

Infertility as a matter of communication: getting the message from sperm to oocyte

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Fertile and infertile states of reproductive health

Birth is often referred to as “the miracle of life” and very rightly so, since successful pregnancy represents a complex, highly coordinated succession of biological processes. In this regard, it is remarkable that pregnancy ever occurs at all.

In order for pregnancy to occur, the two gametes (sperm and oocyte) must meet (Figure 1) and the oocyte must be released from meiotic arrest. However, when sperm is deposited inside the female reproductive tract, an acidic vaginal pH, the presence of viscous cervical mucus and attack by the immune system ensure that only a few sperm make it successfully to the narrow utero-tubal junction. Not surprisingly, sperm suffer considerable damage during this dangerous journey, including DNA fragmentation. Therefore, before moving into the labyrinthine oviduct lumen, sperm undergo a form of biological selection, which is not yet completely understood but is thought to depend upon particular proteins carried on their surface. Sperm then attach to a very specific portion of the Fallopian tubes known as the reservoir, where they undergo a final maturation process called capacitation, in which, amongst other biological features, they acquire a hyper-motile state. After this, sperm detach and respond to chemical and temperature gradients to swim up along the Fallopian tube up into the ampulla, where the arrested oocyte awaits. Herein, sperm must encounter the zona pellucida, a protective glycoprotein coat that surrounds the oocyte. Contact with the zona pellucida elicits a mechanism referred to as the acrosomal reaction, in which the sperm membrane fuses with a cap-like structure in its own head (the acrosome), releasing its enzymic contents to break down the zona pellucida and thus allow the sperm to bind to the oocyte membrane and induce the process of fertilization.

Quite understandably, there are many steps along the way in which this complex process can go wrong, all of which represent a risk to fertility. Generally, infertility can arise because of a problem with the male, the female or a combination of both. Unfortunately, there is also a large proportion of infertility that remains unexplained (Figure 2).

So how can we treat infertility?

Luckily, the answer to this question is continually evolving, as the diagnostic and therapeutic tools at our disposal are constantly expanding and improving. Collectively, these techniques are referred to as Assisted Reproductive Technologies (ARTs) and refer to the *in vitro* handling and manipulation of sperm, eggs or embryos in attempt to achieve successful fertilization, implantation and clinical pregnancy. A brief description of some core ARTs is given in Figure 3.

The application of such techniques has completely revolutionized the way doctors and scientists view conception. ARTs have been responsible for over 5 million live births and may represent our only hope to balance global demographics in the future, particularly in Europe. However, their use has understandably drawn attention from ethical, legal and regulatory bodies across the world. Moreover, while ARTs have answered the dreams of millions of couples, the truth of the matter is that their success rates rarely exceed 35%. Additionally, these techniques often require multiple rounds of treatment in order to be successful, at great personal and economical cost for the couples involved. The reasons underlying this disappointing figure predominantly lie in the artificial methods and environments used, thus creating a significant challenge for the scientific/clinical research community to address. In this context, male infertility stands out as a particularly prominent candidate for improvement,

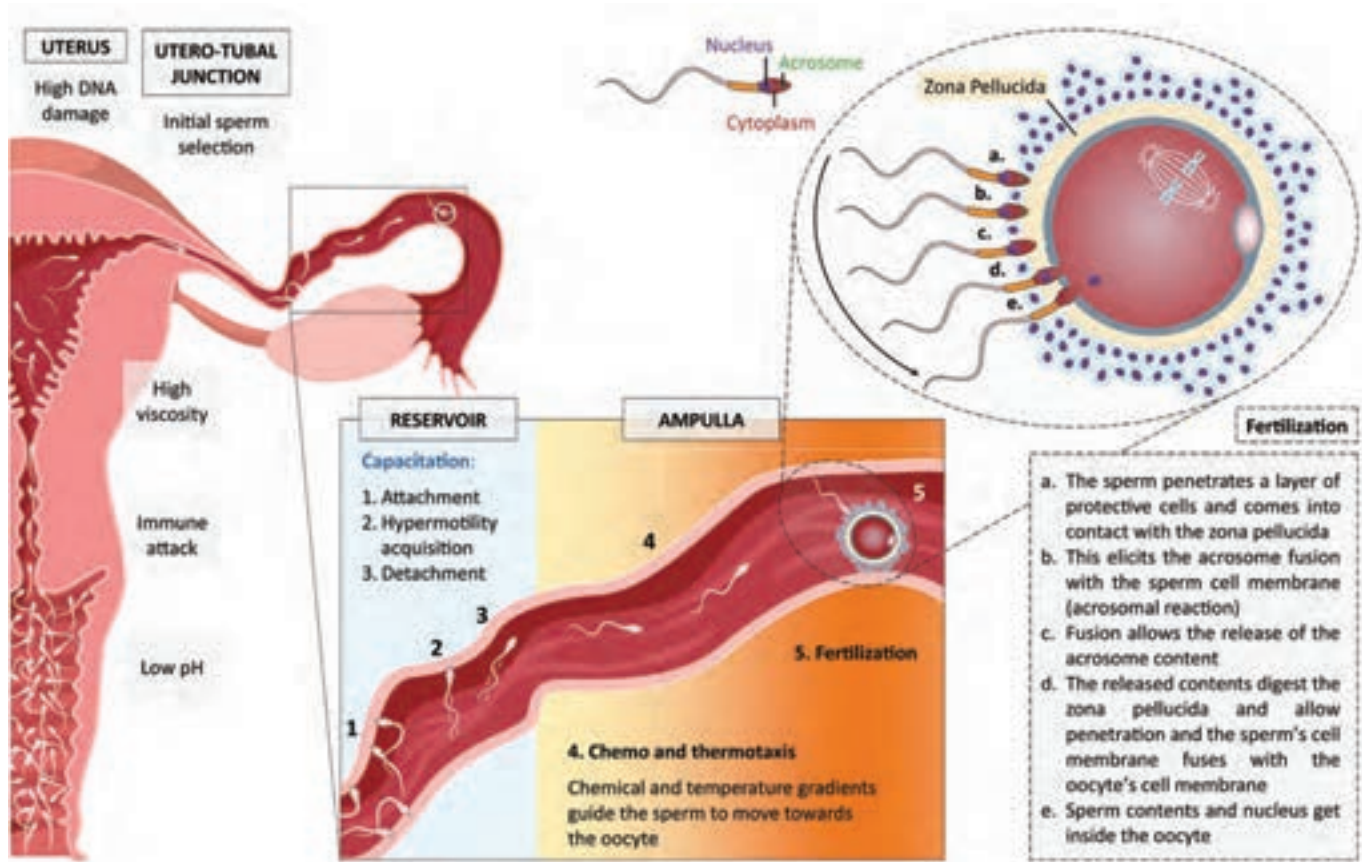


Figure 1. Overview of reproductive events leading to fertilization.

as our ability to diagnose and treat this condition is currently compromised by rather rudimentary morphological or biological parameters, which do not allow us to consider key genes or proteins. A classic example of this is a protein called PLC ζ .

Communication between oocyte and sperm: the critical role of phospholipase C zeta (PLC ζ)

The application of ARTs depends upon diagnosis on a case-by-case basis and also upon a specific couple's response to previous treatment. In terms of assessing male infertility, the first step involves obtaining an ejaculated sperm sample from the patient and carrying out a series of specific tests designed to garner information relating to sperm count, motility, morphology, pH and volume. These parameters are then compared to reference figures published by the World Health Organization (WHO). The patient's sperm can hence be classified into different categories of abnormality. Abnormalities include low sperm count (oligozoospermia), an absence of sperm (azoospermia), reduced motility (asthenozoospermia), a significant proportion of dead sperm (necrozoospermia), and morphology abnormalities (teratozoospermia) (Figure 4). These tests are used in clinics across the world, and generally lead to a clear diagnosis and the advocacy

of a specific treatment, that is, the one that most simply and effectively bypasses the defect presented by the patient. However, there is increasing concern that such assessments are rather superficial and do not provide any specific information on DNA quality or the competence

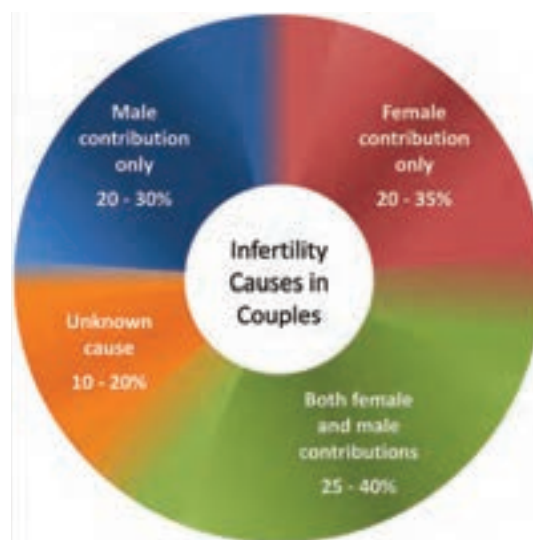


Figure 2. Infertility aetiologies. Pie chart representing the proportion of infertility by causative factor (according to the 2018 ART fact sheet from the European Society for Human Reproduction and Embryology).

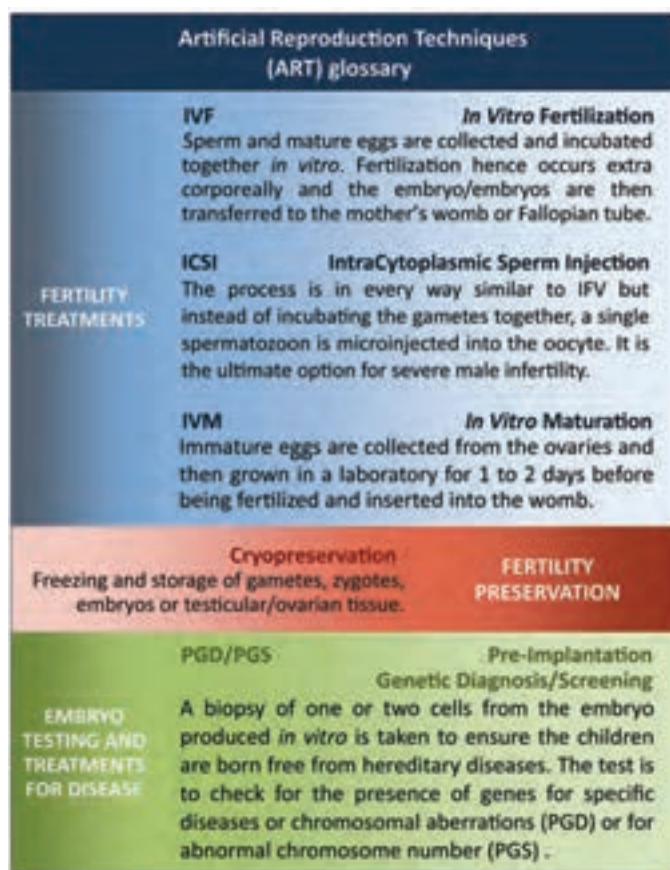


Figure 3. A brief overview of Assisted Reproductive Technologies including fertility treatments, fertility preservation, embryo testing and treatments for disease.

of particular proteins that play critical biological roles. This is especially pertinent in infertile patients who exhibit normozoospermia, which, despite meeting the standard WHO tests' requirements (Figure 4), are still unable to conceive.

Such normozoospermic patients can be infertile because reaching the oocyte is not the end of the race. After penetrating the egg, the sperm then plays a crucial role in alleviating the oocyte from meiotic arrest. Known as oocyte activation, this process also causes the oocyte to release cortical granules (the so-called 'cortical reaction'), which cause the oocyte membrane to harden and thus prevents penetration by any further sperm. The nucleus of each gamete must then fuse to generate a zygote, and divisions must begin in a coordinated manner to produce an early embryo which must travel to the uterus and successfully attach to its wall (implantation).

For over a century now, scientists have debated how a sperm could elicit this absolutely fundamental mechanism of oocyte activation. It is currently accepted that there is a specific trigger within the sperm that initiates conversation between the two gametes when they meet; this is referred to as the "sperm factor" and was identified as a unique

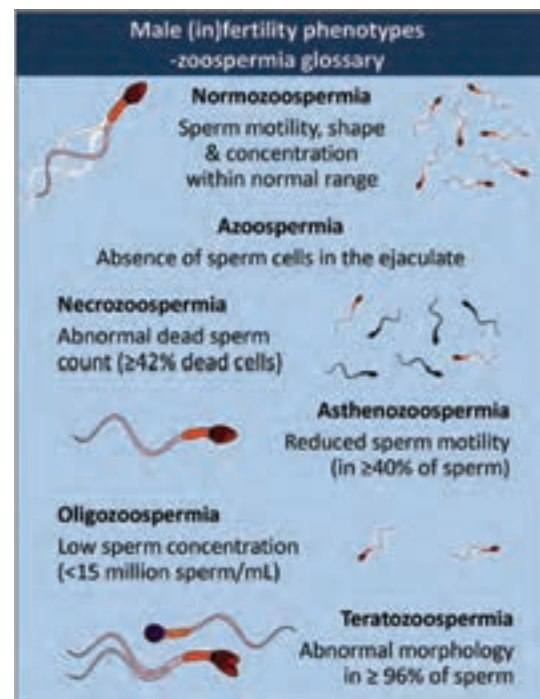


Figure 4. Male sperm phenotypes: a glossary of terms used for the classification of male infertility on the basis of sperm morphology, motility and concentration.

form of phospholipase C, phospholipase C zeta (PLC ζ), which resides within the sperm's cytoplasm. While inside the sperm, PLC ζ remains inactive, but upon fertilization, and via an as yet unknown mechanism, it becomes active and initiates a series of events that ultimately culminate with the release of calcium ions (Ca^{2+}) from the oocyte's endoplasmic reticulum. This ionic release adopts a characteristic, oscillatory pattern, which controls the events that release the oocyte from meiotic arrest, activate it and initiate embryonic development (Figure 5).

Given the fundamental role that PLC ζ plays in activating an oocyte, it is not surprising that male fertility has an implicit relationship with the level, localization and functional activity of PLC ζ . Indeed, our own research, and that of other groups, has shown that the sperm of some infertile patients have low levels of PLC ζ or are completely devoid of PLC ζ . In other patients, PLC ζ shows abnormal localization in the sperm head and/or impaired functionality due to genetic mutation or other problems at the molecular level.

This knowledge has led to the development of a variety of tools which we can use for the diagnosis and treatment of oocyte activation deficiency (OAD). However, PLC ζ

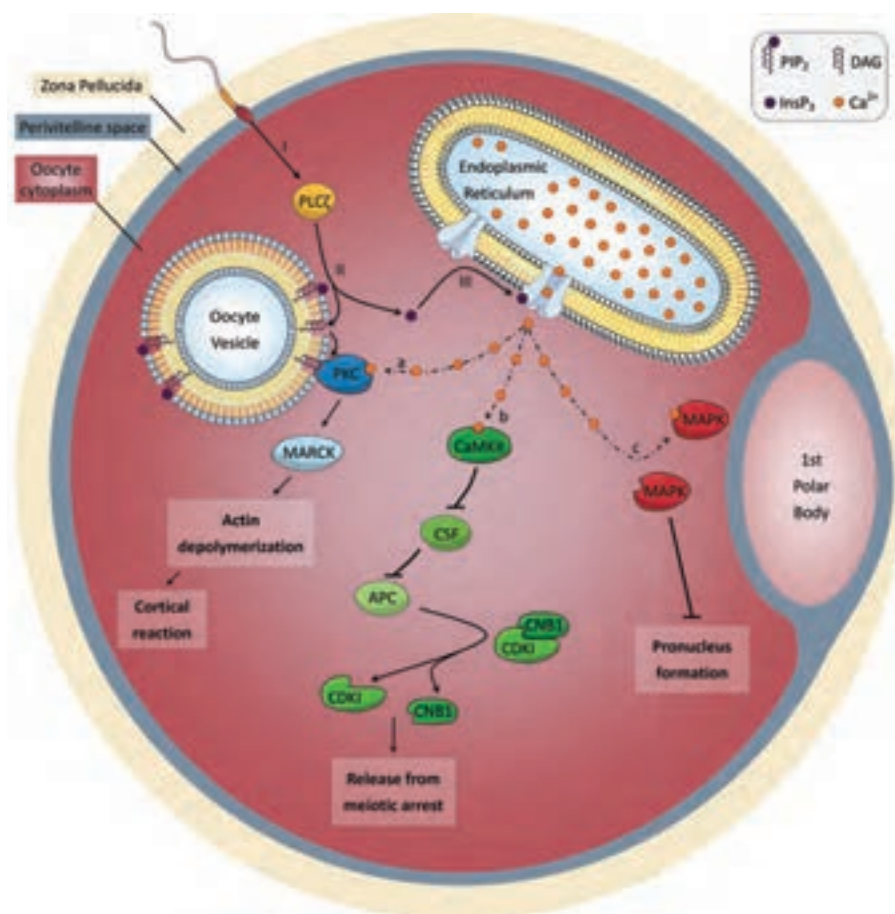


Figure 5. The molecular pathway of PLC ζ . After the sperm penetrates the zona pellucida and the two cytoplasmic membranes fuse, PLC ζ gets inside the oocyte where I) it suffers an as yet unknown mechanism of activation. II) PLC ζ then Hydrolyze oocyte vesicle membrane component PIP₂, yielding InsP₃ (membrane bound) and DAG (soluble). III) InsP₃ goes on to bind the InsP₃ receptor on the endoplasmic reticulum membrane, allowing the release of Ca²⁺ ions. Ca²⁺ release elicits several different responses (a, b and c) within the cell: a) In the presence of Ca²⁺, PKC binds the membrane-bound DAG and becomes active, eliciting a pathway that culminates with the cortical reaction. b) Ca²⁺ activates CaMKII, which in turn inhibits CSF from inhibiting APC. The liberated APC degrades CNB1, which, together with CDK1, forms a complex responsible for maintaining meiotic arrest. CNB1 degradation inactivates the complex, allowing for the meiosis to resume. c) MAPK actively inhibits pronucleus formation; upon binding with calcium ions, MAPK is inactivated, allowing the oocyte's pronucleus to form, which then goes on to fuse with the sperm's pronucleus. (PIP₂ = phosphatidylinositol 4,5-biphosphate; InsP₃ = inositol-1,4,5-triphosphate; DAG = diacylglycerol; PKC = protein kinase C; MARCK = myristoylated alanine-rich C-kinase; CaMKII = calmodulin-dependent protein kinase II; CSF = cytotstatic factor; APC = anaphase-promoting complex/cyclosome; CNB1 = cyclin B1; CDK1 = cyclin-dependent kinase I; MAPK = Mitogen-activated protein kinase).

cannot be investigated in patients using the current suite of WHO-controlled semen analyzes. Patients afflicted by oocyte activation problems have had to wait for the scientific community to 'catch up'.

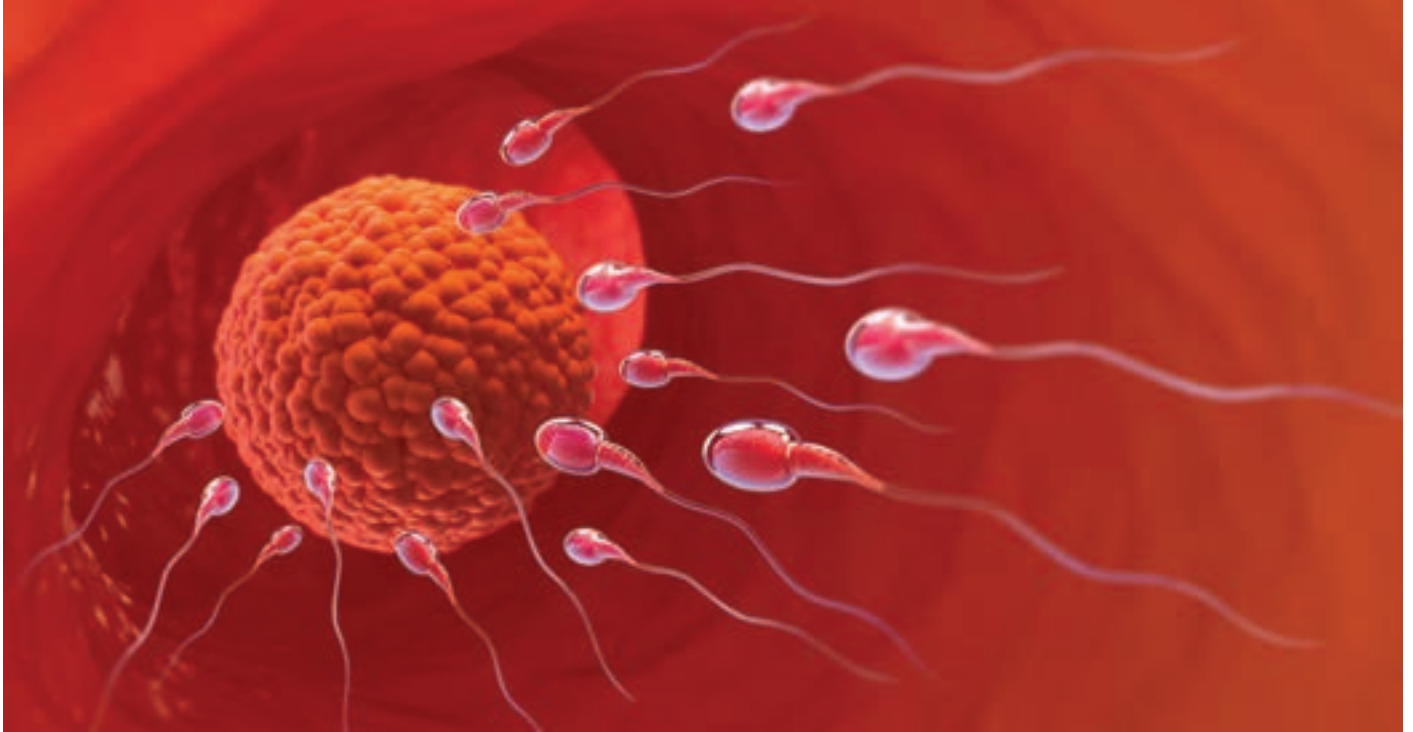
Using PLC ζ to diagnose and treat male infertility

Our group, and others, have already mapped the localization, expression and function of PLC ζ in normal fertile males. But how exactly can our knowledge of PLC ζ be of use in treating infertility? First of all, PLC ζ has great potential as a diagnostic tool, especially for patients who experience recurrent, unexplained intracytoplasmic sperm injection (ICSI) failure (which is estimated to happen 1-5% of the time). Indeed, our team has been able to link abnormalities in the localization, expression and function of PLC ζ to infertility. However, the molecular mechanisms underlying these abnormalities are complex and require further investigative research. As part of a wider research project, and exciting collaborative ventures with Oxford Fertility (Oxford, UK) and the Assisted Conception Unit, Ninewells Hospital (Dundee, UK), we have developed

simple, yet efficient, research assays with which to investigate PLC ζ expression and localization in infertile males who comply with a series of specific inclusion criteria. These assays provide us with important information relating to the oocyte activation ability of a particular patient's sperm, which can then be used by clinicians to develop appropriate clinical management strategies.

But what are the therapeutic options open to such patients? Currently, the only possible option is a technique known as Artificial Oocyte Activation (AOA), in which an artificial agent (commonly a Ca²⁺ ionophore) is applied to the oocyte during conventional ICSI to induce the release of Ca²⁺. While this successfully bypasses the sperm's inability to release Ca²⁺ within the oocyte and therefore initiate activation events, there is a potential problem. Unfortunately, these chemical agents cannot accurately reproduce the specific pattern of Ca²⁺ oscillations that occur naturally. Instead, they lead to a single Ca²⁺ transient, akin to unleashing an uncontrolled tsunami of Ca²⁺ within the oocyte. It is important to remember that the actions of PLC ζ do not just activate the oocyte, they are also involved in regulating gene expression in the early embryo via Ca²⁺-sensitive transcription factors. The

Based on a figure (used with permission) published in: Oocyte activation deficiency: a role for an oocyte contribution? Yeste, M., Jones, C., Amdani, S.N., Patel, S. and Coward, K. (2016), Hum. Reprod. Update, **22**(1), pp. 23–47



characteristic oscillatory pattern of Ca^{2+} release is therefore important in terms of the regulation of gene expression. With this in mind, and despite the fact that artificial oocyte activating agents have been used by some clinics for several years, there are some concerns over the potential effects of abnormal gene expression and research effort is understandably gaining pace in this key area.

A more effective, and safer, approach might therefore be the administration of a recombinant version of PLC ζ which has been pre-tested for its ability to release Ca^{2+} in an appropriate oscillatory manner. However, this is not proving easy. Although PLC ζ was first identified as far back as 2002, there is still no clinically-approved recombinant PLC ζ protein available on the commercial market and a wide array of questions remain for the researchers working in this area: how do we make a pure and functionally active PLC ζ protein? How do we store it without losing activity? How do we test it? How and when do we deliver it to an oocyte? Could there be any negative effects associated with its use? Until these questions are answered, the only way to treat oocyte activation deficiency is by using a Ca^{2+} ionophore. Within the UK, the fertility sector's regulatory body, the Human Fertilisation and Embryology Authority, has consented for the use of AOAs within the UK but only if there is appropriate evidence to justify that there is a specific activation problem, such as PLC ζ deficiency.

While we cannot yet administer an endogenous recombinant PLC ζ protein to oocytes in the clinic, we can at least now screen appropriate patients with our clinical assays and provide clinicians with evidence of PLC ζ deficiency. These current methods, however, are rather crude and

cannot test for the functional ability to cause Ca^{2+} release. Further improvement in our diagnostic assays, for example, by elucidating the three-dimensional structure of PLC ζ and creating PLC ζ antibodies that exhibit far better specificity, will undoubtedly increase our ability to diagnose problems that could potentially be fixed by AOAs, and in future, by recombinant PLC ζ .

Summary

Zeta (ζ) might have been the last letter for the ancient Greeks; but in the infertility arena, the ζ in PLC ζ is rapidly becoming known as the first way-marker to restoring communication between sperm and oocyte. While it is still early days, and our work is purely research-oriented at the moment, our assays may serve to lay down the foundation for new clinical protocols in the future. However, the story is still unfolding: a recombinant PLC ζ is urgently required for clinical testing and it is entirely conceivable that a range of other proteins and molecular pathways are involved in the process of oocyte activation. It is, however, important to highlight that the work described herein relates to just one sperm protein but clearly shows how collaboration between scientists and clinicians can serve to develop new diagnostic and therapeutic options for patients who might otherwise give up their dreams of parenthood. Given that there are over 1700 functional proteins in a single mature human sperm, the PLC ζ story is a reminder for us all that each of these proteins might serve as a way forward and that the established WHO guidelines for sperm analysis provide only a very basic indication of sperm quality. ■

Further reading

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His research interests include oocyte activation deficiency, male factor infertility, development of nanoparticle- and exosome mediated-delivery systems for gametes and embryos, and the use of infra-red laser technology in assisted reproduction. Email: kevin.coward@wrh.ox.ac.uk