Why does a spermatozoon and only one manage to fertilize an oocyte?

Nature and kinetics of sperm neutralization after a first fertilization

Direction : Christine Gourier, Laboratoire de Physique de l'ENS, UMR 8023, Team Molecular Membrane Mechanisms, Paris.

Contact : Christine.gourier@phys.ens.fr, http://www.lps.ens.fr/M3/research/fertilization/

Co-direction : Tzviya Zeev Ben Mordehai, Bijvoet Centre for Biomolecular Research, Team Cellular Structural Biology of Fertilization Utrecht University, the Netherland.
Contact : z.zeev@uu.nl, https://www.uu.nl/en/research/structural-biochemistry/our-research/dr-tzviya-zeev-ben-mordehai/

Context

The thesis is part of our research on fertilization, carried out in a multidisciplinary way within the Molecular Membrane Mechanism team of the Laboratoire de Physique de l'ENS Paris, in collaboration with biologists and physicians of reproduction in France and abroad. We characterize the processes involved in fertilization in terms of mechanical constraints, molecular organization, membrane dynamics and structure by implementing physical approaches (quantification of molecular bonds by picoforce measurements, membrane adhesions by micromanipulation of individual objects, quantitative imaging, microfluidics etc) and by developing models inspired by soft matter physics. The thesis will be carried out in co-supervision with Tzviya Zeev Ben Mordehai, structural biologist who leads complementary research on the cellular structural biology of fertilization using Cryo-electron tomography at the Bijvoet Center for Biomolecular Research of Utrecht University, the Netherlands.

Project

The thesis project focuses on the mechanisms of prevention of polyspermy, a pathological situation resulting from the fertilization of an oocyte by more than one sperm. Polyspermy results in a surplus of paternal genetic material that is fatal to embryo development. Studies in human indicate that 13% of spontaneous abortions result from polyspermy [1-3]. This occurrence is even higher during in vitro fertilization, frequently used in medicine as a method of assisted procreation, but also in the food industry to optimize and control animal birth Elucidating the mechanisms that allow a fertilized oocyte to avoid polyspermy is therefore of fundamental, medical and economical interest.

To fertilize, a sperm must cross 2 successive "barriers". The first one is the zona pellucida, a glycoprotein envelope that surrounds the oocyte and delimits the space (i.e. perivitelline space) in which the oocyte is isolated from its environment. The second barrier is the plasma membrane of the oocyte with which the spermatozoon must fuse to deliver its genetic material. When a first spermatozoon manages to cross these two barriers, fertilization takes place. As early as the 1930s-1950s, the oocytes, observed after mating of mammals in several species, revealed that after fertilization, the number of spermatozoa managing to cross the first barrier (i.e. zona pellucida) decreased over time, and more interestingly, those who passed it were generally unable to pass the second one (i.e. fusion with the plasma membrane) [4-5]. These observations have been interpreted as the evidence of the deployment by the oocyte of "firewalls", triggered by the first sperm, that stop the progression of any further sperm at either of these barriers. These firewalls were imagined as post-fertilization physico-chemical modifications of both the zona pellucida and the plasma membrane making them impassable [5].

This vision of the fertilized oocyte acting to lose its receptivity to sperm, has fueled all the studies published to date on the mechanisms that block polyspermy. Mostly carried out on mice

(considered as a good human model for fertilization), these studies have confirmed an impermeabilization of the zona pellucida following a first fertilization, and have elucidated the involved molecular mechanisms [6]. They also revealed a loss of adhesion and fusion properties of the oocyte plasma membrane after fertilization [7]. Long unexplained, the latter may recently have found a possible molecular origin in the post-fertilization shedding of CD9 and JUNO [8-9], the two oocyte membrane proteins identified to date as essential for the interaction step leading to fusion [8-10]. However, as both the receptivity of the zona pellucida and the receptivity of the oocyte plasma membrane to sperm were shown to gradually decrease within 1 hour after fertilization [7], neither zona pellucida nor plasma membrane inhibition can explain why almost all of the spermatozoa that entered the perivitelline space during this hour were unable to fuse.

Faced with this impasse, we have reconsidered the common vision of the firewalls deployed by the fertilized oocyte and explored the new possibility that it can directly attack and neutralize any spermatozoon that jeopardizes its monospermic status when crossing the zona pellucida and entering the perivitelline space. For that, we have developed a biophysical method to extract the living sperm from the perivitelline space of the fertilized oocytes, bring them in contact with the plasma membrane of an unfertilized oocyte, and quantify the sperm capacity to adhere and fuse with it. Unlike the control sperm (i.e. sperm that had never seen an oocyte before) that strongly adhered and easily fertilized, the sperm from the perivitelline space of fertilized eggs did not adhere at all and therefore did not fertilize. Interestingly, after immunostaining, these sperm were showed to be covered with JUNO and CD9, the two oocyte proteins released by the egg membrane after fertilization. Cryo-electron tomography of these sperm revealed a multitude of vesicles of 50 nm size, bound to their head. These exciting results, obtained in collaboration with Tzviya Zeev Ben Mordehai's group prove the inhibition of the sperm from the perivitelline space of fertilized eggs regarding its capacity to adhere and fuse, and strongly suggests that this inhibition is due to the binding of JUNO- and CD9- bearing vesicles released in the perivitelline space after fertilization that form a repulsive mattress [11].

This study has allowed a significant advance on the almost century-old question of the mechanisms blocking polyspermy: it reveals the existence of a firewall allowing the fertilized oocyte to adopt an offensive position towards undesirable spermatozoa by inhibiting their receptivity to the oocyte plasma membrane and no longer just a defensive position by neutralizing its own receptivity to sperm.

To what extent sperm neutralization outweighs that of the oocyte in actually blocking polyspermy remains an open question that only a study of the kinetics of this neutralization can answer. This is the question to which the thesis will be devoted.

The working hypothesis of the PhD project is that the fertilization-triggered release in the perivitelline space of egg components (among which CD9- and Juno- bearing vesicles than specifically bind to the sperm head) are able within minutes to significantly change the perivitelline space into a "hostile" fertilization environment where any entering spermatozoon behaves like of magnet for these components and is quickly passivated, inhibiting its adhesion and fusion properties.

The working hypothesis will be addressed though 3 complementary approaches mastered in one of the Paris or Utrecht labs:

1-*dynamics approach (Paris)*: the PhD student will study the kinetics of the release of JUNO- and CD9- bearing extracellular vesicles in the perivitelline space to determine the amount of passivating material sperm faces when entering the perivitelline space. The objective is to obtain reference fluorescent curves for JUNO and CD9 concentrations in the perivitelline space as a function of post-fertilization time in *in-vitro* conditions as close as possible to physiological, for typically 3h after

insemination time (which should be enough time to have fertilization + established blockages of the zona pellucida and oocyte's plasma membrane). In a second step, the PhD student will quantify the kinetics of JUNO- and CD9-bearing extracellular vesicles binding on sperm as a function of perivitelline space composition or equivalently post-fertilization time (the former being directly connected to the latter according to previously obtained reference curves). This will be done by incubating sperm for a controlled time with small synthetic unilamelar vesicles of controlled CD9 and JUNO compositions or directly from perivitelline content previously extracted from the perivitelline space of fertilized oocytes, and measuring sperm coverage.

2- *Functional approach (Paris)* The PhD student will correlate through *in vitro* fertilization assays at single cells level, the level of JUNO- and CD9-extracellular vesicles bound to sperm to the inhibition of its capacity to bind and/or fuse with a ZP-free egg. The objective is to determine a threshold level among which it is not anymore able to fuse and, by cross-checking results from approach 1, the time required for that.

3- *Structural approach (Utrecht)*: The PhD student will apply Cryo-electro tomography to image the extracellular vesicles bound to their head and to study vesicle interaction with the sperm membrane. Gold labelling of JUNO and CD9 will be done. If multiple copies of a protein appear on the vesicles bound to the sperm (which will probably be the case especially at the interface between the vesicles and the sperm head), sub-volumes containing the proteins will be computationally extracted, aligned and averaged (sub-tomogram averaging), with the aim to obtain the first structure characterization of these proteins on membranes.

To conclude, this project is designed to approach from a new perspective the longstanding debate on the polyspermy prevention in mammals. We expect the results to significantly advance fundamental knowledge and ultimately to have applications ranging from treatment of human infertility to enhancement of livestock production.

References

[1] Hassold et al (1980). Ann Hum Genet 44, 151-178.
[2] McFadden et al (2002). Hum Reprod 17, 3037-3038.
[3] Zaragoza et al(2000) Am J Hum Genet 66, 1807-1820.
[4] Lewis, W.H. (1935) Contrib. Embryol. 25, 113-143.
[5] Braden, A.W.H et al (1954) Aust. J. Biol. Science,7, 391-409.
[6] Fahrenkamp, E. et al (2020) Mol Reprod Dev, 1-15.
[7] Evans, J.P. (2020) Mol Reprod Dev, 1-9.
[8] Ravaux, B. et al (2018) J Mol Cell Biol 10, 494-502.
[9] Bianchi, E. et al (2014) Nature 508, 483-487
[10] Jegou, A. et al (2011). Proc Natl Acad Sci USA 108, 10946-10951.
[11] Article in preparation