central y derecha). Además, algunos artefactos durante el procesamiento de muestras y también que los espermatozoides podrían ser forzados artificialmente a tener interacciones adicionales con el material de la microesfera en comparación con la configuración fisiológica de la zona pelúcida, no se descartan, como una forma ligeramente "enrollada" en las piezas intermedias o deformaciones de la cabeza que se observa en las imágenes SEM. En la mayoría de los mamíferos, los ovocitos cuando son ovulados están rodeados por una capa sustancial de células del cúmulo incrustadas en una matriz de ácido hialurónico. El complejo de ovocitos y células del cúmulo porcino (COC) típicamente contiene ~ 3000 células del cúmulo (Okudaira, Wakai, and Funahashi 2017) y forma una barrera que los espermatozoides deben penetrar a medida que se acercan a la ZP. La B_{ZP} esférica y la característica superficie dan como resultado la adhesión de las células del cúmulo *de novo*. Esto recrea la apariencia de los COCs nativos en los que las células del cúmulo adoptan formas y proyecciones en forma de lágrima que penetran en la matriz de la ZP (Tanghe et al. 2002). La presencia de glicoproteínas recombinantes de la ZP aumentó el número de células del cúmulo fuertemente unidas a la superficie de la B_{ZP}. Hasta donde sabemos, este es el primer modelo 3D de reconocimiento entre gametos que incluye células del cúmulo y proporciona un sistema robusto para estudiar la actividad fisiológica de los espermatozoides fecundantes. Para evaluar el papel de las proteínas de la ZP en el reconocimiento de gametos de manera

individual, los investigadores han intentado evaluar la actividad de unión de los espermatozoides y la capacidad de inducir la reacción acrosómica. Dos ensayos de unión de espermatozoides-ZP son, el ensayo de hemizona (Burkman et al. 1988) y una prueba competitiva de unión de espermatozoides a la ZP intacta (Liu et al. 1988), pero los resultados reportados han sido contradictorios (Waberski et al. 2005). Las proteínas recombinantes de la ZP de manera individual aparentemente proporcionan más simples, más reproducibles y fisiológicamente relevantes. (Franken, Bastiaan, and Oehninger 2000). Aprovechando las B_{ZP}, hemos validado un ensayo de unión de esperma-B_{ZP} usando gametos porcinos. Cada B_{ZP} fue tratado como un ovocito individual usando la misma relación espermatozoides:microesfera comúnmente utilizada en estudios de FIV porcina (Raquel Romar, Funahashi, and Coy 2016) y se ha observado una unión dependiente del tiempo tal y como se observa en los gametos nativos (Coy et al. 1993; R Romar, Coy, and Rath 2012). El estado del acrosoma de los espermatozoides unidos se determinó fácilmente mediante tinción con PNA-FITC (acrosoma reaccionado) y se confirmó por la presencia dependiente del tiempo restos acrosomales en la superficie de la B_{ZP} (R Romar, Coy, and Rath 2012; Katayama, Koshida, and Miyake 2002). Si bien se realiza con gametos porcinos, el mismo diseño se puede trasladar fácilmente a otras especies. El método de preparación de los espermatozoides afecta la viabilidad de los gametos, la motilidad, la capacitación, la capacidad de unión a la ZP y la reacción acrosómica en animales (Matas et al. 2003; Abraham, Johannisson, and Morrell 2016; Canovas et al. 2017) y humanos (Thijssen et al. 2014; Kim et al. 2017). La selección mediante swim-up es más fisiológica que la doble centrifugación y los espermatozoides seleccionados por este método tienen una motilidad progresiva más baja, una velocidad de capacitación más lenta, menor contenido de ROS y niveles de fragmentación de ADN

que los espermatozoides seleccionados por doble centrifugación (Canovas et al. 2017; Menkveld et al. 1990; Zini et al. 2000).

Usando las B_{ZP}, los espermatozoides centrifugados se unen a las perlas a una velocidad mayor que los espermatozoides seleccionados por swim-up tal y como se observa con los gametos nativos (Canovas et al. 2017). Desde un punto de vista estructural, las B_{ZP} imitan estrechamente los complejos de cúmulo-ovocitos nativos al mantener una forma esférica, respaldar la adhesión de las células del cúmulo y proporcionar una superficie abigarrada específica de glicoproteína análoga a la ZP nativa. Desde un punto de vista biológico, las B_{ZP} admiten la unión de los espermatozoides y recapitula la inducción de la exocitosis del acrosoma con la aparición de restos acrosomales y la dependencia de la unión de los espermatozoides con diferentes métodos de capacitación. A partir de estas observaciones, concluimos que las microesferas recubiertas con glicoproteínas recombinantes de la ZP proporcionarán una herramienta valiosa para explorar la base molecular del reconocimiento entre gametos en una amplia gama de mamíferos.

5. Referencias

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CHAPTER 2

Sperm bind preferentially to ZP2 coated beads and improve *in vitro* fertilization efficiency in pigs

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ABSTRACT

The extracellular matrix surrounding the mammalian oocyte, zona pellucida (ZP), is essential for a successful fertilization and further embryo development. Many studies have tried to clear up the implication of specific ZP glycoproteins in gamete interaction, but their specific role has not been elucidated yet among species. We have recently developed a 3D model based on magnetic sepharose beads (B) conjugated to recombinant ZP glycoproteins (B_{ZP}) and cumulus cells (CB_{ZP}) that allows the study of isolated ZP proteins in gamete recognition. The objective of this study is to apply this model to porcine species to gain knowledge about the specific roles of ZP2, ZP3 and ZP4 in inducing acrosome reaction, sperm interaction with ZP and cumulus cells adhesion. ZP proteins coated beads were incubated with fresh ejaculated boar spermatozoa and with isolated cumulus cells (24 h) obtained from *in vitro* matured cumulus-oocyte complexes (COCs). Sperm binding, acrosomal shrouds on bead's surface and acrosome integrity (by means of PNA-FITC lectin staining) of bound and unbound sperm were studied. Moreover, fertilization results were assessed after insemination of *in vitro* matured COCs in presence of B_{ZP2}. Results at 2 h incubation showed that over 60 % of beads had at least one sperm bound, being B_{ZP2} model the highest (8.82 ± 0.63, N=289 sperm bound per bead). However, B_{ZP3} and B_{ZP4} models presented a higher number of acrosomal shrouds than B_{ZP2}. IVF output and monospermy rate increased when porcine COCs were inseminated in presence of $B_{ZP2.}$ Cumulus cells attached to the surface of the

B_{ZP} acquiring a roundish shape, emitting membrane projections and vesicles-like structures through the sepharose coat. In conclusion, ZP3 and ZP4 mainly induce acrosome reaction whereas ZP2 is involved in sperm-ZP binding in porcine species. B_{ZP2} model might be a useful tool to improve the final IVF efficiency in pigs and a first step to generate artificial COCs to be used in the laboratory.

1. Introduction

The zona pellucida ZP is an extracellular matrix composed by 3 or 4 glycoproteins (ZP1, ZP2, ZP3 and ZP4), depending on the species (Goudet et al. 2008), surrounding the mammalian oocytes from oogenesis to preimplantational embryo (Wassarman and Litscher 2018). Sperm-ZP binding is the first event in fertilization, which allows sperm penetration trough the matrix to reach the plasma membrane and fuse. Once a single sperm fertilizes the oocyte, ZP participates in the blockage of polyspermy. Additionally, the ZP is involved in other crucial mechanisms such as the protection of the oocytes and embryos prior implantation and the nutrition of the oocytes by stabilizing the intercellular connections between the oocyte and the corona radiata allowing the proper transfer of nutrients (Wassarman and Litscher 2018).

Recent studies have cleared up the implication of specific ZP glycoproteins in gamete interaction of mice and human where successful fertilization requires sperm binding to ZP2 (Avella, Baibakov, and Dean 2014). Furthermore, in studies performed in genetic edited mice, the authors demonstrated that the sperm binding domain of the ZP2 is cleaved by the enzyme ovastacin, which is exocyted after fertilization thus preventing polyspermy (Gahlay et al. 2010; Burkart et al. 2012). However, specific ZP glycoproteins' role remains controversial in other mammalian species and the knowledge available in relevant livestock species like pigs, where incidence of polyspermy after *in vitro* fertilization remains a substantial problem, is scarce (Tanihara et al. 2014). In porcine species, the ZP is comprised by ZP2, ZP3 and ZP4 proteins (Hedrick and Wardrip 1987), being ZP4 the one proposed as the responsible for sperm-ZP binding in this species. This proposal is based on sperm binding assays performed with solubilized and purified ZP proteins or recombinant proteins expressed in non-mammalian cells (Yonezawa et al.

2012). Moreover, many studies have described the ZP's role as an inductor of the sperm acrosome reaction, but non-consensus have been achieved among different species. So while ZP3 glycoprotein induces the acrosome reaction (AR) in mice sperm (Gupta and Bhandari 2011), several assays in humans suggest ZP1 (Ganguly et al. 2010), ZP3 (Bray et al. 2002) and ZP4 (Chiu et al. 2008) as the responsible for AR induction in this species. Nonetheless, ZP2 seems to bind only acrosome reacted sperm in both species (Gupta 2018).

In addition to ZP, the granulosa cells comprising the *cumulus oophorus* when ovulation occurs also have an important role in fertilization, being still present at this moment in most mammalian species (Yanagimachi 1988). They confer beneficial effects by means of i) creating the microenvironment required for fertilization and probably exercising a chemoattractant effect, and ii) preventing changes in the oocyte which can alter the successful fertilization (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 and Franken 2007; Franken and Bastiaan 2009). Furthermore, their role on gamete interaction cannot be ruled out despite in porcine *in vitro* fertilization (IVF) systems, cumulus cells are classically removed from COCs which are inseminated partially or completely denuded (Romar et al. 2019).

The establishment of new strategies to study gamete interaction and therefore the sperm-ZP binding in wide range of mammalian species is necessary. The currently available techniques such as the use of double or triple transgenic animals are time- are time-consuming, expensive and not always extrapolable to livestock species. A deeper knowledge on the molecular basis of gametes interaction would improve the final

success of assisted reproductive techniques (ART) what has an special interest in pigs, where the final output after IVF rarely exceeds 45% (Romar et al. 2019).

Recently, we have developed a new strategy to study the role of single proteins involved in gamete recognition. This new model, easily transferable among mammals, stable over time and scalable, is based on magnetic sepharose beads (B) conjugated to recombinant ZP glycoproteins (ZP2, ZP3 and ZP4). The models, named $B_{ZP2,}$ B_{ZP3} and B_{ZP4}, allow the individual study of these proteins over a spherical 3D surface mimicking the oocyte's shape to support sperm binding (Hamze et al. 2019). In addition, the model of sepharose beads conjugated to other relevant proteins can be used to predict sperm fertilizing ability in bovine species (Hamze et al. 2020). The model can now be used to enlarge the knowledge on porcine gametes interaction, so the objectives of this study are i) to investigate the direct role of each ZP protein coated bead to decoy sperm, ii) to corroborate the sperm binding ability to ZP protein coated beads by using complexes cumulus:ZP coated beads, iii) to analyze the acrosomal status of sperm cells bound to ZP coated beads; and iv) to study the likely modulating effect of ZP coated beads when added to the porcine IVF system.

2. Material and Methods

All chemicals were purchased from Sigma-Aldrich Química (Madrid, Spain), 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).

2.1. Recombinant zona pellucida protein expression and conjugation to magnetic sepharose beads

The expression and characterization of the recombinant proteins as well as its conjugation to the magnetic sepharose beads (His Mag Sepharose Excel™; GE Healthcare) was performed as described in Hamze et al (2019). Briefly, the expression plasmids encoding the porcine ZP proteins ZP2: UniProt P42099; ZP3: UniProt P42098; and ZP4: UniProt Q07287 were amplified and purified using Library Efficiency DH5α™ Competent cells (Thermo Fisher Scientific) and GenEluted Plasmid Kit, respectively. Transient transfections were performed to Chinese Hamster Ovary cells (CHO cells, ECACC, The European Collection of Authenticated Cell Cultures) with lipotransfectin (Solmeglas, Madrid, Spain). A ratio of 1 µg template plasmid per 2 µL of lipotransfectin was pre-dissolved in a final volume of 200 µL Opti-MEM reduced-serum medium (Gibco-Invitrogen, Gaithersburg, USA) and incubated 15 min at room temperature (RT). After incubation period, the mix was added to 2 ml Opti-MEM, overlaid on growing cells (37°C, 5% CO_2 and 95% humidity), and incubated for 48 h. The secreted proteins were collected and concentrated in 10.000 Da Vivaspin® Turbo 4(Sartorius, Gottingen, Germany), obtaining a final volume of 200-300 µL of concentrated proteins in 20 mM sodium phosphate buffer, pH 7.4.

Concentrated protein was incubated overnight at 4ºC with 10 µL sepharose beads in 500 µL binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.1% Tween-20, pH 7.4) previously washed with 500 µL washing buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4) and 500 µL binding buffer. After incubation period, 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 4ºC until use. Beads incubated with non-transfected CHO

cells media and processed as the B_{ZP} were used as control (B_{Ctrl}). Successful protein expression and conjugation were analysed by electrophoresis and western blot.

2.2. Sperm collection and processing

Semen was hand-gloved collected from mature fertile boars (1-2 years old, Landrace x Largewhite; CEFU, SA, Pliego, Murcia, Spain). Once in the laboratory, the semen was diluted in Beltsville Thawing Solution (BTS) (1:5 v/v) (Pursel, Johnson, and Schulman 1973) Heterospermic samples prepared by double centrifugation as described in Hamze et al. (2019) were used for all the experiments. Briefly, sperm were centrifuged 300 x g for 3 min, supernatant collected and centrifuged 800 x g for 5 min. Then, sperm pellet was resuspended in modified TALP medium (Rath et al. 1999) supplemented with 1 mM sodium pyruvate, 0.6% BSA and 50 µg/mL gentamycin (IVF-TALP) and sperm concentration adjusted for each experiment.

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

Groups of 50-55 B_{ZP} conjugated to recombinant ZP proteins (B_{ZP2}, B_{ZP3} and B_{ZP4}) were washed twice and coincubated in 500 µl IVF-TALP medium (38.5ºC, 20% O_2, 5% CO_2, and saturated humidity) with 200,000 sperm/mL prepared as mentioned above. Acrosomal integrity was evaluated just before and immediately after sperm preparation, as well as at 0.5, 1 and 2 h after insemination of beads. As for sperm-B_{ZP} binding assay it was assessed at 2 h of co-incubation. After coculture times, B_{ZP} were washed three times in PBS with 0.1% PVA, fixed in glutaraldehyde (0.5%, v/v) in PBS and stained for 30 min with 0.01 mM Hoechst 33342 and 4 µg/mL fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC) (Matas et al. 2010). Besides, the unbound spermatozoa were aspirated from the insemination well and stained to evaluate acrosome reaction at the different times (0.5, 1 and 2 h). Mounted B_{ZP} were observed under an

epifluorescence microscopy (Leica, DMLS, Barcelona, Spain) and the number of sperm cells bound to each bead and its acrosome status (green stained acrosome equivalent to reacted sperm) was recorded, as well as the number of acrosomal shrouds on the bead surface. The percentage of beads with at least one sperm bound ($B_{ZP}SB$), the mean number of sperm per bead (S/B_{ZP}), and the percentage of acrosome reacted sperm from total bound sperm was calculated. In the unbound sperm collected from insemination wells, acrosome reaction was assessed in 200 cells per co-incubation period and replicate. Data from 4 and 3 replicates, respectively for sperm-B_{ZP} binding assay and assessment of the acrosome integrity, were collected.

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

After *in vitro* maturation of porcine cumulus-oocyte complexes (COCs) as previously described (Coy et al. 2010), batches of COCs cultured for 44 h were repeatedly pipetted in IVF-TALP medium to strip the associated cumulus cells. Oocytes were removed and medium containing cumulus cells was centrifuged twice at 1,200 g for 10 min. The resultant pellet was resuspended in IVF-TALP medium and concentration of cumulus cells adjusted using a hemocytometer.

Groups of 50-55 B_{ZP} were washed twice in IVF-TALP medium and coincubated for 24 h in 500 µL IVF-TALP medium (38.5ºC, 20% O_2, 5% CO_2, and saturated humidity) containing 2,500 cumulus cells per B_{ZP} (based on the reported number of cells per porcine COC; (Campos et al. 2001; Munakata et al. 2016)). Cumulus mass plugs around B_{ZP} (CB_{ZP}) were observed under the stereomicroscope after co-incubation period. CB_{ZP} were pipetted three times in IVF-TALP medium to retain only the tightly attached cumulus cells. CB_{ZP} were then coincubated for 2 h with boar sperm (200,000 sperm/mL) prepared as mentioned above (38.5ºC, 20% O_2, 5% CO_2, and saturated humidity). CB_{ZP} with bound

sperm were fixed in glutaraldehyde (0.5%, v/v) in PBS and stained with 0.01 mM Hoechst 33342 and 4 µg/mL fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC) for 30 min. Once mounted on slides, CB_{ZP} were observed by epifluorescence microscope. Three replicates were done. The percentage of beads with at least one sperm bound ($B_{ZP}SB$) and the total number of sperm bound to B_{ZP} (TS/B_{ZP}) were recorded.

2.5. Evaluation of beads and oocytes by field emission scanning electron microscopy

Groups of 50-100 B_{ZP}, CB_{ZP} and CB_{ZP} coincubated with sperm and IVM porcine oocytes after being partially denuded were processed for field emission scanning electron microscopy (FESEM) to better describe the nature of cumulus cells adhesion and sperm binding. Samples were fixed in 2% glutaraldehyde in Dulbecco's PBS w/o Ca-Mg at 4ºC for 2 h. After fixation, three washes in Dulbecco's PBS w/o Ca-Mg (PBS-w/o) were made. Samples were then processed by the Electron Microscopy Service Section of the University of Murcia and imaged in ApreoS (Thermo Fisher Scientific. Eindhoven, The Netherlands).

2.6. Evaluation of beads and oocytes by confocal microscopy

Groups of 30-40 B_{ZP} with sperm bound were washed 3 times (10 min) in PBS-w/o and incubated for 1 h at RT with antibodies (anti-FLAG for ZP2 (1:200 v/v); anti-ZP3 for ZP3 (1:200 v/v); and V5 Epitope Tag Antibody for ZP4 (1:200 v/v)) in PBS-w/o supplemented with 1% BSA. Then, B_{ZP} were incubated for 1 h at RT with secondary antibodies, anti-rabbit IgG-FITC conjugate (1:100 v/v) for ZP2 and ZP4; Alexa Fluor 488 Donkey anti-goat IgG (1:200 v/v) for ZP3. After that, samples were fixed in 4% paraformaldehyde in PBS at RT, and stained with 0.01 mM Hoechst 33342 for 30 min.

Groups of 30-40 CB_{ZP} and IVM oocytes partially denuded were fixed in 4% paraformaldehyde in PBS at RT for 30 min. Samples were then permeabilized for 20 min in PBS supplemented with 0.1% PVA and 1% Triton X-100. In order to observe whether the cumulus cells contacted with the B_{ZP} forming projections or not, the samples were stained with Rhodamine phalloidin (Invitrogen, Carlsbad, CA, USA) 1:50 (v/v) in PBS with 0.1% PVA and 0.01 mM Hoechst 33342 for 30 min at RT. All samples were placed on a chambered slide (25-µL cavity) with Gene Frame (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, Spain). Rhodamine Phalloidin, FITC and Alexa 488, and Hoechst 33342 were excited at 540, 488 nm and 350 respectively.

2.7. *In vitro* maturation of porcine COCs

In vitro maturation of cumulus-oocyte complexes (COCs) was carried out as described by Coy et al. (Coy et al. 2010). Briefly, ovaries from gilts (Landrace x Large-White) were collected within 30 min of slaughtering and transported in saline with 100 mg/mL kanamycin sulphate at 38.5ºC. Once in the laboratory, the ovaries were washed once in 0.04% cetrimide solution and twice in saline. Antral follicles of 3 to 6 mm diameter were aspirated and COCs collected under the stereomicroscope. COCs with dark granulated cytoplasm and several layers of cumulus cells were washed twice in Dulbecco's PBS with 1 mg/mL PVA, and twice in maturation medium consisting of NCSU-37 medium (Petters and Wells 1993) supplemented with 0.57 mM cysteine, 1 mM dibutyryl 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) equilibrated for

3 h at 38.5ºC and 5% CO_2. After washing, 50-55 COCs were transferred to 500 µL maturation medium and cultured for 22 h (38.5ºC, 20% O_2, 5% CO_2 and saturated humidity). COCs were then washed twice in maturation medium without dibutyryl cAMP and hormones for another 22 h.

2.8. Simultaneous insemination of COCs and B_{ZP2}

In vitro matured COCs obtained after 44 h of culture were partially denuded by pipetting, washed twice in IVF-TALP medium and transferred in groups of 25-30 to a well of a 4-well multidish containing 250 µL equilibrated IVF-TALP medium and 25-30 B_{ZP2} or B_{Ctrl} previously washed twice in IVF-TALP medium. Control group consisted of 50-55 partially denuded COCs in each well without beads. The heterospermic sperm samples prepared by double centrifugation were diluted in IVF-TALP medium to adjust the concentration at 10,000 sperm/mL that were added to each well containing the denuded oocytes and beads in a final volume of 500 µL. Gametes and beads (B_{ZP2} or B_{Ctrl}) were co-cultured for 18-20 h (38.5ºC, 20% O_2, 5% CO_2, and saturated humidity). After incubation, samples were mechanically stripped of cumulus cells and sperm, fixed with glutaraldehyde (0.5%, v/v) in PBS and stained in 0.01 mM Hoechst 33342. Oocytes and beads (B_{ZP2} or B_{Ctrl}) were observed under an epifluorescence microscopy (Leica, DMLS, Barcelona, Spain). In the oocytes, sperm penetration (%), monospermy (calculated from penetrated oocytes) (%), output (%), male pronucleus formation (%), mean number of spermatozoa per penetrated oocyte, number of decondensed spermatozoa and male pronucleus per penetrated oocyte were assessed. In the beads, the number of sperm bound per bead, percentage of beads with at least one sperm bound ($B_{ZP}SB$) and mean number of sperm per bead (S/B_{ZP}) were calculated. Data from 5 replicates are shown.

2.9. Statistical analysis

Data are presented as mean ± SEM and all percentages were modelled following a binomial model of variables and arcsine transformation in order to achieve a normal distribution. The analysed parameters such as the percentage of beads with at least one sperm bound ($B_{ZP}SB$), the mean number of sperm bound to each B_{ZP} (S/B_{ZP}), the percentage of reacted spermatozoa bound to B_{ZP}, the percentage of reacted spermatozoa unbound to B_{ZP}, the percentage of B_{ZP} with at least one acrosomal shroud, the mean number of acrosomal shrouds per B_{ZP}, the sperm penetration (%), monospermy (%), output (%), male pronucleus formation (%), mean number of spermatozoa per penetrated oocyte, the number of decondensed spermatozoa and male pronucleus per penetrated oocyte were analysed by one-way ANOVA. When a significant effect was revealed, a Tukey's test was applied. Statistics differences were considered when a P value <0.05 was obtained. Systat v13.1 (Systat Software, Inc San Jose, CA, USA) software was used.

3. Results

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

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 (Figure 2.1) (Hamze et al. 2019).

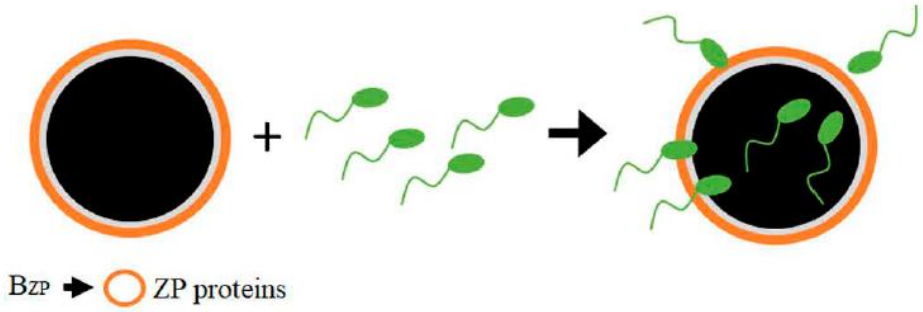

Figure 2.1. *Schematic representation of sperm-B_{ZP} binding assay. Recombinant ZP glycoprotein coated beads (B_{ZP}) and beads incubated with non-transfected CHO cells media (B_{Ctrl}) were incubated with double centrifuged porcine sperm for 2 h.*

The uniform distribution of the protein over the whole 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 (Figure 2.2).

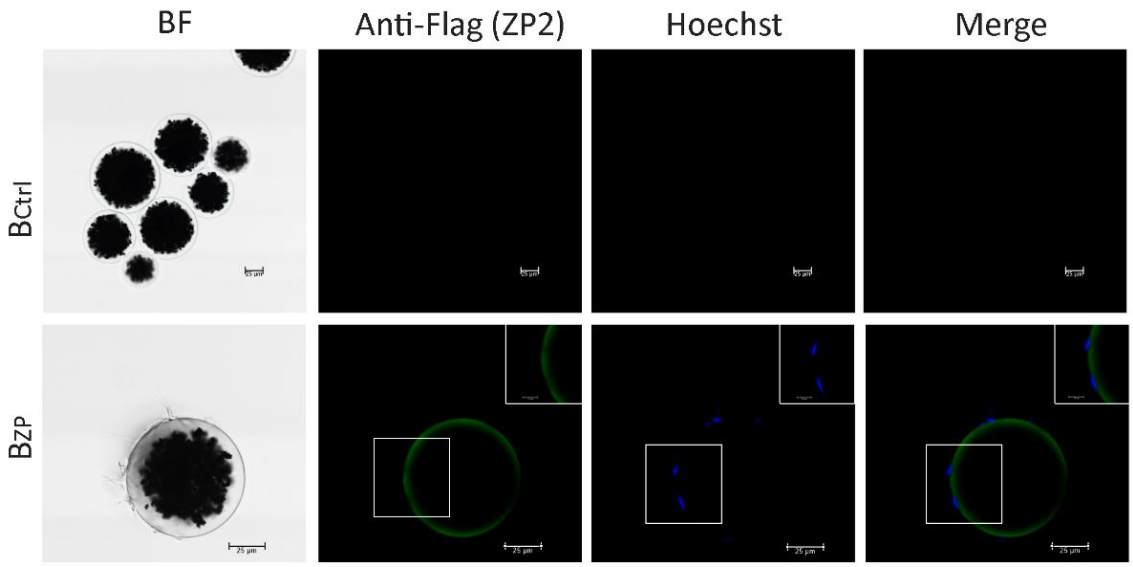

Figure 2.2. *Confocal microscopy images of B_{Ctrl} and B_{ZP2} showing uniform coating of beads with recombinant ZP glycoproteins and sperm stained with (0.01 mM bisbenzimide). The uniform distribution of the protein over the bead's surface was observed for the three models, but only B_{ZP2} is shown as an example. Scale bar, 25 µm.*

The ability of the porcine sperm to bind to B_{ZP} was recorded as the percentage of B_{ZP} with at least one sperm bound (B_{ZP}SB%) and as the mean number of sperm bound to each B_{ZP} (S/B_{ZP}) after 2 h co-incubation. Regarding B_{ZP}SB%, all models showed more than 64.00% of beads with at least one sperm bound (Figure 2.3, left). However, B_{ZP2} had a significantly higher S/B_{ZP} (8.82 ± 0.63, N=289) than B_{Ctrl} (4.90 ± 0.33, N=270), B_{ZP3} (6.72 ± 0.64, N=253) or B_{ZP4} (3.90 ± 0.30, N=297) models ($P<0.01$) (Figure 2.3, right panel), showing that B_{ZP2} decoy highest number of sperm cells.

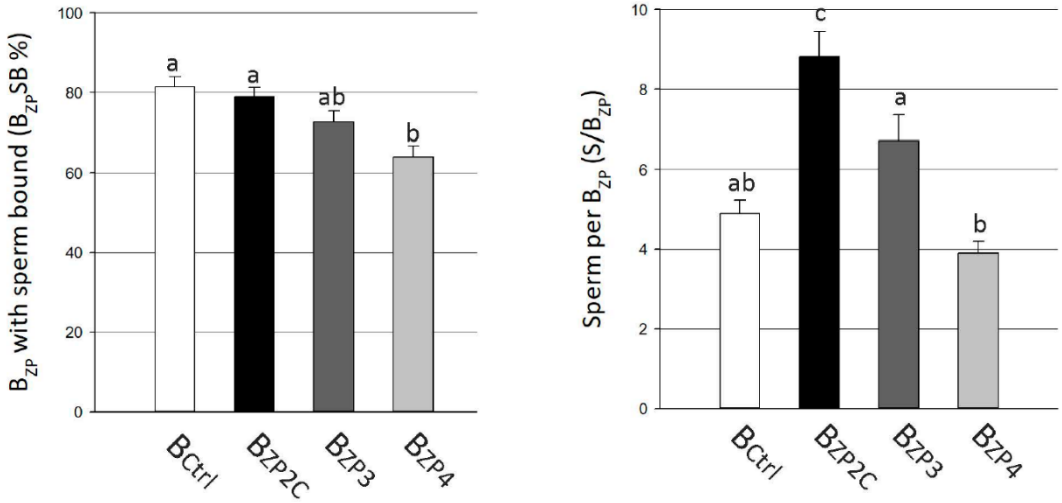

Figure 2.3. *The percentage of B_{ZP} and B_{Ctrl} with at least one sperm bound (B_{ZP}SB%) (left) and the average number of sperm per bead (S/B) (right) were recorded after incubation period. A significantly higher mean number of sperm per bead was observed in B_{ZP2} compared with B_{Ctrl}, B_{ZP3} and B_{ZP4} (P<0.01). Different letters (a, b, c) show significant differences (P<0.05).*

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

Cumulus ZP-beads complexes were generated and incubated for 2 h with double centrifuged porcine sperm (Figure 2.4).

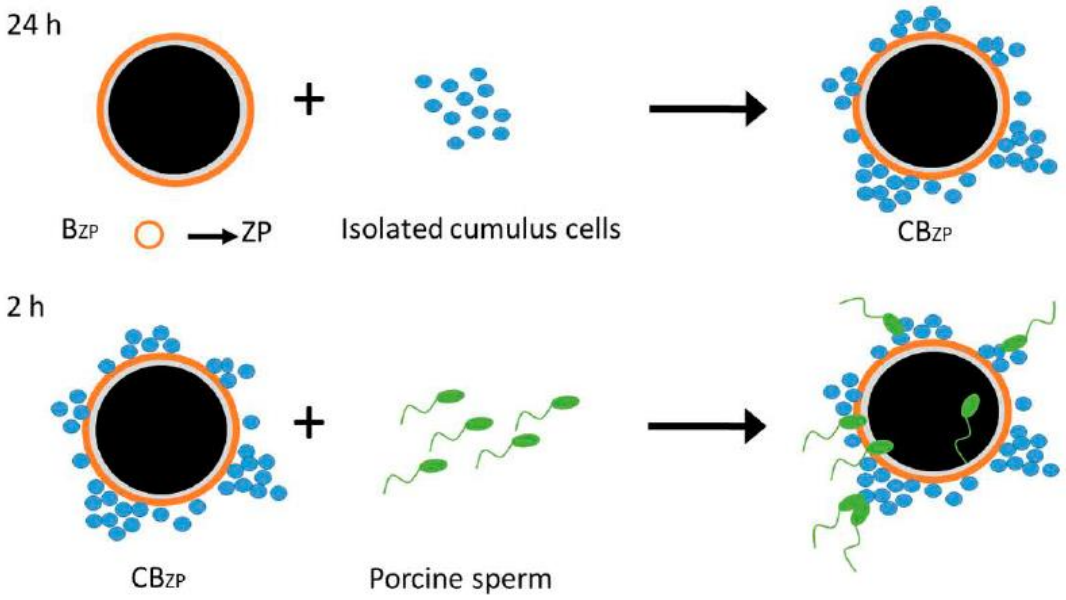

Figure 2.4. *Schematic representation of sperm-CB_{ZP} sperm binding assay. Attachment of cumulus cells to B_{ZP} (upper panel) and coincubation (2 h) of CB_{ZP} with double centrifuged porcine sperm (lower panel).*

The complex formed by cumulus cells membrane and the carbohydrate sepharose (CB_{ZP}) was confirmed by field emission scanning electron microscopy (Figure 2.5) where it was clearly observed that cumulus cells showed membrane projections 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.

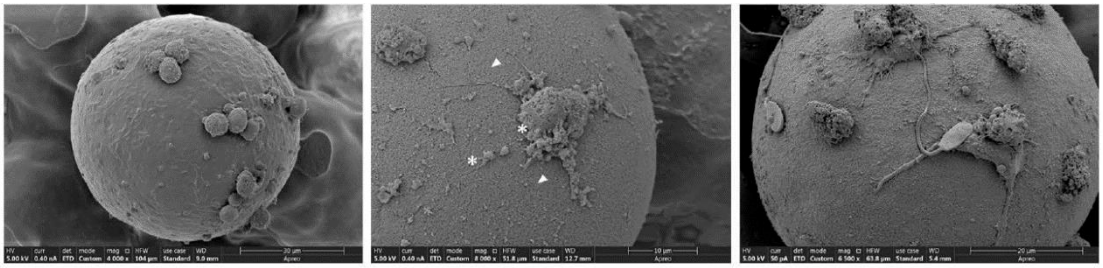

Figure 2.5. *Images from FESEM of an in vitro matured oocyte (left) and a CB_{ZP} (middle) and CB_{ZP} with attached sperm. Vesicle-like structures (*) and cell projections can be observed (Δ).*

Observation of CB_{ZP} under confocal microscopy after staining with Rhodamine Phalloidin showed that attached cumulus cells emit projections with ramifications penetrating on the bead's surface (Figure 2.6).

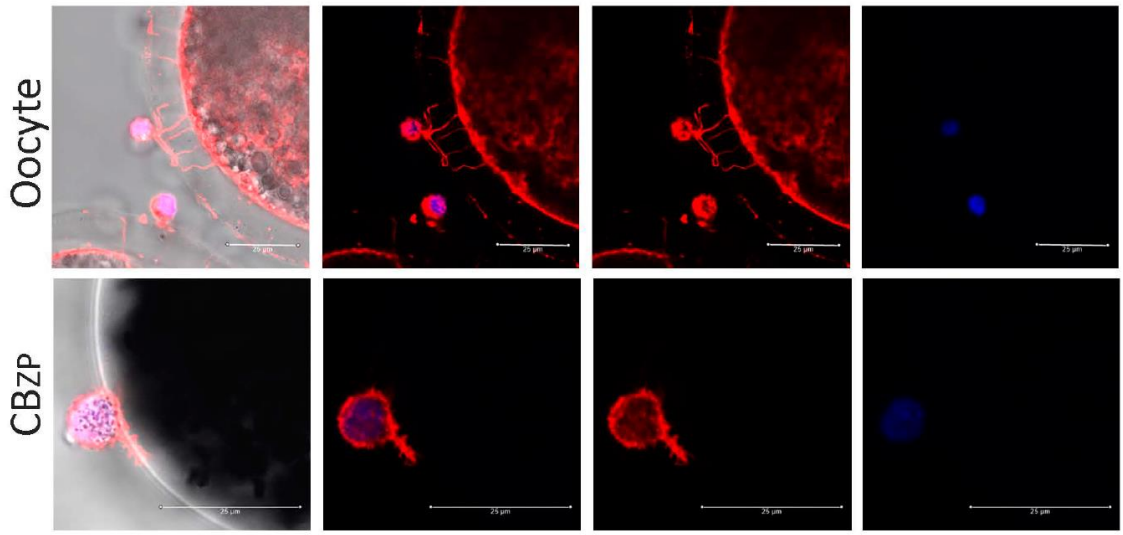

Figure 2.6. *Images taken by confocal microscopy of an in vitro matured oocyte and CB_{ZP} stained with Rhodamine Phalloidin. By this approach, it was observed that attached cumulus cells emit projections with ramifications penetrating on the bead's surface.*

As above, the percentage of CB_{ZP} with at least one sperm bound ($B_{ZP}SB\%$) and the total sperm bound per B_{ZP} (TS/B_{ZP}) were assessed. Regarding $B_{ZP}SB\%$, more than 84.00% of CB_{ZP} in all models showed at least one sperm bound (Figure 2.7, left), being significantly higher for CB_{ZP2} model (96.60 ± 1.40, N=176, P<0.001) over the other two models, CB_{ZP3} and CB_{ZP4}. Additionally, CB_{ZP2} was also the model with a higher TS/B_{ZP} (Figure 2.7, right), being 13.86 ± 0.74 (N=173), 6.52 ± 0.47 (N=168) and 10.08 ± 0.62 (N=158) for CB_{ZP2}, CB_{ZP3} and CB_{ZP4}, respectively (P<0.001). Showing that, same as before, CB_{ZP2} decoy the highest number of sperm.

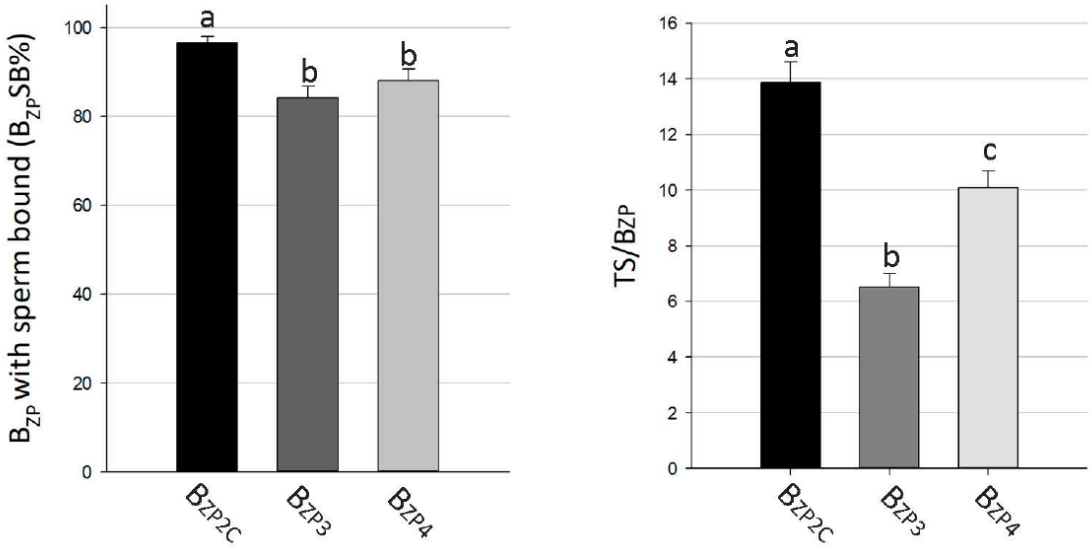

Figure 2.7. *The percentage of CB_{ZP} with at least one sperm bound (B_{ZP}SB%) (left) and the average number of total sperm per bead (TS/B_{ZP}) were recorded after coincubation period. A significantly higher mean number of total sperm per bead was observed in CB_{ZP2} compared with CB_{ZP3} and CB_{ZP4} ($P<0.001$). Different letters (a, b, c) show significant differences ($P<0.05$).*

3.3. ZP2 is involved in sperm binding, whereas ZP3 and ZP4 in acrosome reaction

Acrosome integrity was assessed in the sperm cells bound to B_{ZP} and the sperm that remained unbound in the well after co-incubation with B_{ZP}. Acrosome status just before and immediately after sperm preparation was 4.70 ± 0.90% (N=600) and 29.30 ± 1.90% (N=600) respectively. The percentage of reacted sperm bound to B_{ZP} increased over time in all groups (Figure 2.8).

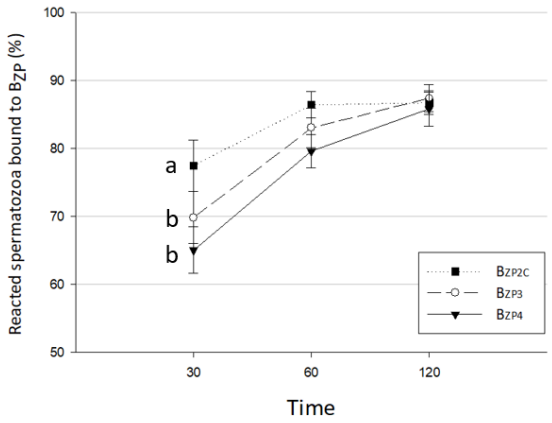

Figure 2.8. *Percentage of acrosome reacted sperm bound to B_{ZP} (B_{ZP2}, B_{ZP3} and B_{ZP4}) over time (0.5, 1 and 2 h).*

After 0.5 h co-incubation, the B_{ZP2} model showed a higher rate of reacted bound sperm (77.50 ± 3.76%, N=154) than B_{ZP3} (69.84 ± 3.84, N=158) and B_{ZP4} (65.04 ± 3.43, N=155) ($P<0.05$) (Figure 2.9, left). 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} (45.20 ± 2.00, N=600) compared to B_{ZP3} and B_{ZP4} groups (57.70 ± 2.00 N=600; and 52.80 ± 2.00 N=600, respectively) ($P<0.05$) (Figure 2.9, right).

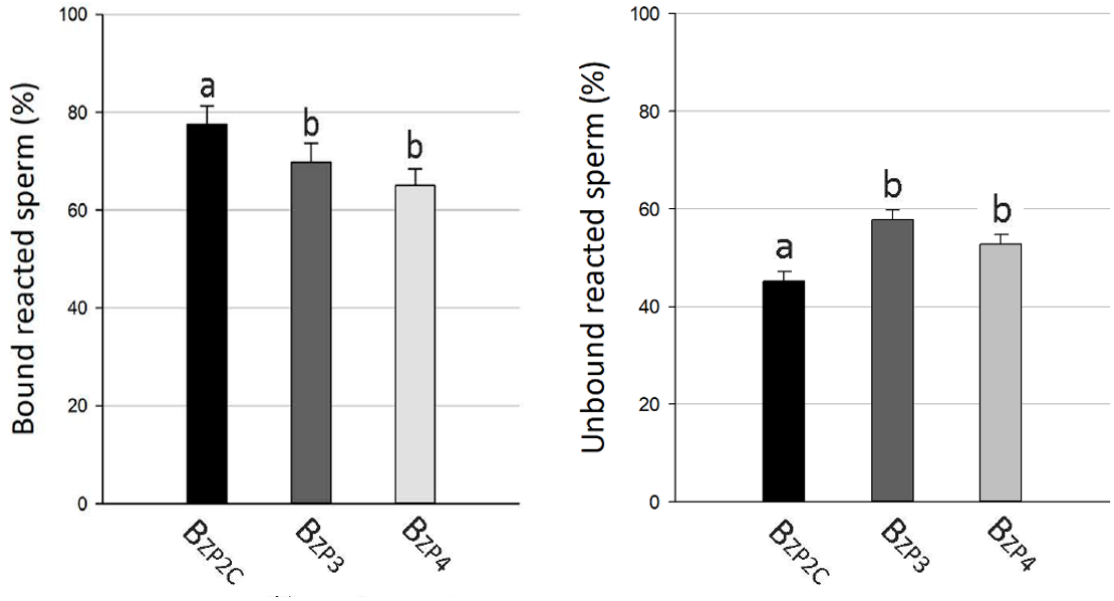

Figure 2.9. *Percentage of acrosome reacted sperm bound to B_{ZP} at 0.5 h (left). B_{ZP2} showed a higher percentage of acrosome reacted sperm bound to beads. Percentage of acrosome reacted sperm unbound to B_{ZP} at 0.5 h (right), being the sperm coincubated with B_{ZP2} the ones presenting the lowest percentage of acrosome reaction.*

Altogether the data show that the highest percentage of bound reacted sperm is found when coincubation is performed with B_{ZP2} model, meanwhile the percentage of unbound acrosome reacted sperm, is the lowest in this group, being higher in B_{ZP3} and B_{ZP4}. Moreover, 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, 2.67 ± 0.14 (N=153), compared to B_{ZP3} and B_{ZP4} (3.77 ± 0.17 N=156; and 3.48 ± 0.15 N=153 respectively) ($P<0.01$) (Figure 2.10).

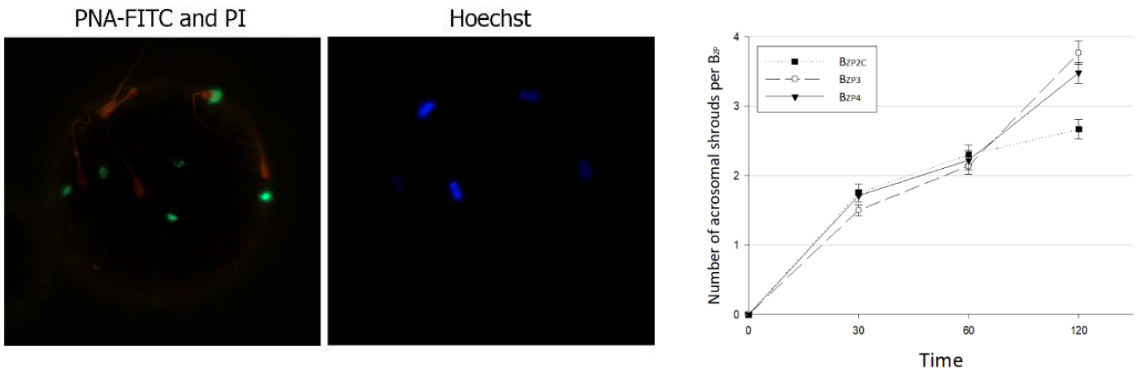

Figure 2.10. *Acrosomal shrouds on the surface of B_{ZP}, which were fixed (0.5% glutaraldehyde) and stained with 4 µg/mL PNA-FITC (fluorescein isothiocyanate-conjugated peanut agglutinin) and 0.01 mM bisbenzimide. Images were taken in an epifluorescence microscope. To improve acrosomal shrouds visualization, sperm were also stained with propidium iodide. Number of acrosomal shrouds per B_{ZP} over time. At 2 h co-incubation, B_{ZP2} group presented a lower number of acrosomal shrouds per bead. Different letters (a, b, c) show significant differences (P<0.05).*

3.4. 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} had or not an effect on *in vitro* fertilization (IVF) in porcine species three experimental groups were proposed: oocytes alone, oocytes together with B_{ZP2} (B_{ZP2} group) and oocytes together with B_{Ctrl} (B_{Ctrl} group) (Figure 2.11).

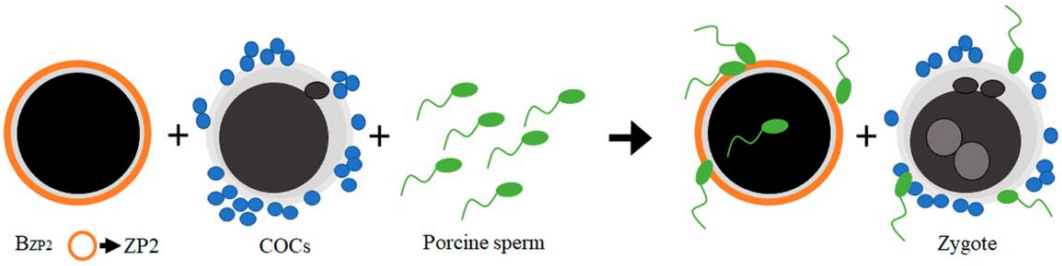

Figure 2.11. *Schematic representation of porcine in vitro fertilization with B_{ZP2} model.*

After oocyte *in vitro* fertilization and 24 h of co-incubation, B_{ZP2} model presented a higher percentage of beads with at least one sperm bound (B_{ZP}SB%) (Figure 2.12, left) and a higher number of sperm bound per bead (S/B_{ZP}) than B_{Ctrl} (Figure 2.12, right).

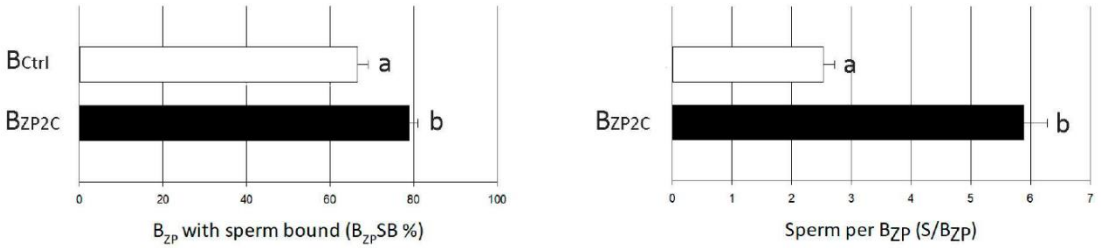

Figure 2.12. *The percentage of B_{ZP2C} and B_{Ctrl} with at least one sperm bound (B_{ZP}SB%) (left) and the mean number of sperm per bead (S/B) (right) were recorded after co-incubation period. A significantly higher mean number of sperm per bead was observed in the B_{ZP2C} compared with B_{Ctrl}. Different letters (a, b) show significant differences (P<0.05).*

Fertilization rate of eggs exposed to B_{ZP2} were assessed (Table 1). Penetration rate was similar in all experimental groups being around 75.00%. The monospermy rate was higher when COCs were simultaneously inseminated with B_{ZP2} (43.90 ± 3.30%, N=303) than with B_{Ctrl} (33.10 ± 3.00%, N=315) and COCs inseminated in absence of beads (34.20 ± 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} (2.33 ± 0.11 and 0.73 ± 0.08, 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 pronucleus per oocyte showing that presence of B_{ZP} had not a harmful effect on fertilizing spermatozoa (Table 1).

Table 1. *Penetrability results of in vitro matured porcine oocytes inseminated together with ZP2 beads, control beads or without beads (Oocytes group). Results were assessed 18 h post insemination. MPN (male pronuclear formation rate), MPN (number of male pronucleus in each penetrated oocyted). Data from 5 replicates.*

GROUP	N	Penetration (%)	Monospermy* (%)	Output** (%)	MPNf* (%)	Mean number spermatozoa/oocyte*	Decondensed spermatozoa/oocyte*	MPN/oocyte*
Oocytes	350	76.0±2.3	34.2±2.9 a	26.0±2.3	99.2±0.5	2.932±0.123 a	1.173±0.099 a	1.759±0.061
Oocytes + BZP2C	303	75.2±2.5	43.9±3.3 b	33.0±2.7	100	2.325±0.109 b	0.728±0.085 b	1.596±0.058
Oocytes + BCtrl	315	75.9±2.4	33.1±3.0 a	25.1±2.4	98.3±0.8	2.925±0.129 a	1.310±0.116 a	1.615±0.053
P value			0.029	0.049		<0.001	<0.001	

**From penetrated oocytes. Different letter in the same column indicate significant differences (P<0.05).*

***From total number of inseminated oocytes.*

4. Discussion

The ZP is essential for a successful fertilization but the role of each ZP protein among mammalian species is still controversial giving as a result different proposed models to explain the sperm-ZP binding, such as the glycan-release model (Bleil and Wassarman 1980; Vazquez, Phillips, and Wassarman 1989) and the ZP2 cleavage model in mice (Avella, Baibakov, and Dean 2014). The lack of consistent models to study gamete interaction is one of the reasons why the molecular basis of this process remains unclear in mammalian species. Recently, we have 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 among mammals and scalable. Specifically, porcine recombinant ZP glycoproteins (ZP2, ZP3 and ZP4) were incubated with magnetic sepharose beads (B_{ZP}) and the correct conjugation was confirmed by SDS-PAGE and western blot (Hamze et al. 2019). In the 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 seems to have a key role in sperm-ZP binding in pigs, as it has been also reported in mice and humans (Avella, Xiong, and Dean 2013; Avella et al. 2016). 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). However, ZP2 was not included in the mentioned study so 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. However, is not clear whether an association between the spermatozoa and the

cumulus cells surrounding the unfertilized oocyte further affect the spermatozoa activity (Jin et al., 2011, for review see Gadella, 2013). In the current study, cumulus cells and sperm bind to the models (CB_{ZP}) allowing the study of the different ZP glycoproteins over a spherical 3D surface offering a new tool to reconstruct artificial cumulus-oocyte complexes. Firstly, it has been shown the nature of this interaction by means of FESEM and confocal microscopy observing that cumulus cells bind the rugose surface of the bead emitting cytoplasmic prolongations, vesicle-like structures and trying to penetrate the bead's surface. In the native oocyte, cumulus cells emit transzonal projections (Del Collado et al. 2017) and extracellular vesicles (Avila et al. 2019) in order to communicate and cooperate metabolically with the oocyte. The current observations might reflect an attempt of cumulus cells to reflect their functional aspects. 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. 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 compare to ZP3 and ZP4.

In the oocyte (ZP)-sperm interaction, acrosome reaction is a crucial event necessary for sperm penetration trough the ZP. Different studies suggest that ZP3 is the glycoprotein inducing the acrosome reaction in mice sperm (Gupta and 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 bind to human ZP2 (Gupta 2018). Accordingly, results presented in the current study showed a higher percentage of bound reacted sperm in the B_{ZP2} group whereas the percentage of unbound reacted sperm are 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, Coy, and Rath 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 similar pattern of acrosomal content deposition on oocyte's ZP (Romar, Coy, and Rath 2012) indicating that the 3D model reproduced this feature of native ZP and suggesting 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 scale mainly due to the low efficiency of the IVF system (Romar et al. 2019) being the high rates of polyspermy 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 avoiding the likely interactions among them, has many potential applications. A study carried out in mice found that 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 their interaction with oocytes and reducing polyspermy levels. 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 worthy 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 allow 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 outcome of this technique in pigs.

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CHAPTER 3

JUNO protein coated beads: a potential tool to predict sperm fertilizing ability

ABSTRACT

Considerable variation in fertility exists between bulls in artificial insemination (AI) centres, despite passing minimum post-thaw quality control checks. The development of a reliable *in vitro* test to predict bull fertility could enable the identification and selection of high fertility bulls, without the need to resort to test inseminations. An in-depth knowledge of the molecular basis of fertilization is a prerequisite to the development of such a test or combination of tests. To date, JUNO is the only oocyte plasma membrane receptor described to be involved in gamete binding for which the partner in the sperm, IZUMO1, is known. Despite the fact that this interaction appears to be conserved among mammals, it has not been confirmed yet in some species including cattle. Furthermore, an association between binding and fertility has not been tested. Here, we propose a sperm-binding assay based on magnetic sepharose beads coated with bovine recombinant JUNO protein (B_{JUNO}) to study sperm binding. Bull sperm bound specifically to B_{JUNO} demonstrating that the JUNO-IZUMO1 interaction is conserved in cattle. Moreover, the assay was able to distinguish between epididymal and ejaculated sperm. Lastly, the number of sperm cells bound to B_{JUNO} was significantly lower for frozen-thawed sperm from bulls of low vs high field fertility. In conclusion, our findings document a novel valid sperm-binding assay to predict mammalian sperm function and to investigate the role of specific proteins involved in gamete recognition and fusion.

1. Introduction

At fertilization, a capacitated sperm penetrates the zona pellucida (ZP), binds and fuses with the oolemma of the mature oocyte to ultimately generate a new and unique individual. Although we have considerable knowledge about the molecular basis of gamete interaction in some species, including cattle (Avella et al., 2013; Georgadaki et al., 2016; Sutovsky, 2018), much remains to be learned. Gamete interactions can be explored *in vitro* to develop novel reliable and consistent methods to predict semen fertility.

Regarding the molecular basis of fertilization, two proteins have been described as essential in gamete recognition and fusion, JUNO (Bianchi et al., 2014) in the oocyte and IZUMO1 in the sperm (Inoue et al., 2005). Male IZUMO1-deficient mice (Izumo-/-) are healthy but infertile; while Izumo-/- sperm are able to penetrate the ZP and bind the oolemma, they are unable to fuse with it (Inoue et al., 2005). IZUMO1 has been also identified in bull sperm and, as in mice, it exhibits a translocation to the equatorial segment after the sperm undergoes the acrosome reaction (Satouh et al., 2012; Fukuda et al., 2016).

The essential role of JUNO in fertilization was demonstrated using an *in vitro* approach designed to identify low affinity interactions between extracellular proteins (Bushell et al., 2008) and using IZUMO1 as a bait (Bianchi et al., 2014). Female JUNO-deficient mice (Juno-/-) are healthy but infertile as wild-type sperm are unable to fuse to Juno-/- oocytes (Bianchi et al., 2014). To date, JUNO-IZUMO1 is the first and only protein-protein gamete interaction described in which the oocyte ligand and the sperm receptor

are known. However, knowledge of the JUNO-IZUMO1 interaction in economically important livestock species such as porcine and bovine is lacking.

Artificial insemination (AI) centres have traditionally relied upon classical microscopy-based sperm assessment to assess motility (total and progressive) and morphological parameters. However, bulls whose semen pass these minimum post-thaw quality control checks can still vary in fertility by up to 20 to 25% (Fair and Lonergan, 2018). Therefore, a reliable *in vitro* test, or a combination of tests, predictive of bull field fertility would potentially enable AI companies to identify high fertility bulls and also eliminate bulls with low fertility prior to their widespread use in the field. Currently, the most commonly used method to estimate fertility is based on the non-return rate (NRR) i.e., the proportion of inseminated females not returning to oestrus (Fazeli et al., 1997; Puglisi et al., 2010). *In vitro* fertilization offers a test of the functional capacity of sperm; however, it does not mimic all aspects of the process in vivo including sperm transport and capacitation and in general only poorly correlates with field fertility. Other methods of sperm function including the hemi-zona assay (Fazeli et al., 1997) and the cervical mucus penetration test (Tas et al., 2007) have been reported to be correlated with bull fertility, but they are not used routinely. In recent years, putative fertility biomarkers have been identified by comparing high and low fertile bulls using a proteomic approach (Muhammad Aslam et al., 2018; Menezes et al., 2020). In addition, a sperm selection assay has been established based on sperm chemotaxis towards progesterone (Dominguez et al., 2018). Moreover, parameters such as reactive oxygen species (ROS) generation (Celeghini et al., 2019), high esterase activity, plasma membrane integrity, intracellular Ca^{+2} levels, acrosomal status and mitochondrial membrane potential (Bucher et al., 2019) may be associated with bull fertility. Although there are a plethora

of published studies which have used a wide range of *in vitro* assessments to predict the fertility of bulls (Utt, 2016) there is still no test, or combination of tests, which can reliably predict bull fertility. For example, in a recent comprehensive study by Sellem et al. (2015) who correlated bull fertility (based on individual frozen-thawed ejaculates) with a combination of flow cytometric and computer-assisted sperm analysis (CASA) parameters, at best, no more than 40% of the variation in sire fertility could be explained.

Recently, we developed a 3D *in vitro* model of sperm-oocyte interaction that mimics the oocyte's shape by means of magnetic sepharose beads coated with individual porcine recombinant ZP proteins (Hamze et al., 2019). The study of the ZP proteins using this system highlighted the important role of ZP2 in porcine fertilization, corroborating previous data described in mice and humans (Avella et al., 2014, 2016). Here, a model based on magnetic sepharose beads (B) coated with bovine recombinant JUNO protein (B_{JUNO}) was tested using semen from different sources and characteristics. In order to validate the B_{JUNO} model, we assessed: i) the ability of recombinant JUNO protein to be conjugated to the sepharose beads and its stability over time: ii) the potential of B_{JUNO} to bind bull sperm; iii) the ability to distinguish between epididymal and fresh ejaculated sperm; and iv) the behavior of the model when coincubated with frozen-thawed semen from bulls with different fertilizing capacity.

2. Material and Methods

Unless otherwise stated, all reagents and chemicals were purchased from Merck (Madrid, Spain). This study was exempt from ethical approval. All of the semen (fresh and frozen-thawed) used in this study was donated by an EU approved Artificial

Insemination Centre (National Cattle Breeding Centre, Naas, Kildare, Ireland). All of the oocytes and epididymal sperm used were obtained from an EU approved commercial abattoir following collection of ovaries and testes, respectively, after slaughter.

2.1. Recombinant JUNO protein design and expression

The predicted sequence encoding the Bos taurus IZUMO1 receptor, Juno (NCBI Reference Sequence: XM_024975624.1), was used to construct and design the expression plasmid (pcDNA3.1[+]) (GeneArt, Life Technology, Thermo Fisher Scientific, Carlsbad, CA, USA). A V5 tag was added to the N-terminus (23 to 36 aa) and a histidine tag (6x) was added to the C-terminus (239 to 244 aa). To amplify the expression plasmid, the Library Efficiency DH5α Competent cells (Thermo Fisher Scientific) were used. Recombinant JUNO (rbJUNO) expression plasmid was purified using the GenEluted Plasmid Kit. rbJUNO protein was expressed in Chinese Hamster Ovary cells (CHO cells, The European Collection of Authenticated Cell Cultures). The cells were grown at 37ºC, 95% humidity and 5% CO2 for 48 to 72 h until 80 to 90% confluence was reached in Ham's F-12 (Biowest, Nuaillé, France) supplemented with fetal bovine serum (10%) and penicillin-streptomycin (100 U/mL) (GibcoBRL-Life Technologies, Gaithersburg, USA). Transient transfections were performed with Lipotransfectin (Solmeglas, Madrid, Spain) following the manufacturer's protocol. Briefly, 4 µL of Lipotransfectin were used in a volume of 200 µL of Opti-MEM reduced-serum medium (Gibco-Invitrogen) with 2 µg of template plasmid. The complex was incubated at room temperature for 15 min before being diluted in 2 mL Opti-MEM and overlaid on CHO growing cells (37°C, 95% humidity and 5% CO2). After 48 h, the secreted protein was recovered and centrifuged (4,000 x g, 5 min, 4ºC) to remove cell debris. Then, the protein was concentrated in Vivaspin

Turbo 4 of 10,000 Da (Sartorius, Göttingen, Germany). The final protein volume obtained was 200 to 300 µL in 20 mM sodium phosphate buffer, pH 7.4 with protease inhibitor (EDTA-free EASYpack, Roche, Mannheim, Germany).

2.2. Conjugation of recombinant (rb) JUNO glycoprotein to magnetic beads

The conjugation of rbJUNO to the magnetic beads was performed following the protocol previously described in Hamze et al. (2019). Briefly, 10 µL of magnetic sepharose beads (His Mag Sepharose Excel GE Healthcare, Uppsala, Sweden) were resuspended in a microcentrifuge tube with 500 µL of sodium phosphate buffer. Then, the beads were washed once with 500 µL of washing buffer and once with 500 µL binding buffer. The washed beads and rbJUNO concentrate were incubated overnight at 4ºC with orbital agitation (1:1 v/v). The beads coated with rbJUNO (B_{JUNO}) were then washed twice and resuspended in sodium phosphate buffer. B_{JUNO} were stored at 4ºC until use. As a control, beads processed as described above, but incubated with non-transfected CHO cell media were used (B_{Ctrl}).

2.3. Western blot

Concentrated rbJUNO was separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were probed with Penta-Histidine antibody, BSA-free (Qiagen, Hilden, Germany) before chemiluminescence visualization (Pierce ECL-Plus, Thermo Fisher Scientific).

To confirm the successful conjugation of rbJUNO to the magnetic beads, B_{JUNO} were analyzed by SDS-PAGE. The stability of B_{JUNO} following storage at 4ºC was assessed at 0, 24, 48, 72 and 144 h. At each time-point, B_{JUNO} were washed and resuspended in sodium

phosphate buffer. The samples were solubilized under reducing conditions (4X SDS sample buffer, Millipore, USA) for 10 min at 100ºC. Then, the supernatant was separated by SDS-PAGE, transferred to PVDF membranes and incubated with the Penta-Histidine antibody mentioned above.

2.4. Confocal microscopy

After conjugation, B_{JUNO} and B_{Ctrl} were incubated with the Penta-Histidine antibody (1:100 v/v) diluted in calcium and magnesium free PBS supplemented with 1% BSA for 1h at RT. The beads were then incubated (1 h at RT) with secondary antibody Alexa Fluor 488 Goat anti-mouse IgG (1:200 v/v). After incubation, B_{JUNO} and B_{Ctrl} were washed and fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, Philadelphia, USA). Stained beads were placed on a (25-µL cavity) chambered slide with Gene Frame (Advanced Biotechnologies, Leatherhead, UK) and covered with a coverslip. Samples were observed and images were taken with a Leica TCS SP8 confocal microscope. Alexa Fluor 488 was excited at 488 nm. Image analysis was performed using LAS X Core software analysis software (Leica Microsystems, Spain).

2.5. Sperm preparation

As described below, sperm from various sources (epididymal, fresh ejaculated, frozen-thawed ejaculated) and of various characteristics (high and low fertility) were used.

2.5.1. Fresh ejaculated sperm

Fresh ejaculated sperm from the same Charolais bull of proven fertility were collected with an artificial vagina (1:1 v/v in BullXcell extender; IMV Technologies, L'Aigle, France) and were selected by centrifugation (700 x g, 9 min) through a 45/90% discontinuous

Percoll gradient (Pharmacia, Uppsala, Sweden). A total of 20x106 sperm were placed on the top of the Percoll gradient and after the first centrifugation, the pellet was recovered in HEPES-buffered Tyrode's medium (Parrish, 2014) and centrifuged for 5 min at 100 x g. Only samples with motility > 85% after selection were used. Concentration was adjusted using a hemocytometer.

2.5.2. Epididymal sperm

Sperm were obtained from the corpus and the cauda of the epididymis as described by Fernández-Fuertes et al. (2016). Briefly, testes were recovered from 3 different mature beef bulls at slaughter in a commercial abattoir and transported to the laboratory within 2 h. Cauda epididymis sperm was recovered by making a small incision in the cauda and cannulating the lumen of the deferent duct (blunted 22-gauge needle). Sperm were flushed through the cauda with a syringe (5 mL) loaded with PBS at 38ºC. To obtain corpus sperm, the corpus epididymis was isolated and minced with a scalpel blade in a dish containing PBS (38ºC). Sperm was washed in HEPES-buffered Tyrode's medium at 200 x g for 5 min. As before, the concentration was adjusted using a hemocytometer.

2.5.3. Frozen-thawed sperm

Semen straws were thawed at 37ºC in a water bath for 30 s. A 45/90% Percoll gradient was used to select motile sperm as described before. Only samples with motility > 85% were used. Sperm concentration was adjusted as necessary with a hemocytometer.

2.6. Sperm binding assay

Groups of 50 to 55 magnetic sepharose beads were washed twice in fertilization media (113.16 mM NaCl, 3.19 mM KCl, 0.2 mM NaH2PO4, 2 mM CaCl2, 0.487 mM MgCl2,

0.5mM pyruvate, 12.79 mM NaHCO3, 6 mg/ml BSA and 10 µg/ml heparin). Then, the beads were placed in 4-well dishes (Nunc, Roskilde, Denmark) containing 250 µL of the same medium. A sperm dilution in 250 µL of fertilization medium was added to each well to yield a final concentration of 200,000 sperm/mL. The beads and the sperm were incubated for 0.5, 1 and 2 h or just for 2 h depending on the assay at 39ºC, 5% CO2, 20% O2 in air with maximum humidity. After the co-incubation period in each experiment, the beads with the sperm attached were washed 3 times in PBS supplemented with 0.1% PVA, fixed with glutaraldehyde in PBS (0.5 % v/v) and stained with Hoechst 33342 (0.01 mM).

2.7. Fluorescence microscopy

In order to assess the number of sperm cells bound to each bead after co-incubation, the beads were mounted on slides and evaluated under an epifluorescence microscope (Nikon Eclipse TE2000s). The percentage of beads with at least one sperm bound (B_{JUNO} SB) and the mean number of sperm per bead (S/B_{JUNO}) were recorded and calculated for each assay. Images were taken under an epifluorescence microscope (Leica DMI8).

To assess the acrosome reaction of sperm incubated in fertilization medium for 0.5, 1 or 2 h, sperm were fixed with glutaraldehyde in PBS (0.5 % v/v) and stained with 4 □g/mL fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC) (Grullón et al., 2013). 200 sperm cells were counted in 4 fields at 40x magnification.

2.8. Statistical analysis

Statistical analysis was performed using Systat v13.1 (Systat Software Inc., San Jose, CA, USA). The percentages were modelled following the binomial model of variables and

arcsine transformation in order to achieve a normal distribution. Data are presented as mean ± SEM. The $B_{JUNO}SB$ and the S/B_{JUNO} were analyzed by one-way ANOVA. When the effect was significant, a Tukey's test was performed. A P value <0.05 was considered statistically significant.

3. Results

3.1. Secreted rbJUNO is expressed and stably conjugated to magnetic beads

The plasmid encoding bovine rbJUNO glycoprotein (Figure 3.1, left) was expressed in CHO cells. The secreted protein was successfully collected and isolated. rbJUNO glycoprotein was detected by SDS-PAGE and western blot obtaining the expected molecular weight (30 kDa) when probed with Penta-Histidine antibody (Figure 3.1, right).

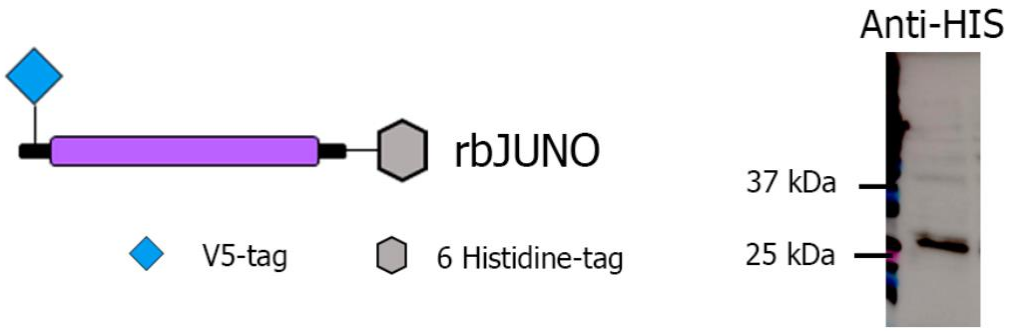

Figure 3.1. *Schematic representation of bovine recombinant JUNO glycoprotein. V5-tag (blue) and 6 histidine-tag (grey) (left). rbJUNO was expressed in CHO cells, separated by SDS-PAGE and analysed by western blot. The protein was probed with Penta-Histidine antibody (right). Molecular mass marker on the left.*

rbJUNO glycoprotein was successfully conjugated to beads as confirmed by immunofluorescence and confocal microscopy; the uniform coating of beads' surface by rbJUNO was confirmed (Figure 3.2).

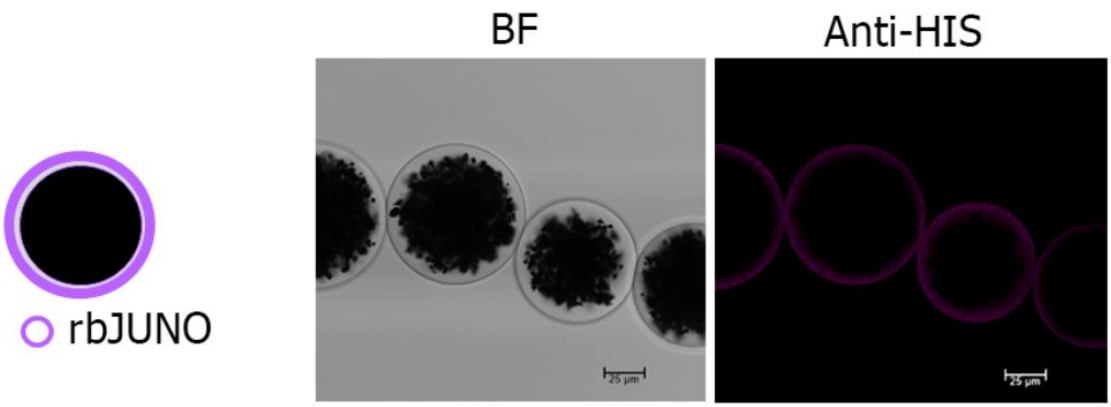

Figure 3.2. *Schematic representation of bovine recombinant JUNO glycoprotein coated beads (B_{JUNO}). Confocal microscopy images of beads conjugated beads (B_{JUNO}) showing uniform coating of beads with rbJUNO protein. Scale bar, 25 µm.*

rbJUNO was detected with its expected molecular weight (30 kDa) in the secreted medium before bead conjugation (Figure 3.3, left, lane 1) and after elution from the magnetic beads (Figure 3.3, left, lane 2). No protein was detected in the medium where conjugation occurred (Figure 3.3, left, lane 3), thus confirming that all available rbJUNO glycoprotein was successfully bound to the magnetic beads. Additionally, the protein was stably bound to the beads during 144h (Figure 3.3, right).

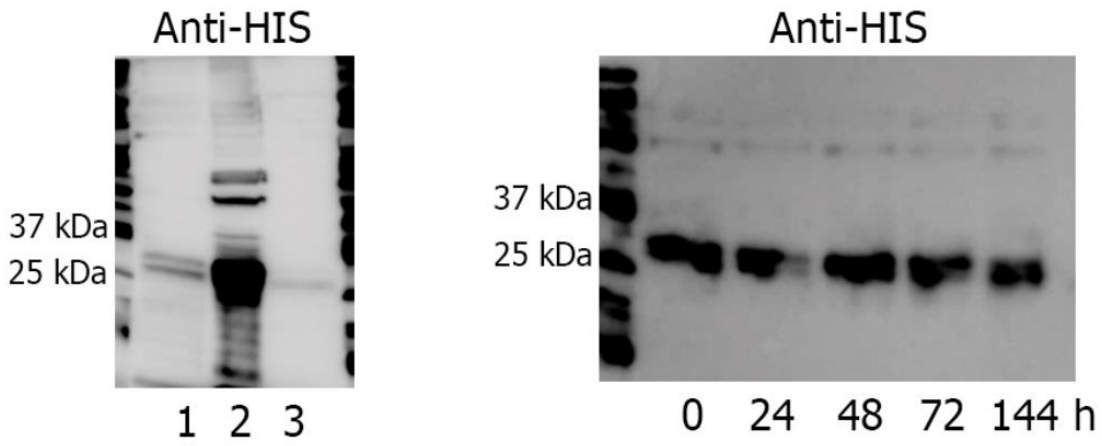

Figure 3.3. *SDS-PAGE and western blot of rbJUNO conjugated to magnetic beads. Medium with secreted rbJUNO before conjugation (lane 1), in the eluted fraction (lane 2) and media after conjugation (lane 3) (left). SDS-PAGE and western blot of rbJUNO conjugated to beads after storage for 0, 24, 48, 72, and 144 h (right). The protein was probed with Penta-Histidine antibody. Molecular mass marker on the left.*

3.2. Recombinant bovine JUNO beads support sperm binding

The aim of this experiment was to validate a sperm-B_{JUNO} binding assay. Diluted sperm from 4 ejaculates from the same Charolais bull were used and four replicates were performed. The ability of ejaculated sperm to bind B_{JUNO} and B_{Ctrl} after 0.5. 1 and 2 h co-incubation was expressed as the percentage of magnetic beads with at least one sperm bound (BSB) and as the mean number of sperm bound to each bead (S/B) (Figure 3.4).

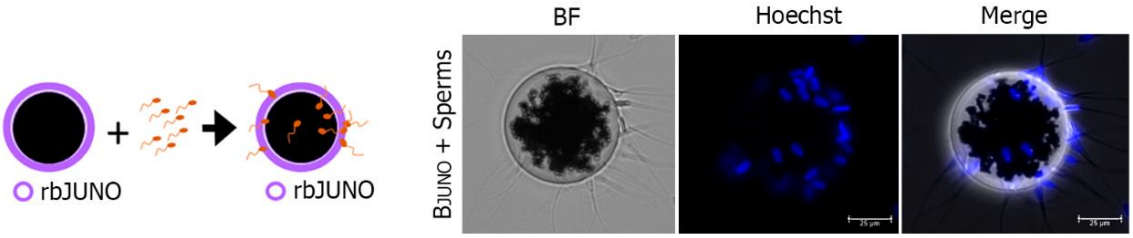

Figure 3.4. *Schematic representation of the sperm-B_{JUNO} binding assay where magnetic beads coated with bovine recombinant JUNO glycoprotein and beads incubated with non-transfected CHO cells media (B_{Ctrl}) are coincubated with bull sperm. B_{JUNO} with bull sperm attached observed under epifluorescence microscope after being fixed (0.5% glutaraldehyde) and stained (0.01 mM bisbenzimide). Scale bar 25 µm.*

The BSB increased over time, from 49.2% and 49.8% at 0.5 h to 90.5% and 85.4% at 2 h for B_{JUNO} and B_{Ctrl}, respectively. No differences were found between groups at each specific time (Figure 3.5, left). As for S/B, B_{JUNO} showed a significantly higher number of sperm bound to each bead than B_{Ctrl} at all time-points (Figure 3.5, right). The greatest differences ($P<0.001$) in sperm bound were observed at 2 h of co-incubation with 13.4 ± 0.71 (N=277) for B_{JUNO} and 5.69 ± 0.32 (N= 257) for B_{Ctrl}.

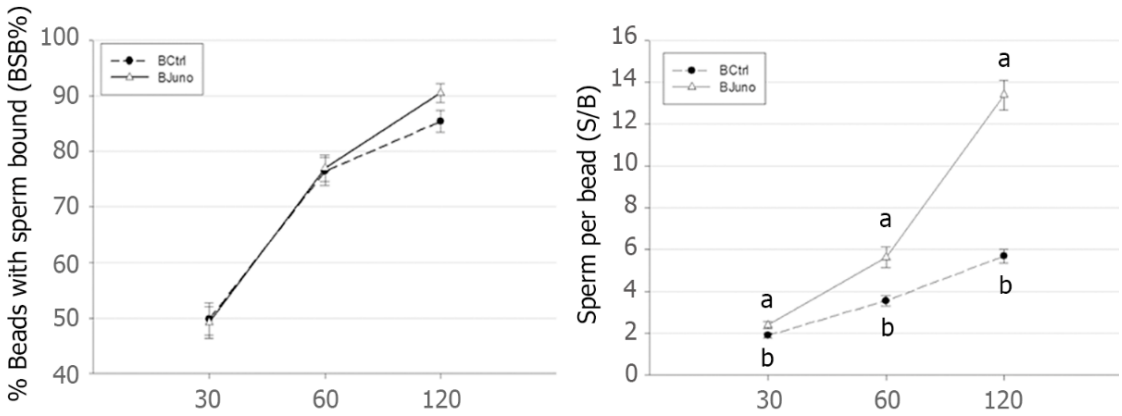

Figure 3.5. *The percentage of B_{JUNO} with at least one sperm bound (B_{JUNO}SB) (left) and the mean number of sperm per bead (S/B) (right) was recorded after 30, 60 and 120 min of co-incubation. Both parameters increased over time and a significantly higher mean number of sperm per bead was observed in the B_{JUNO} compared with B_{Ctrl} at all the studied times (P<0.001).*

The S/B increased with time as well as the percentage of acrosome-reacted sperm when incubated in fertilization medium (Figure 3.6).

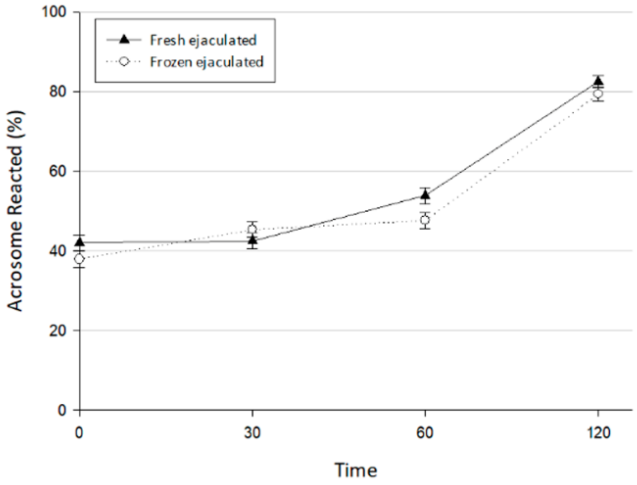

Figure 3.6. *Fresh and frozen-thawed ejaculated sperm were incubated for 0.5, 1 or 2 h in fertilization media. After incubation, sperm were fixed with 0.5% glutaraldehyde in PBS (v/v) and stained with 4 µg/mL fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC). The percentage of acrosome-reacted sperm increased with incubation time for both groups.*

3.3. Recombinant bovine JUNO beads bind more fresh ejaculated sperm than fresh epididymal sperm

The objective of this experiment was to compare sperm-B_{JUNO} binding when performed with (i) immature sperm from the corpus of the epididymis, (ii) mature sperm from the cauda of the epididymis, which has not been exposed seminal plasma, or (iii) freshly ejaculated sperm, which had been exposed to seminal plasma. Epididymal sperm from the corpus and the cauda and fresh ejaculated sperm used as a control were coincubated for 2 h with B_{JUNO}. Four replicates were performed. Total subjective motility was 78.3 ± 6% and 21.7 ± 1.7% respectively for sperm from the cauda and the corpus of the epididymis. A higher percentage of beads with at least one sperm bound ($B_{JUNO}SB$) and a higher number of sperm bound per bead (S/B_{JUNO}) were found in the group incubated

with ejaculated sperm (99.0 ± 0.70, N=199 for B_{JUNO}SB and 19.4 ± 1.10, N=197 for S/B_{JUNO}) (P<0.001) (Figure 3.7).

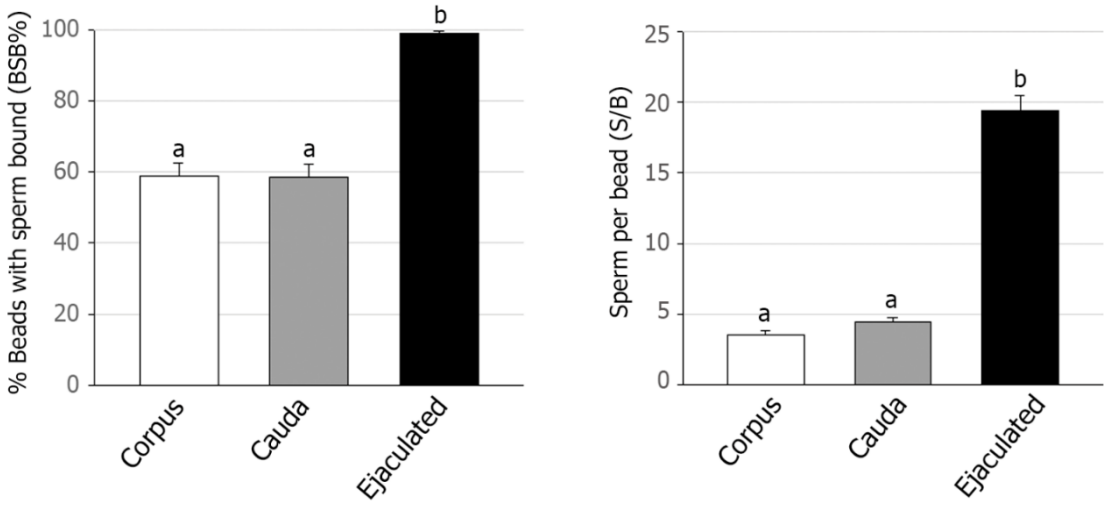

Figure 3.7. *B_{JUNO} were incubated for 2 h with 200,000 sperm/mL collected from the corpus or cauda of the epididymis or after ejaculation. After co-incubation, B_{JUNO} were fixed (0.5% glutaraldehyde), stained (0.01 mM bisbenzimide) and evaluated by epifluorescence microscope to calculate the percentage of B_{JUNO} with at least one sperm bound (B_{JUNO}SB) (left) and to count the number of sperm bound per bead (S/B) (right). A higher percentage of B_{JUNO} with at least one sperm bound and a higher number of sperm bound per B_{JUNO} were observed when incubated with ejaculated sperm (P<0.001).*

No differences were found in B_{JUNO}SB and S/B_{JUNO} between epididymal sperm from the corpus (58.9 ± 3.50, N=202 for B_{JUNO}SB; and 3.53 ± 0.29, N=119 for S/B_{JUNO}) or cauda (58.7 ± 3.40, N=206 for B_{JUNO}SB; and 4.45 ± 0.33, N=121 for S/B_{JUNO}).

3.4. Recombinant bovine JUNO beads bind more sperm cells from wild type than TMEM95 deficient bulls

A nonsense mutation in the transmembrane 95 protein (TMEM95) has been described in Fleckvieh bulls exhibiting subfertility (Pausch et al. 2014) which has been shown to be due to failure to interact with the oocyte vestments (Fernandez-Fuertes et al. 2017). The aim of this experiment was to characterize sperm-B_{JUNO} binding ability of sperm from wild type (wt/wt), heterozygous (wt/mt), and homozygous (mt/mt) TMEM95 bulls. Two bulls of each genotype were used and three replicates were performed.

B_{JUNO} were incubated for 2 h with frozen-thawed ejaculated sperm from wt/wt, wt/mt and mt/mt Fleckvieh bulls (Bayern-Genetik GmbH, Kumhausen, Germany). Following incubation, the B_{JUNO}SB was greater than 97 % for the three groups (Figure 3.8, left) demonstrating that the three genotypes had the ability to bind to B_{JUNO}. Regarding the S/B_{JUNO}, differences were found between groups being higher for B_{JUNO} incubated with wt/wt sperm (24.5 ± 1.10; N=211) than wt/mt (20.5 ± 1.10; N=198) and mt/mt sperm (16.6 ± 0.70; N=192) ($P<0.001$) (Figure 3.8, right). These results show that the 3D model can distinguish between sperm from different genotype and that sperm cells from homozygous TMEM95 deficient bulls exhibit an impaired ability to bind B_{JUNO}.

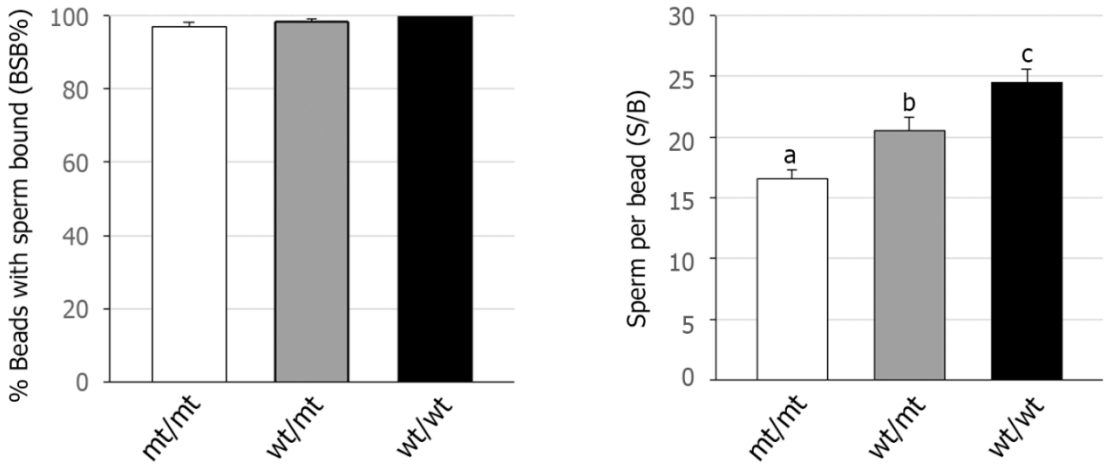

Figure 3.8. *B_{JUNO} were incubated for 2 h with 200,000 sperm/mL from wild type (wt/wt), heterozygous (wt/mt) and mutant (mt/mt) bulls. B_{JUNO} were fixed (0.5% glutaraldehyde), stained (0.01 mM bisbenzimide) and evaluated by epifluorescence microscope to calculate the percentage of B_{JUNO} with at least one sperm bound (B_{JUNO}SB) and to count the number of sperm bound per bead (S/B). B_{JUNO}SB was greater than 97% for the three groups (left) and the mean number of sperm per bead was significantly lower in the mt/mt group (P<0.001).*

3.5. Recombinant bovine JUNO beads bind more sperm cells from high fertility bulls

The objective of this experiment was to characterize sperm-B_{JUNO} binding in bulls of divergent field fertility. Holstein bulls (National Cattle Breeding Centre, Naas, Ireland) were categorized as Low or High fertility following a multiple regression mixed model which evaluates male fertility based on field factors (Berry, Evans, and Mc Parland 2011). Three replicates were performed. In each replicate, one straw from two low fertility and one straw from two high fertility bulls were selected according to cleavage rate results

when IVF was performed. High fertility bulls showed a cleavage rate >80% and low fertility bulls <40%.

Once selected, sperm was incubated with B_{JUNO}. For the two experimental groups, low and high fertility bulls, the percentage of beads with at least one sperm bound was similar and nearly 100%. Regarding the S/B_{JUNO}, the number of sperm bound per bead was significantly higher in the high fertility bull group (19.2 ± 0.9; N=310) when comparing with the low fertility bulls (13.6 ± 0.8; N=288) ($P<0.05$) (Figure 3.9).

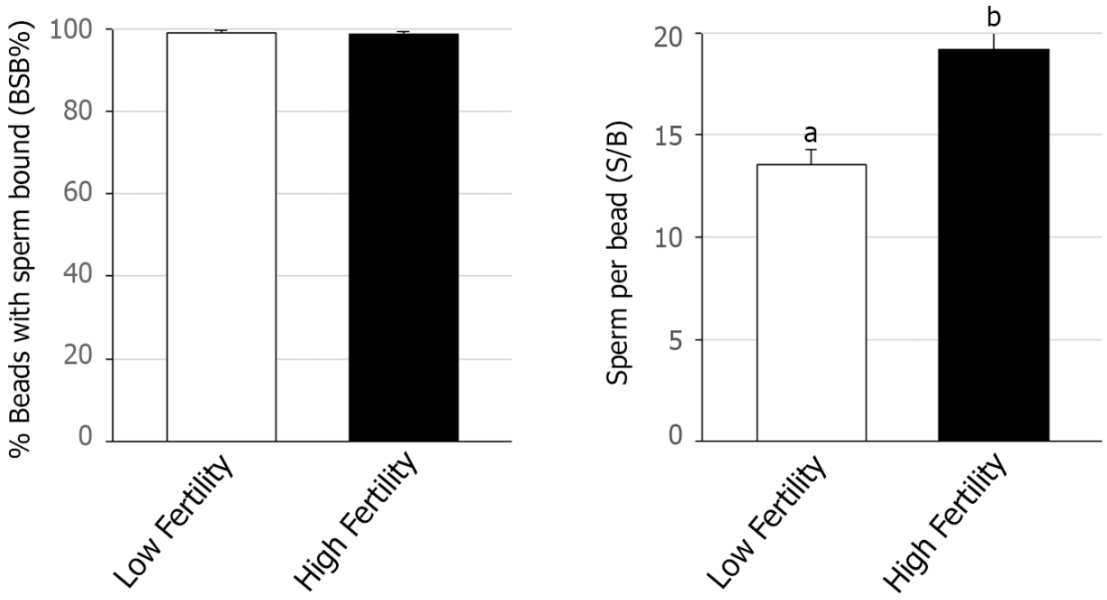

Figure 3.9. *B_{JUNO} were incubated for 2 h with 200,000 sperm/mL from Low and High Fertility bulls. After incubation, B_{JUNO} were fixed (0.5% glutaraldehyde), stained (0.01 mM bisbenzimide) and evaluated by epifluorescence microscope to calculate the percentage of B_{JUNO} with at least one sperm bound ($B_{JUNO}SB$) and to count the number of sperm bound per bead (S/B). The percentage of beads with at least one sperm bound was similar and nearly 100% for both groups. The S/B was significantly higher in the high fertility bulls group ($P<0.05$).*

4. Discussion

The molecular basis of sperm-egg recognition and fusion remains poorly described in most species. The interaction of JUNO (Bianchi et al. 2014) and IZUMO1 (Inoue et al. 2005) proteins and their essential role in fertilization has been well described in mice (Bianchi et al. 2014; Han et al. 2016; Aydin et al. 2016) and recently has been reported in humans (Jean et al. 2019). Furthermore, it is believed that the JUNO-IZUMO1 interaction is conserved within mammals since *Izumo1* and *Juno* orthologues have been found in all mammalian genomes sequenced to date (Bianchi et al. 2014). Elucidation of the molecular mechanisms involved in sperm-egg recognition in different species would benefit many applications that rely on assisted reproduction techniques, such as methods to evaluate the fertilizing ability of sperm, and consequently livestock production. However, the study of gamete interaction is challenging mainly because of the transient nature of this event and the lack of consistent assays to assess this complex process.

Here, we propose a new method to predict sperm fertilizing capacity in cattle. The method is based on the recently described 3D model using magnetic sepharose beads that mimic the shape of the oocyte, conjugated to recombinant proteins potentially involved in gamete interactions (Hamze et al. 2019). Moreover, this system is easily translated among mammals and is scalable, representing an important advance in the validation of the role of individual proteins associated with gamete recognition or fusion. Here, recombinant JUNO glycoprotein was used to evaluate the capacity of bovine sperm to bind to the beads.

JUNO is a cysteine-rich glycosylated protein (Aydin et al. 2016); therefore, heterologous mammalian cell lines are preferred to express and secrete a biologically active rbJUNO

glycoprotein to maintain functionality and allow the correct expression of the glycoprotein. To date, only recombinant murine JUNO (Bianchi et al. 2014) and recombinant human JUNO (Jean et al. 2019) glycoproteins have been designed and expressed in HEK cells. To the best of our knowledge, JUNO has not been expressed in CHO cells to date. Here, rbJUNO was successfully secreted and detected by commercial antibodies and we ensured that rbJUNO conjugation to the magnetic sepharose beads was successful and stable over time, allowing the performance of the experiments over a wide window of time. The glycoprotein conjugation to the beads and its stability has been previously described by Avella et al. (2016) and Hamze et al. (2019).

To validate the B_{JUNO} model for widespread use, sperm binding assays were performed with sperm from different origins (ejaculated, epididymal and different genotypes). The ability of the model to discriminate different types of sperm is relevant and might help to objectively test and select suitable bulls for commercial *in vitro* embryo production programs without the need to perform IVF. The binding ability of the sperm to the magnetic sepharose beads conjugated with JUNO was scored, revealing a similar binding pattern to that described in our previous study (Hamze et al. 2019). A higher mean number of sperm per bead was observed in the group including JUNO beads when compared with the group containing control beads, showing that bovine sperm specifically recognize JUNO protein. Additionally, both the mean number of sperm per bead and the percentage of sperm that had undergone the acrosome reaction increased over incubation time in a capacitating medium. Therefore, if IZUMO1 is translocated and available after the acrosome reaction (Satouh et al. 2012; Fukuda et al. 2016), the sperm will be more likely to bind to the sepharose beads conjugated to rbJUNO. This is consistent with reports that mice sperm bind to the oocyte plasma membrane by means

of JUNO-IZUMO recognition (Bianchi et al. 2014) once the sperm is acrosome-reacted (Jin et al. 2011; Satouh et al. 2012).

The acquisition of motility and fertilizing ability develops along the process of sperm maturation that takes place during the transit of sperm along the epididymis (Dacheux, Gatti, and Dacheux 2003; Cornwall 2014). To determine whether sperm ability to bind to JUNO- conjugated beads is related to its maturation stage or exposure to the seminal plasma, ejaculated sperm and sperm from the corpus and the cauda of the epididymis where incubated with JUNO beads. Although the motility of corpus sperm is lower than that of sperm collected from the cauda of the epididymis (Fernandez-Fuertes et al. 2016), no differences were observed in the evaluated parameters, suggesting that the binding ability of the sperm to the beads depends on more variables than just motility. Our results indicate that ejaculated sperm, which have been exposed to seminal plasma, have a higher binding ability to B_{JUNO}. It is known that some proteins from the seminal plasma such as PLA2, osteopontin, tissue inhibitor of metalloproteinase 2, clusterin or spermadhesin Z13 play important roles to prepare the sperm to recognize the oocyte (Moura et al. 2007).

In cattle, a putative receptor in sperm-egg interaction was described when bulls homozygous for a nonsense mutation in the gene encoding the transmembrane protein 95 (TMEM95) exhibited poor fertility (Pausch et al. 2014). IVF assays performed later suggested that the fertilization failure observed was due to the inability of the mt/mt sperm to interact with the oocyte vestments (Fernandez-Fuertes et al. 2017). Interestingly, the mean number of sperm per bead was higher for the wt/wt group than for wt/mt and mt/mt groups. In addition, the mean number of sperm per bead was

higher for the wt/mt group than for mt/mt. These results demonstrate that the model is capable of distinguishing between ejaculates with different fertilizing ability. It is important to note that the number of sperm bound to JUNO beads of the mt/mt group was higher than that observed in the control group and those recovered from the epididymis, suggesting that these sperm cells still maintain the ability to bind JUNO beads albeit in lower numbers than the wt/wt group. This would support the validation of the proposed model as indicative of the sperm fertilizing capacity, even though the reason for the infertility is not directly related to JUNO protein. Moreover, ejaculated frozen-thawed sperm from low and high fertility Holstein bulls based on field factors and selected according their cleavage rates when IVF was performed showed different ability to bind to the JUNO beads. Although no differences were found in the $B_{JUNO}SB$ parameter, with both groups having a percentage close to 100%, the mean number of sperm per bead was significantly higher in the group incubated with ejaculates from high fertility bulls.

In conclusion, results demonstrate that bull sperm bind specifically to JUNO and the proposed model supports sperm binding and it is able to discern between epididymal and ejaculated sperm. Furthermore, the assay is able to distinguish between sperm from bulls with different fertilizing capacity, and thus could be implemented as a sperm selection method to predict fertility.

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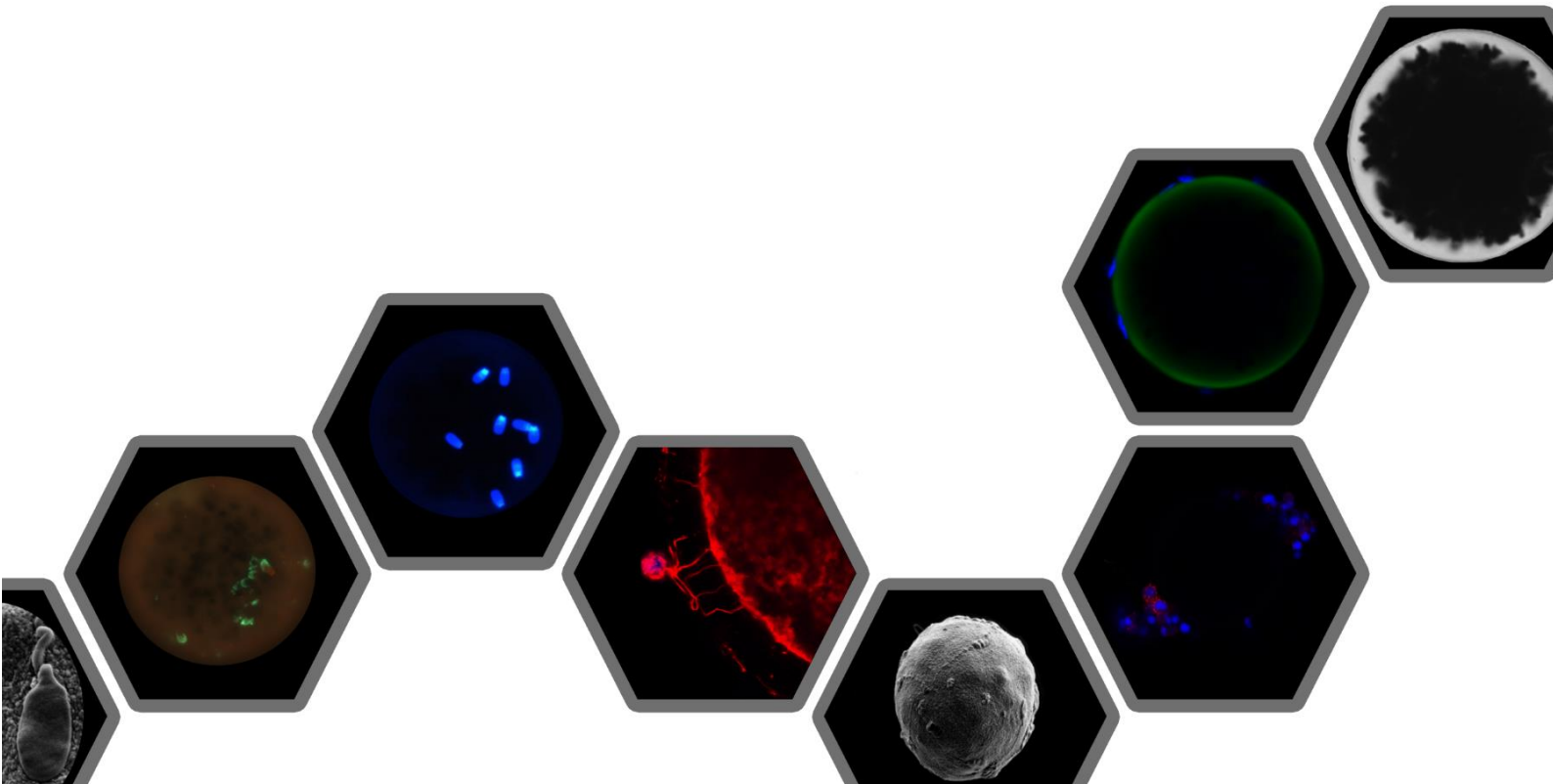
CONCLUSIONS/CONCLUSIONES



CONCLUSIONS

1. The designed recombinant porcine zona pellucida glycoproteins (ZP2, ZP3 and ZP4) and bovine JUNO were expressed and secreted in mammalian CHO cells.
2. All the above-mentioned glycoproteins were uniformly and over time stable conjugated to magnetic sepharose beads.
3. A 3D model by means of magnetic sepharose beads coated with porcine ZP2, ZP3, ZP4 and JUNO glycoproteins has been developed.
4. The 3D model mimics to some extent a native cumulus-oocyte complex by maintaining the spherical shape, supporting cumulus cells adhesion, providing a glycoprotein-specific surface, allowing sperm binding and further induction of acrosome reaction.
5. ZP2 coated beads bind the highest number of spermatozoa with the highest rate of acrosome reaction thus suggesting a key role of ZP2 protein in gamete interaction in porcine species.
6. ZP3 and ZP4 coated beads showed the higher number of acrosomal shrouds on their surface and the higher rate of unbound reacted sperm indicating that these proteins induce acrosome reaction in boar spermatozoa.
7. The efficiency of porcine IVF increases when cumulus-oocyte complexes are inseminated in presence of recombinant ZP2 coated beads.
8. Bull sperm bind specifically to JUNO coated beads suggesting that JUNO-IZUMO1 interaction is conserved in bovine species.
9. JUNO coated beads bind more ejaculated than epididymal sperm.

10. JUNO coated beads decoy a greater number of sperm cells from high fertility than low fertility bulls thus being a potential tool to predict fertility in seminal samples.

CONCLUSIONES

1. Las glicoproteínas recombinantes de la zona pelúcida porcina diseñadas (ZP2, ZP3 y ZP4) y JUNO bovino fueron expresadas y secretadas en células de mamífero (CHO) correctamente.

2. Todas las glicoproteínas mencionadas anteriormente fueron conjugadas de manera uniforme y estable en el tiempo a microesferas de sefarosa magnéticas.

3. Un modelo 3D consistente en microesferas de sefarosa magnéticas conjugadas a las glicoproteínas porcinas ZP2, ZP3 y ZP4, así como a JUNO bovino ha sido desarrollado.

4. El modelo imita en cierta medida un complejo cúmulo-ovocito nativo al mantener la forma esférica, permitiendo la unión de células del *cumulus oophorus,* presentando una superficie glicoproteica, permitiendo la unión de espermatozoides y la inducción de la reacción acrosómica.

5. Las microesferas de sefarosa magnéticas conjugadas a ZP2 unen el mayor número de espermatozoides y presentan el mayor porcentaje de espermatozoides reaccionados unidos, sugiriendo que la proteína ZP2 tiene un papel clave en la interacción entre gametos en la especie porcina.

6. Las microesferas conjugadas a las glicoproteínas ZP3 y ZP4 presentan un mayor número de restos acrosomales y el mayor porcentaje de espermatozoides no unidos reaccionados, lo que indica que estas proteínas están implicadas en la inducción de la reacción acrosómica en la especie porcina.

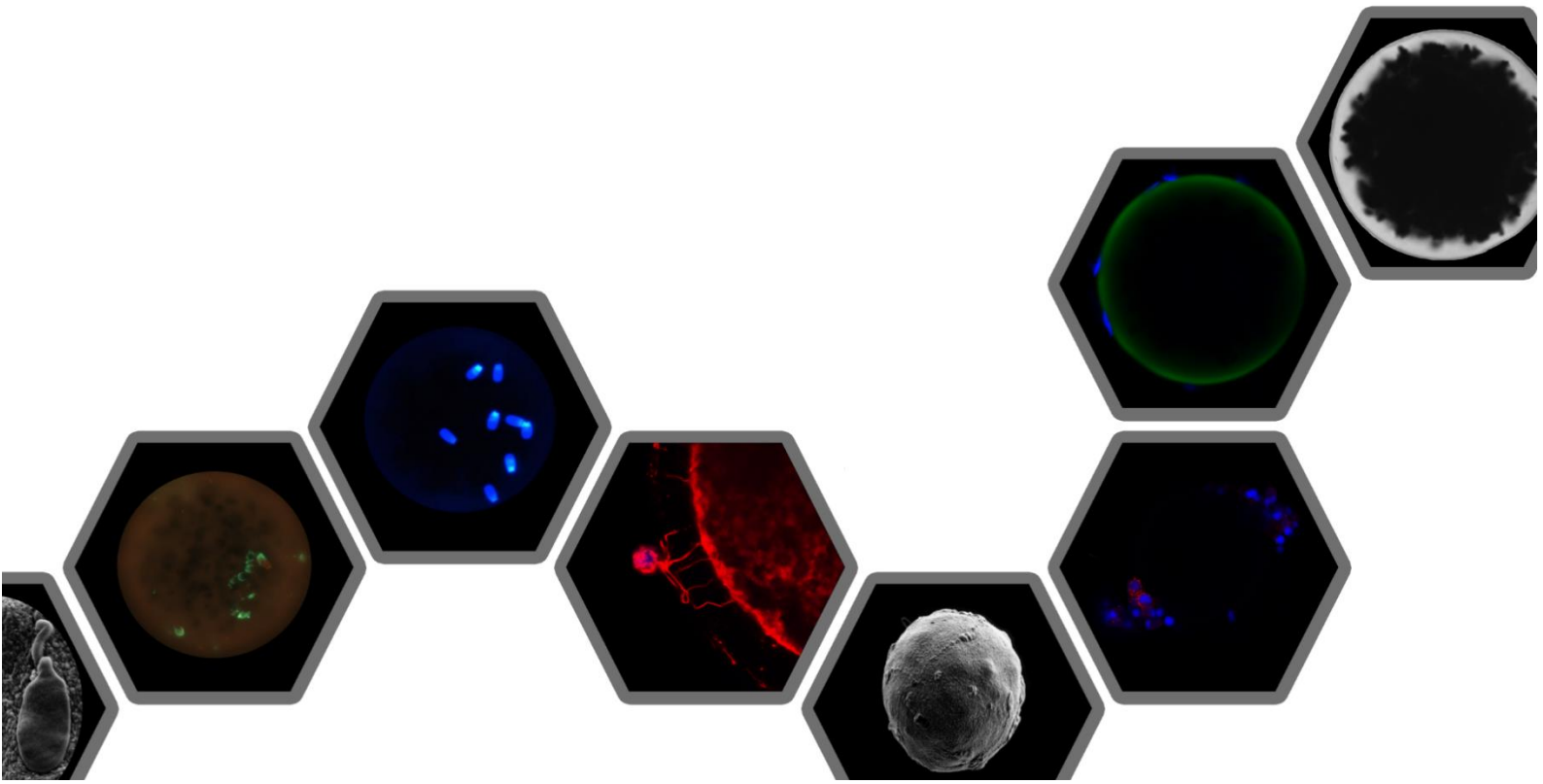
7. El rendimiento de la FIV porcina aumenta cuando los complejos cúmulo-ovocito se inseminan en presencia de microesferas conjugadas a ZP2.

8. Los espermatozoides de toro se unen a las microesferas conjugadas a JUNO, sugiriendo que la interacción JUNO-IZUMO1 está conservada en la especie bovina.

9. Las microesferas conjugadas a JUNO unen más espermatozoides eyaculados que epididimarios.

10. Las microesferas conjugadas a JUNO atraen a un mayor número de espermatozoides de toros de alta fertilidad que de baja, por lo que suponen una potencial herramienta para predecir la capacidad fecundante de una muestra seminal.

APPENDIX



APPENDIX I

Additional data

1. Details of the peptides identified and validated from the trypsin digest by MS in Chapter 1

Protein Name	Score	SPI%	Sequence
Zona pellucida sperm-binding protein 2	17.99	89.0	(K)VHSHQTKPALNLDTLR(V)
Zona pellucida sperm-binding protein 2	14.95	89.7	(K)VIYENEIHALWADPPSAVSR(D)
Zona pellucida sperm-binding protein 2	13.42	83.7	(K)APAQGLVQFR(I)
Zona pellucida sperm-binding protein 2	15.95	97.9	(K)SVNLGSGNIAVSQLHK(H)
Zona pellucida sperm-binding protein 2	9.19	96.8	(R)MVVEFPR(I)
Zona pellucida sperm-binding protein 2	8.99	83.1	(R)QPIYLEVR(I)
Zona pellucida sperm-binding protein 2	8.22	98.1	(R)LIDDNAALR(Q)
Zona pellucida sperm-binding protein 2	7.70	95.4	(R)qEALmYHISCPVmGAEGPDQHSGSTIcmKDFmSFTFNF FPGmADENVKR(E)
Zona pellucida sperm-binding protein 2	5.30	73.5	(R)VGDSScQPTFK(A)

Protein Name	Score	SPI%	Sequence
Zona pellucida sperm-binding protein 3	14.44	88.3	(R)AEVPIEcHYPR(Q)
Zona pellucida sperm-binding protein 3	14.17	92.9	(R)WSPVEGPAVIcR(C)
Zona pellucida sperm-binding protein 3	11.34	79.5	(K)cEPLVSQDTDAVVR(F)
Zona pellucida sperm-binding protein 3	8.02	71.5	(R)NTIYITcHLK(V)
Zona pellucida sperm-binding protein 3	6.53	71.0	(K)MTPTFQLGDR(A)
Zona pellucida sperm-binding protein 3	5.44	70.2	(K)ACsFSKsSNR(W)
Zona pellucida sperm-binding protein 3	5.19	84.3	(R)LMEENWSAEK(M)

Protein Name	Score	SPI%	Sequence
Zona pellucida sperm-binding protein 4	17.55	91.5	(R)LPcAPPPITQGEcK(Q)
Zona pellucida sperm-binding protein 4	15.21	91.3	(R)VTGNQAVYENELVAAR(D)
Zona pellucida sperm-binding protein 4	9.55	94.2	(K)ASNLLFPSHYQR(F)
Zona pellucida sperm-binding protein 4	7.99	92.9	(R)FPFSScGTAK(R)
Zona pellucida sperm-binding protein 4	5.09	88.3	(R)EPIYVEVSIR(H)

APPENDIX II

Publications derived from the Doctoral Thesis

The results obtained in the present Doctoral Thesis have been included in the following publications:

1. **ARTICLES IN JOURNALS INCLUDED IN THE SCIENCE CITATION INDEX (SCI) OF THE INSTITUTE FOR SCIENTIFIC INFORMATION (ISI):**
    - **Hamze JG**, Canha-Gouveia A, Algarra B, Gómez-Torres MJ, Olivares MC, Romar R, Jiménez-Movilla M. (2019). Mammalian spermatozoa and cumulus cells bind to a 3D model generated by recombinant zona pellucida protein-coated beads. Sci Rep 2019;9:17989. https://doi.org/10.1038/s41598-019-54501-7.
2. **ABSTRACTS IN JOURNALS INCLUDED IN THE SCIENCE CITATION INDEX (SCI):**
    - **Hamze JG**, Jimenez-Movilla M, Romar R. Cumulus-oocyte complexes-like 3D models to analyze sperm binding. 35th AETE conference. 13th-14th September 2019, Murcia (Spain). Poster. Anim. Reprod., v.16, n.3, p.779, Jul./Sept.
    - **Hamze JG**, Romar R, Jiménez-Movilla M. Cumulus cells projections on ZP coated beads. XX Congreso de la Sociedad Española de Histología e Ingeniería Tisular, VIII International. Congress of Histology and Tissue Engineering, VI Congreso Iberoamericano de Histología. 4th-6th September 2019, Murcia (Spain). Poster. Histol Histopathol 34 (Suppl. 1)-S243.
    - **Hamze JG**, Jiménez-Movilla M, Romar R. Cumulus-oocyte complexes-like 3D models to study gamete interaction in porcine species. IX International

Conference on Boar Semen Preservation (ICBSP). 11th-14th August 2019, Sidney (Australia). Poster. Theriogenology 2019, 137: 133. Abstract 4.5.

- **Hamze JG**, Romar R, Jimenez-Movilla M. Design of pJuno-beads to study the molecular mechanisms of sperm-oocyte interaction. 34th AETE conference. 7th-8th September 2018. Nantes (France). Poster and Oral communication. Animal Reproduction v.15, n.3, p.594, Jul./Sept.
- **Hamze JG**, López-Úbeda R, Canha-Gouveia A, Jimenez-Movilla M, Romar R. *In vitro* assessment of acrosomal status of boar sperm bound to beads conjugated to ZP proteins. 2017. 33rd AETE conference. Bath (United Kingdom). 8th-9th September 2017. Oral communication and poster. Winner of the best poster. Animal Reproduction 14 (3): 974.
- **Hamze JG**, Canha A, Zamorano L, Algarra B, Olivares MC, Romar R, Jimenez-Movilla M. Desarrollo de un modelo en 3D para estudiar la interacción entre gametos. Información Técnica Económica Agraria (ITEA) XVII Jornadas sobre producción animal de la Asociación Interprofesional para el Desarrollo Agrario (AIDA). 30th-31st May 2017. Zaragoza (Spain). "Young researcher's prize 2017" to the best oral communication in the Reproduction sessions. ISBN: 978-84-697-3065-2; pp 338-340.
- **Hamze JG**, Canha A, Zamorano L, Algarra B, Olivares MC, Romar R, JimenezMovilla M. Porcine sperm bind to beads conjugated to ZP2 protein under *in vitro* conditions. 32nd AETE meeting. September 2016. Barcelona (Spain). Oral communication and poster. Animal Reproduction, [S.l.], v. 13, n. 3, p. 647-647, mar. 2016. ISSN 1984-3143.

3. COMMUNICATIONS TO CONGRESS

- Hamze JG, López-Úbeda R, Olivares MC, Jiménez-Movilla M, Romar R. Evaluación *in vitro* de espermatozoides porcinos unidos a esferas magnéticas conjugadas a proteínas de la Zona Pelúcida. II Jornadas científicas del IMIB-ARRIXACA. November 2017. Murcia (Spain). Oral communication.
- **Hamze JG**, Canha A, Zamorano L, Algarra B, Olivares MC, Romar R, JimenezMovilla M. ZP proteins conjugated with magnetic beads to generate 3D models to study gamete interaction. I Jornadas científicas del IMIB-ARRIXACA. November 2016. Murcia (Spain). Oral communication

APPENDIX III

List of abbreviations

ZP	Zona pellucida
3D	Three-dimensional
B	Magnetic sepharose beads
B_{ZP}	Magnetic sepharose beads coated with single recombinant porcine ZP glycoproteins
B_{ZP2}	Recombinant ZP2 glycoprotein coated beads
B_{ZP3}	Recombinant ZP3 glycoprotein coated beads
B_{ZP4}	Recombinant ZP4 glycoprotein
PNA-FITC	Peanut agglutinin lectin - Fluorescein isothiocyanate
B_{JUNO}	Recombinant bovine JUNO glycoprotein coated beads
ART	Assisted reproductive techniques
AR	Acrosome reaction
COCs	Cumulus-oocyte complexes
LH	Luteinizing hormone
aa	Amino acids
THP	Tamm-Horsfall protein

GP-2	Glycoprotein-2
(TGF)-β	Transforming growth factor
TD	Trefoil domain
CFCS	Consensus furin cleavage site
TMD	Transmembrane-like domain
WT	Wild type
IVF	*In vitro* fertilization
ICSI	Intracytoplasmic sperm injection
GPI	Glycosylphosphatidylinositol
GV	Germinal vesicle
MII	Metaphase II
TSSK	Testis Specific Kinase
SPACA4	Sperm acrosome membrane-associated 4
TMEM95	Transmembrane protein 95
AI	Artificial insemination
PtdSer	Phosphatidylserine
TZP	Transzonal projections
3R	Principle of Replacement, Reduction and Refinement

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cas	CRISPR-associated proteins
B_{Ctrl}	Beads control
rbJUNO	Recombinant bovine JUNO glycoprotein
DNA	Deoxyribonucleic acid
CHO	Chinese hamster ovary
RT	Room temperature
CB_{ZP}	Cumulus mass plugs around ZP coated beads
AIC	Aritificial insemination center
BTS	Belstville thawing solution
PBS	Phosphate buffered saline
PI	Propidium iodide
$B_{ZP}SB$	Percent of beads with at least one sperm bound
S/B_{ZP}	Mean number of sperm per bead
SPI	Scored Peak Intensity
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TS/B_{ZP}	Total number of sperm bound to B_{ZP}

FESEM	Field emission scanning electron microscopy
eCG	Equine chorionic gonadotropin
hCG	Human chorionic gonadotropin
NRR	Non-return rate
ROS	Reactive oxygen species
NCBI	National Center for Biotechnology Information
BSB	Percentage of magnetic beads with at least one sperm bound
S/B	Mean number of sperm bound to each bead
wt/wt	Wild type
wt/mt	Heterozygous
mt/mt	Homozygous

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«Lo único que sabemos es lo que nos sorprende: que todo pasa, como si no hubiera pasado»

-Silvina Ocampo