Male Fertility

Re: Is PAWP the 'real' sperm factor?

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This letter is in response to an article written by Michail Nomikos, Karl Swann and F. Anthony Lai in the Research Highlights section of the *Asian Journal of Andrology* (AJA). The article is entitled, "Is PAWP the 'real' sperm factor?" and was written in response to our article entitled, "Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in human and mouse," published recently in *FASEB J.*[1] According to the Science Editor of AJA, "Research Highlight" pieces are brief articles that are meant to report on publications from the primary literature. Along those lines, we were delighted to read an insightful comment on our *FASEB* article prepared for AJA by Dr. George L Gerton. In stark contrast, we found that the article by Nomikos *et al.* was a promotion of phospholipase C zeta (PLCζ) as the "real" sperm factor, a molecule Dr. Lai's group has been working on for over a decade. In this article we found several omissions, inaccuracies and mistakes that highlight shortcomings in the study of PLCζ which point to major criteria of a "real" sperm factor that have not yet been addressed for PLCζ.

Throughout their article Nomikos *et al.* reiterate that, "since the discovery of PLCζ in 2002, many research laboratories across the world (Supplementary Table 1) have reported experimental evidence supporting the proposition that PLCζ is the 'sperm factor' that causes Ca^{2+} oscillations at fertilization." On careful examination, these labs have only shown that PLCζ induces calcium oscillations and artificial parthenogenetic oocyte activation when microinjected into oocytes; however, unlike the data for PAWP,[1–4] PLCζ has never been shown to enter the oocyte cytoplasm with the fertilizing spermatozoon during or after gamete fusion, nor has sperm-induced oocyte activation ever been shown to be blocked by specific inhibitors or antibodies to PLCζ. Therefore, no evidence has been presented that PLCζ is required, or even delivered to the oocyte during natural fertilization.

Nomikos *et al.* also argue that mounting clinical evidence suggest that the factor responsible for the initiation of Ca^{2+} oscillations during mammalian fertilization is a testis-specific isoform of PLC, named PLCζ. In regards to the clinical evidence presented in *J Clin Invest*,[5] the absence of PLCζ in sperm samples from infertility patients that are unable to activate oocytes by intracytoplasmic sperm injection (ICSI) does not necessarily support PLCζ in this role because many other proteins including PAWP could be missing, as is the case in globozoospermia, a presumed heritable sperm defect in which spermatozoa lack both the sperm perinuclear theca that harbors PAWP and the acrosome that appears in the predominant site of PLCζ localization. In fact in accompanying articles[6,7] to our 2014 *FASEB J* article,[1] we show intriguing correlative data from both spermatozoa of infertile men and livestock spermatozoa used in commercial artificial insemination programs indicating that inadequate amounts of PAWP affect male fertility, possibly due to the ability of spermatozoa to fertilize/activate oocytes. With respect to a recent article by Nimikos *et al.* in *Fertil Steril*,[8] we agree with them that the potential exists for rescuing failed oocyte activation with PLCζ but at present, this would not be considered natural as PLCζ has not yet been shown to diffuse from the sperm head into the oocyte cytoplasm and to be required for sperm induced oocyte activation.

In response to the criticism that it took us 7 years from our initial publication in 2007 to show that PAWP induces calcium signaling in oocytes it is important to point out that we published a paper in 2010 showing that PAWP in fact induces calcium release in *Xenopus* oocytes.[2] Additionally, our overall research was based on several other themes unrelated to PAWP and sperm-borne oocyte activating factor (SOAF) candidates. In other words, we did other research than just SOAF research! Despite this, we managed to make steady progress and publish several papers on this topic, including the aforementioned one. Following the disproval of "oscillin," first proposed as the candidate "sperm factor" by Parrington *et al.*[9] in *Nature*, and its quick replacement with PLCζ by Saunders *et al.*[10] in development,the identity of the 'sperm factor' became a contentious issue in fertilization biology and any publication of data that did not support PLCζ's role in oocyte activation was bound to meet with disapproval, reducing a chance that any alternative "sperm factor" candidate would be given fair consideration. In hindsight, and perhaps not coincidentally, "oscillin's" protein mass of 33 kDa is less than half of PLCζ's mass, but very close to that of PAWP, meaning that protein mass-separated cytosolic sperm fractions used by Parrington *et al.*[9] to activate oocytes likely had high PAWP content.

Nomikos *et al.* argue that there have been no other research groups that have independently verified PAWP's ability to activate oocytes or cause calcium oscillations. It is important to emphasize that the activation results obtained in humans in our recent *FASEB* paper were done independently from our lab by a group of investigators at the CReATe Fertility Center and the Department of Obstetrics and Gynecology, University of Toronto, who specialize in human fertilization. They became co-authors after confirming independently that PAWP induces the entire repertoire of oocyte activation events in oocytes, including calcium oscillations.

In a recent paper, Nomikos *et al.*[11] microinjected mouse oocytes with tagged recombinant mouse PAWP protein, or the complementary RNA encoding either untagged PAWP, or YFP-PAWP, or PAWP-luciferase, but consistently failed to observe any Ca^{2+} release. In our experience, tagging on a protein to PAWP prevents its binding or interaction with oocyte WWI domain containing proteins, which is a compulsory first step in the signal cascade that PAWP initiates in the oocyte cytoplasm. Furthermore, the concentrations of microinjected PAWP cRNAs and recombinant proteins were much higher than we would recommend. In fact our working injection concentration of human PAWP cRNA into both swine and human oocyte was 0.002 $\mu g\ \mu l^{-1}$, about 600 times less PAWP cRNA than was injected by Nomikos *et al.* In our initial dilution trials, we found that higher concentrations such as 0.1 $\mu g\ \mu l^{-1}$ failed to produce calcium oscillations. Although the paper is suggested as a first attempt to confirm our findings, it uses the mouse rather than human PAWP cRNA/protein, which was used in our *FASEB J* article. In addition, Nomikos *et al.* showed that PAWP was unable to hydrolyze PIP_2 *in vitro* and also did not act as a generic activator of PLC activity. Since PAWP follows a different signaling pathway than PLCζ, it is difficult to understand the rationale behind testing the PIP_2 hydrolyzing activity of PAWP!

In their critique of our *FASEB J* article, Nomikos *et al.* state that PLC gamma (PLCγ) does not mediate calcium oscillations in fertilizing oocytes. This claim is a misinterpretation of results presented by Mehlmann *et al.*[12] This article only shows that the SH2 domain of PLCγ is not required in mediating calcium oscillations in *Xenopus* and mice. It should also be noted that calcium oscillations and parthenogenetic oocyte activation can be induced by a number of means not involving PLCζ, demonstrating that intrinsic oocyte PLCs alone are able to convey and sustain calcium oscillations.

Nomikos *et al.* reflect back on previous "sperm factor" candidates making the claim that none of these molecules stood the test of time, mainly because subsequent research either could not validate, or else did not build upon, the original data. Again this is a misleading statement. Astonishingly, the aforementioned 33 kDa protein, "oscillin," described by a group led by Dr. F.A. Lai in a high profile *Nature* article,[9] which was never retracted but omitted from the present "rebuttal" attempt by Nomikos *et al.*, is the only sperm factor candidate that has been clearly disproved by independent investigators. It is important to reflect back on a protein in the 33 kDa range that Dr. Lai and co-investigators semi-purified from a sperm extract and demonstrated was responsible for inducing calcium oscillations in oocytes. The question remains which protein in that fraction was responsible for inducing the calcium oscillations? Neither oscillin, which was proven later to be a hexose phosphate isomerase nor PLCζ, which migrates in the 70 kDa range, were the SOAF candidate isolated in the *Nature* work.

Finally, Nomikos *et al.* state that immunolocalization analysis has indicated that PLCζ, like PAWP, is also present in the perinuclear matrix of the spermatozoa. In the reference[13] they used to back up this statement, the spermatozoa that were used to immuno-localize PLCζ were acrosome-intact and no mention was made of whether the localizations were similar in permeablized versus nonpermeablized

spermatozoa and after triton extraction. As well, no ultrastructural localization was made making it difficult to resolve at what level the labeling was. Since then, PLCζ has been detected in various compartments of mature spermatozoa in different species, including surface, perinuclear material, equatorial region, acrosome and tail,[14–19] most of which are not consistent with the proposed role of PLCζ as the "sperm factor." In the *PloS One* paper[15] it was shown that that PLCζ is first seen in the acrosome of the developing round spermatid, that is, long before the spermatids acquire the ability to activate oocytes after ICSI; it was never seen in the forming postacrosomal sheath of the perinuclear theca after the acrosome is formed. Moreover, it was shown that PLCζ is no longer detectable by immunofluorescence on the sperm head after acrosomal exocytosis and during sperm-zona pellucida penetration and sperm incorporation into the oocyte cytoplasm. Surprisingly, when mouse sperm heads and tails were separated from each other it was found by immunoblotting that the 74 kDa functional isoform of PLCζ was in the sperm tail and not in the perinuclear theca of the sperm head as had been previously speculated by Young *et al.*[18] It is important to emphasize that all the work mentioned above was documented with anti-PLCζ antibodies obtained from Dr. Lai's group and further confirmed by other commercial/home-made antibodies. The inconsistency in immuno-labelling among different researchers makes it difficult to confirm precisely whether sperm-derived PLCζ fits the criteria set for an SOAF candidate protein. However, the fact that PLCζ is an acrosomal protein cannot be denied as even Dr. Lai's co-investigators have shown this to be the case; however, little attention has been paid to its role in acrosome formation or capacitation/acrosome reaction where PLCs are needed. In contrast, it is clear that PAWP originates in the cytosol during spermatid elongation and resides in the postacrosomal part of the sperm perinuclear theca after sperm-zona penetration during *in vitro* fertilization.[3,4,20]

As mentioned in the introductory paragraph, Dr. Lai and co-investigator have ignored addressing major criteria needed to establish that PLCζ is the "real" sperm factor. The most important of these criteria, since the PLCζ KO indicates an alternative role for this enzyme,[21,22] is to show that sperm-induced oocyte activation can be blocked by specific inhibitors or antibodies to PLCζ, as was done for PAWP. Second, evidence should be provided that PLCζ enters the oocyte cytoplasm with the sperm head during or after gamete fusion, as was done for PAWP. Finally, evidence should be provided that PLCζ assembles along the postacrosomal sheath during the elongating spermatid phase of spermiogenesis, which again was done for PAWP. As for the clinical aspect of their study, they should be able to correlate some aspects of male factor fertility with levels of PLCζ, which was done most recently for PAWP.[6] Recent genomic studies show that PLCζ gene polymorphisms and transcript abundance slightly affect sperm quality in livestock, not sperm-oocyte activating ability! As correctly pointed out in the commentary by G. Gerton, a mutant mouse lacking *Pawp* gene could help address the issue of "sperm factor," although no general conclusions for all mammalian species should be based solely on a rodent mutant. The issue of the "essential" nature of *Pawp* gene, which is being worked on, may be further complicated by high homology between PAWP/WBP2NL and its testis-expressed, presumed somatic cell orthologue WBP2.

In conclusion, the hunt for the elusive "sperm factor" is still on and collaboration instead of fierce competition and persecution of those searching for alternatives should be pursued by groups involved in it. It is unfortunate that in this single-minded chase of "sperm factor," we have lost the sight of facts and alternative hypotheses. Importantly, proponents of the soluble "sperm factor" discount possible contribution of sperm-oolemma adhesion and fusion events to oocyte activation (see our review[23]). This premature rejection of "contact/membrane addition hypothesis" of oocyte activation may have thwarted a potentially important line of investigation.[24–26] One should bear in mind that in the absence of sperm-oolema/oocyte cortex interactions, sperm and by extension sperm extract injection into the oocyte cytoplasm is not sufficient to trigger all aspects of oocyte activation and anti-polyspermy defense seen after natural fertilization.[27,28]

REFERENCES

1 Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, *et al*. Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. *FASEB J* 2014; 28: 4434–40.
2 Aarabi M, Qin Z, Xu W, Mewburn J, Oko R. Sperm-borne protein, PAWP, initiates zygotic development in *Xenopus laevis* by eliciting intracellular calcium release. *Mol Reprod Dev* 2010; 77: 249–56.
3 Wu AT, Sutovsky P, Manandhar G, Xu W, Katayama M, *et al*. PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J Biol Chem* 2007; 282: 12164–75.
4 Wu AT, Sutovsky P, Xu W, van der Spoel AC, Platt FM, *et al*. The postacrosomal assembly of sperm head protein, PAWP, is independent of acrosome formation and dependent on microtubular manchette transport. *Dev Biol* 2007; 312: 471–83.
5 Yoon SY, Jellerette T, Salicioni AM, Lee HC, Yoo MS, *et al*. Human sperm devoid of PLC, zeta 1 fail to induce Ca (2+) release and are unable to initiate the first step of embryo development. *J Clin Invest* 2008; 118: 3671–81.
6 Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, *et al*. Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. *Fertil Steril* 2014; 102: 440–7.
7 Kennedy CE, Krieger KB, Sutovsky M, Xu W, Vargovic P, *et al*. Protein expression pattern of PAWP in bull spermatozoa is associated with sperm quality and fertility following artificial insemination. *Mol Reprod Dev* 2014; 81: 436–49.
8 Nomikos M, Yu Y, Elgmati K, Theodoridou M, Campbell K, *et al*. Phospholipase Cζ rescues failed oocyte activation in a prototype of male factor infertility. *Fertil Steril* 2013; 99: 76–85.
9 Parrington J, Swann K, Shevchenko VI, Sesay AK, Lai FA. Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. *Nature* 1996; 379: 364–8.
10 Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, *et al*. PLC zeta: a sperm-specific trigger of Ca (2+) oscillations in eggs and embryo development. *Development* 2002; 129: 3533–44.
11 Nomikos M, Sanders JR, Theodoridou M, Kashir J, Matthews E, *et al*. Sperm-specific post-acrosomal WW-domain binding protein (PAWP) does not cause Ca2+ release in mouse oocytes. *Mol Hum Reprod* 2014; 20: 938–47.
12 Mehlmann LM, Carpenter G, Rhee SG, Jaffe LA. SH2 domain-mediated activation of phospholipase Cgamma is not required to initiate Ca2+ release at fertilization of mouse eggs. *Dev Biol* 1998; 203: 221–32.
13 Fujimoto S, Yoshida N, Fukui T, Amanai M, Isobe T, *et al*. Mammalian phospholipase Czeta induces oocyte activation from the sperm perinuclear matrix. *Dev Biol* 2004; 274: 370–83.
14 Nikiforaki D, Vanden Meerschaut F, De Gheselle S, Qian C, Van den Abbeel E, *et al*. Sperm involved in recurrent partial hydatidiform moles cannot induce the normal pattern of calcium oscillations. *Fertil Steril* 2014; 102: 581–8.e1.
15 Aarabi M, Yu Y, Xu W, Tse MY, Pang SC, *et al*. The testicular and epididymal expression profile of PLCζ in mouse and human does not support its role as a sperm-borne oocyte activating factor. *PLoS One* 2012; 7: e33496.
16 Bedford-Guaus SJ, McPartlin LA, Xie J, Westmiller SL, Buffone MG, *et al*. Molecular cloning and characterization of phospholipase C zeta in equine sperm and testis reveals species-specific differences in expression of catalytically active protein. *Biol Reprod* 2011; 85: 78–88.
17 Kashir J, Jones C, Mounce G, Ramadan WM, Lemmon B, *et al*. Variance in total levels of phospholipase C zeta (PLC-ζ) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation capability. *Fertil Steril* 2013; 99: 107–17.
18 Young C, Grasa P, Coward K, Davis LC, Parrington J. Phospholipase C zeta undergoes dynamic changes in its pattern of localization in sperm during capacitation and the acrosome reaction. *Fertil Steril* 2009; 91: 2230–42.
19 Grasa P, Coward K, Young C, Parrington J. The pattern of localization of the putative oocyte activation factor, phospholipase Czeta, in uncapacitated, capacitated, and ionophore-treated human spermatozoa. *Hum Reprod* 2008; 23: 2513–22.

20 Oko R, Sutovsky P. Biogenesis of sperm perinuclear theca and its role in sperm functional competence and fertilization. *J Reprod Immunol* 2009; 83: 2–7.
21 Ito M, Nagaoka K, Kuroda K, Kawano N, Yoshida K, *et al*. Arrest of Spermatogenesis at Round spermatids in PLCZ1-Deficient Mice. 11th International Symposium on Spermatology 2010b. [abstract].
22 Ito J, Parrington J, Fissore RA. PLCzeta and its role as a trigger of development in vertebrates. *Mol Reprod Dev* 2011; 78: 846–53.
23 Sutovsky P. Sperm-egg adhesion and fusion in mammals. *Expert Rev Mol Med* 2009; 11: e11.
24 White KL, Passipieri M, Bunch TD, Campbell KD, Pate B. Effects of arginine-glycine-aspartic acid (RGD) containing snake venom peptides on parthenogenetic development and *in vitro* fertilization of bovine oocytes. *Mol Reprod Dev* 2007; 74: 88–96.
25 Tatone C, Carbone MC. Possible involvement of integrin-mediated signalling in oocyte activation: evidence that a cyclic RGD-containing peptide can stimulate protein kinase C and cortical granule exocytosis in mouse oocytes. *Reprod Biol Endocrinol* 2006; 4: 48.
26 Iwao Y, Fujimura T. Activation of *Xenopus* eggs by RGD-containing peptides accompanied by intracellular Ca2+ release. *Dev Biol* 1996; 177: 558–67.
27 Wortzman-Show GB, Kurokawa M, Fissore RA, Evans JP. Calcium and sperm components in the establishment of the membrane block to polyspermy: studies of ICSI and activation with sperm factor. *Mol Hum Reprod* 2007; 13: 557–65.
28 Maleszewski M, Kimura Y, Yanagimachi R. Sperm membrane incorporation into oolemma contributes to the oolemma block to sperm penetration: evidence based on intracytoplasmic sperm injection experiments in the mouse. *Mol Reprod Dev* 1996; 44: 256–9.

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