Antigen-unmasking enhances visualisation efficacy of the oocyte activation factor, phospholipase C zeta, in mammalian sperm.

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Running title: Antigen unmasking of PLCzeta in mammalian sperm

Abstract

Study question: Is it possible to improve clinical visualization of phospholipase C zeta (PLCζ) as a diagnostic marker of sperm oocyte activation capacity and male fertility?

Summary answer: Poor PLCζ visualization efficacy using current protocols may be due to steric or conformational occlusion of native PLCζ, hindering antibody access, and significantly enhanced using antigen unmasking/retrieval protocols.

What is known already: Mammalian oocyte activation is mediated via a series of intracellular calcium (Ca^{2+}) oscillations induced by sperm-specific PLCζ. PLCζ represents not only a potential clinical therapeutic in cases of oocyte activation deficiency (OAD), but also a diagnostic marker of sperm fertility. However, there are significant concerns surrounding PLCζ antibody specificity and detection protocols.

Study design, size, duration: Two PLCζ polyclonal antibodies, with confirmed PLCζ specificity, were employed in mouse, porcine, and human sperm. Experiments evaluated PLCζ visualisation efficacy, and whether antigen-unmasking/retrieval (AUM) improved this. Antibodies against two sperm-specific proteins **[**post-acrosomal WW-binding protein (PAWP) and acrosin] were used as controls.

Participants/materials, setting, methods: Aldehyde- and methanol-fixed sperm were subject to immunofluorescence analysis following HCl exposure (pH=0.1-0.5), acid Tyrode's solution exposure (pH=2.5), or heating in 10mM sodium citrate solution (pH=6.0). Fluorescence intensity of at least 300 cells was recorded for each treatment, with three independent repeats.

Main results and the role of chance: Despite high specificity for native PLCζ following immunoblotting using epitope-specific polyclonal PLCζ antibodies in mouse, porcine, and human sperm, immunofluorescent visualization efficacy was poor. In contrast, sperm markers PAWP and acrosin exhibited relatively impressive results. All methods of AUM on aldehyde-fixed sperm enhanced visualization efficacy for PLCζ compared to visualization efficacy before AUM ($p<0.05$ for all AUM interventions), but exerted no significant change upon PAWP or acrosin immunofluorescence following AUM ($p=0.644$ and $p=0.1633$, respectively). All methods of AUM enhanced PLCζ visualization efficacy in mouse and human methanol-fixed sperm compared to without AUM ($p<0.05$ for all AUM interventions), while no significant change was observed in methanol fixed porcine sperm before and after AUM ($p>0.05$ for all AUM interventions). In the absence of aldehyde-induced cross-linkages, such results suggest that poor PLCζ visualization efficacy may be due to steric or conformational occlusion of native PLCζ, hindering antibody access. Importantly, examination of sperm from individual donors revealed that AUM differentially affects observable PLCζ fluorescence, and the proportion of sperm exhibiting detectable PLCζ fluorescence in sperm from different males.

Limitations, reasons for caution: Direct correlation of fertility outcomes with the level of PLCζ in the sperm samples studied was not available. Such analyses would be required in future to determine whether the improved methodology for PLCζ visualization we propose would indeed reflect fertility status.

Wider implications of the findings: We propose that AUM alters conformational interactions to enhance PLCζ epitope availability and visualization efficacy, supporting prospective application of AUM to reduce misinterpretation in clinical diagnosis of PLCζ-linked male infertility. Our current results suggest that it is perhaps prudent that previous studies investigating links between PLCζ and fertility parameters are re-examined in the context of AUM, and may pave the way for future work to answer significant questions such as how PLCζ appears to be kept in an inactive form in the sperm.

Large scale data: not applicable.

Study funding/competing interest(s): JK is supported by a Health Fellowship award from the National Institute for Social Care and Health Research (NISCHR). MN is supported by a Marie Curie Intra-European Research Fellowship award. This work was also partly funded by a research grant from Cook Medical Technologies LLC. There are no competing financial interests to declare.

Key words: Oocyte activation, Phospholipase C zeta, male infertility, antigen unmasking/retrieval, biomarker, sperm, fertilisation, diagnostic

Introduction

Following gamete fusion, mammalian oocytes elicit a series of intracellular calcium (Ca^{2+}) oscillations mediated by the inositol 1,4,5-trisphosphate (IP_3) signalling pathway, resulting in a series of events collectively termed 'oocyte activation'. Ultimately, activation permits alleviation of oocyte meiotic arrest, allowing embryogenesis to begin. In mammals, oocyte activation begins soon after gamete fusion and persists beyond the completion of meiosis. Current scientific evidence supports the sperm factor theory of oocyte activation, whereby the fertilising sperm releases a soluble 'sperm factor' into the oocyte following gamete fusion, to explain the occurrence of this phenomenon (Stricker, 1999; Whitaker, 2006; Berridge, 2009; Kashir *et al*., 2014; Swann & Lai, 2016).

Considerable experimental evidence indicates that the agent responsible for inducing Ca^{2+} oscillations within activating mammalian oocytes is a testis-specific phospholipase C, termed phospholipase C zeta (PLCζ) (Cox *et al*., 2002; Saunders *et al*., 2002; Fujimoto *et al*., 2004; Knott *et al*., 2005; Kouchi *et al*., 2005; Kurokawa *et al*., 2005; Kashir *et al*., 2014; Swann & Lai, 2016). Most importantly, injection of recombinant PLCζ cRNA and purified protein into mouse oocytes initiates Ca^{2+} oscillations, with cRNA and protein injections supporting embryonic development to the blastocyst stage (Saunders *et al*., 2002; Kouchi *et al*., 2005). Intriguingly, sperm extracts and PLCζ cRNA of one species elicit Ca^{2+} release upon microinjection into oocytes from other species (Cox *et al*., 2002; Bedford-Guaus *et al*., 2006; Ito *et al*., 2008). Indeed, non-mammalian testis-specific PLCζ homologues have also been identified (Coward *et al*., 2005; Ito *et al*., 2008; Mizushima *et al*., 2008; Coward *et al*., 2011), suggesting PLCζ-induced Ca^{2+} release may be a universal feature of vertebrate oocyte activation.

Recently, debate has surrounded PLCζ as the sperm factor, with studies proposing that the soluble sperm factor responsible for eliciting Ca^{2+} oscillations at oocyte activation may be a post-acrosomal sheath WW domain-binding protein, termed PAWP (Wu *et al*., 2007a; Aarabi *et al*., 2010; 2014). However, subsequent studies from independent research groups (including our own) now strongly suggest that PAWP is not involved in eliciting Ca^{2+} oscillations at oocyte activation, while PLCζ remains the valid candidate for the soluble sperm factor (Nomikos *et al*., 2014b; 2015; Kashir *et al*., 2015; Satouh *et al*., 2015; Escoffier *et al*., 2016).

Infertile human sperm, unable to activate human and mouse oocytes even following ICSI (whereby a single sperm is injection into an oocyte), are deficient in their ability to elicit Ca^{2+} oscillations, resulting in either reduced fidelity of oscillations or their complete absence (Yoon *et al*., 2008). Collectively, such examples strongly suggest that certain manifestations of male infertility are linked to PLCζ deficiency indicating that therapeutically, PLCζ is a strong candidate for an endogenous alternative for current assisted oocyte activation protocols (Kashir *et al*., 2010; 2011; Nomikos *et al*., 2013; Sanusi *et al*., 2015). Thus, in addition to a therapeutic role, PLCζ may represent a prognostic biomarker of sperm and male fertility (Kashir *et al*., 2010).

Numerous studies report that PLCζ assessment in human sperm being used in fertility treatment may represent a useful diagnostic tool. Indeed, sperm showing oocyte activation deficiency (OAD) is associated with reduced/absent levels of PLCζ (Yoon *et al*., 2008; Heytens *et al*., 2009; Kashir *et al*., 2010; 2013; Yelumalai *et al*., 2015), while PLCζ deficiencies have been associated with multiple male-specific conditions (Heytens *et al*., 2009; Kashir *et al*., 2010;

2013; Escoffier *et al*., 2015; Park *et al*., 2015) and abnormal pregnancies, namely recurrent partial hydatidiform moles (Nikiforaki *et al*., 2014). Furthermore, males presenting morphologically normal sperm that lack oocyte activation capacity exhibit mutations in the PLCζ gene, which is consistent with the absence of Ca^{2+} oscillatory ability of such sperm (Heytens *et al*., 2009; Nomikos *et al*., 2013; Kashir *et al*., 2012a; 2012b; Escoffier *et al*., 2016).

However, numerous issues persist pertaining to PLCζ localization in mammalian sperm, particularly in humans. PLCζ generally appears localised to distinct regions within the sperm head in mammals, with potentially differential functional roles suggested for each population (Grasa *et al*., 2008; Young *et al*., 2009; Kashir *et al*., 2014). Populations of PLCζ have been identified in acrosomal and post-acrosomal regions of mouse and porcine sperm, with a tail population also identified in porcine sperm (Fujimoto *et al*., 2004; Young *et al*., 2009; Nakai *et al*., 2011; Kaewmala *et al*., 2012). In equine sperm, PLCζ was reportedly localised to the acrosome, equatorial segment, and head mid-piece, as well as the principal piece of the flagellum (Bedford-Guaus *et al*., 2011; Sato *et al*., 2013). In humans, three distinct populations of PLCζ have been identified in the acrosomal, equatorial and post-acrosomal regions of the sperm head, with additional potential tail localization (Grasa *et al*., 2008; Yoon *et al*., 2008; Heytens *et al*., 2009; Kashir *et al*., 2011a; 2011b; 2013).

Such conundrums are further compounded by the fact that, with the exception of mouse, all PLCζ antibodies used to date do not appear to be entirely specific to native PLCζ following sperm immunoblotting. Perhaps a significant causative factor underlying such issues is that most antibodies do not seem entirely specific to PLCζ, identifying additional proteins alongside native

PLCζ in human sperm, with different studies failing to exhibit consistent blotting profiles even with the same antibody (Yoon *et al*., 2008; Heytens *et al*., 2009; Escoffier *et al*., 2015; 2016). It is not yet apparent whether such differences are a reflection of antibody specificity and laboratory protocol, or whether such discrepancies are due to individual antibody specificity between different antibodies, and between individual animals/men. Indeed, at least in human sperm, significant variance in predominant PLCζ localization patterns has been reported, with no consistent motif reflective of fertility status (Kashir *et al*., 2013). However, such variance in antibody specificity following immunoblotting directly impacts localisation studies, as one cannot attribute localisation patterns observed in human sperm to PLCζ if multiple bands are recognised following immunoblotting.

Recently, our laboratory has developed epitope-specific polyclonal antibodies to human PLCζ, with high consistency in results throughout multiple studies for both recombinant and native human PLCζ (Nomikos *et al*., 2013; 2014a; 2014b; 2015; Theodoridou *et al*., 2013). Herein, to explore concerns surrounding variable PLCζ localization patterns in mammalian sperm, we use these highly specific PLCζ antibodies against human, porcine, and mouse sperm in immunoblotting and immunofluorescence applications. Additionally, we have developed a uniform protocol for immunological analysis of PLCζ in mammalian sperm, to aid and synchronise global efforts to apply the diagnostic potential of PLCζ within the fertility clinic.

Materials and Methods

Antibodies

Production of the anti-PLCζ polyclonal antibody used to detect human and murine PLCζ (EF) has been described previously (Nomikos *et al*., 2013), and was raised in rabbits against a 16-mer-peptide sequence (^{8}SKIQDDFRGGKINLEK^{23}) of human PLCζ protein. The anti-PLCζ polyclonal antibody used to detect porcine PLCζ (R1) was as raised in rabbits against a 13-mer-peptide sequence (^{284}LPSPEALKFKILV^{296}) of human PLCζ protein. Anti-PAWP (post-acrosomal WW-binding protein) and anti-acrosin rabbit polyclonal antibodies used as controls were purchased from Proteintech (UK) and Sigma (UK; catalog# SAB1301239) respectively.

Sperm processing

Human sperm collection was performed as previously described (Nomikos *et al*., 2015). Briefly, surplus semen samples were donated by individuals undergoing routine semen assessment at the Wales Fertility Institute, University Hospital Wales, Cardiff, with informed, written consent. This study received ethical approval from the local research ethics committee (REC reference number 08/WSE/02/20), and all procedures performed were carried out in accordance with the local research ethics committee approval and *Molecular Human Reproduction* guidelines. Sperm were isolated using PureSperm 40/80 (Nidacon, Sweden) following the manufacturers protocol. For individual patient analyses, sperm were kept separate, but were otherwise pooled.

Mouse sperm were obtained from the cauda epididymis of euthanized mature male mice (C57xCBA F1 hybrid), and released into T6 media following caudal puncture for ~30 minutes at 37^{o}C. Motile sperm were pooled and washed with PBS+ protease inhibitors (PI) at 500g for 10

minutes each. Porcine sperm was obtained commercially (JSR Genetics, UK), and ejaculated sperm were delivered suspended in diluent. Motile sperm were isolated by centrifugation at 500g for 10 minutes, washed twice with PBS, and resuspension of the pellet in PBS. All procedures using animals were approved by Cardiff University Animals Ethics Committee and carried out under a UK Home Office Project Licence.

Motile sperm were subsequently washed three times in PBS containing a cocktail of protease inhibitors (Roche, UK) by centrifugation at 500g for 10 minutes each, and sperm concentration determined microscopically using a Neubauer improved counting chamber. Sperm were then processed differently depending on intended use (immunoblotting or immunofluorescence).

Sperm immunoblotting

Following sperm washing, single-use aliquots were prepared by addition of appropriate amounts of sperm to 5x sample loading buffer, such that each aliquot contained $1x10^6$sperm/aliquot. Each aliquot was briefly vortexed, snap frozen on dry-ice, and stored at -80^{o}C until required. Single sperm aliquots were thawed and briefly heated at 101^{o}C, before loading onto 4-20% pre-cast gradient gels (BioRad, UK), and performing sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis as previously described (Nomikos *et al*., 2015). Separated proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, UK) using a semidry transfer system (Trans-Blot SD; BioRad, UK) in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% v/v methanol) at 18 V for 1 hour.

Membranes were incubated overnight at 4°C in blocking buffer (Tris-buffered saline, 0.1% Tween 20, 5% non-fat milk powder). Membranes were probed with polyclonal antibodies against PLCζ, PAWP (1:50,000), or acrosin (1:1000). Human and mouse PLCζ were recognised by the EF antibody (1:1500), while porcine PLCζ was recognised by the R1 antibody (1:750) diluted in blocking buffer at room temperature for 1 hour. This was followed by incubating with a horse-radish peroxidase-conjugated anti-rabbit secondary antibody (1:50,000), diluted in blocking buffer at room temperature for 1 hour. Antibody detection was achieved using enhanced chemiluminescence (ECL select; GE LifeSciences, UK).

Sperm immunofluorescence

Following isolation of motile sperm, cells were fixed by incubation in 10% neutral-buffered formalin solution (40% formaldehyde; Sigma, UK) for 10 minutes at room temperature, following which fixative was diluted with an equal volume of PBS, and sperm centrifuged at 500g for 10 minutes. Sperm were also fixed by methanol (MeOH) where appropriate. Isolated motile sperm were suspended in 100% MeOH pre-chilled at -20 ^{o}C and incubated at -20^{o}C for 10 minutes. Following fixation, fixative was diluted with and equal volume of PBS, and sperm centrifuged at 500g for 10 minutes. The supernatant was discarded, and further washed with PBS. Fixed sperm was resuspended in PBS containing protease inhibitors, and stored at 4^{o}C until required.

Immunofluorescence was performed as previously described (Kashir *et al*., 2011b; Nomikos *et al*., 2015). Briefly, fixed sperm were added to hydrophobic moulds, previously drawn with an ImmEdge hydrophobic barrier pen (Vector laboratories, UK) onto slides coated with 0.01% (w/v) poly-L-lysine solution (Sigma). Cells were allowed to settle onto the slide for

~45 minutes, and were then permeabilised with PBS-1% Triton X-100 at room temperature for 1 hour. Non-specific antigen binding sites in permeabilised cells were blocked with PBS-5% bovine serum albumin (BSA; Sigma, UK) for 1 hour at room temperature. Cells were probed with primary antibody diluted (1:50) in PBS-5% BSA overnight at 4^{o}C. Cells were subsequently incubated with AlexaFluor-488-conjugated anti-rabbit secondary antibody, diluted in PBS-5% BSA (1:100; Life Technologies, UK) at room temperature for 1 hour.

Cells were washed 3 times with PBS between each aforementioned step. Cells were mounted with Vectashield mounting medium containing 4′-6-diamidino-2-phenylindole (Vector Laboratories, UK) and slides stored at 4°C until imaging. Slides were imaged using a 63x oil-immersion lens, and a Zeiss Axiovert 200 fluorescence microscope utilising a brightfield filter to image sperm cells, and a fluorescein isothiocyanate filter for PLCζ immunofluorescence. Images were captured using an AxioCam MRc camera (Ziess, UK), and processed using ImageJ. Care was taken to ensure all microscopic, acquisition, and camera settings remained consistent for all sets of analyses.

Antigen unmasking/retrieval

Antigen unmasking/retrieval (AUM) protocols were integrated into the sperm immunofluorescence methodology, and were performed following permeabilisation after sperm had been fixed and settled onto poly-L-lysine-coated slides. Three separate methods of AUM were employed in this study, as described below.

Hydrochloric acid (HCl) AUM: 1M HCl (pH=0.1-0.5) was added directly to hydrophobic moulds containing sperm on the slides, and incubated for 5 seconds at room temperature. HCl

was removed from the slide via aspiration, and acid neutralised by three washes with neutralisation buffer (NB: 100mM Tris, pH=8.5).

Acidic Tyrode's solution (AT) AUM: AT solution (pH=2.5-3.0; Sigma, UK) was applied to hydrophobic moulds, and incubated at room temperature for 5 seconds. AT solution was removed from the slide via aspiration, and acid neutralised by three washes with NB.

Sodium Citrate AUM: Sodium Citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) was applied to hydrophobic moulds such that the buffer covered the entirety of the mould. Slides were then heated in a microwave at full power for 7 minutes, with slides checked every 2 minutes. Buffer levels were topped up where necessary to avoid drying of the slide. Following heating, slides were allowed to cool at room temperature for 10 minutes, after which sodium citrate buffer was removed via aspiration, and hydrophobic moulds washed thrice with NB.

Hydrophobic moulds were further washed thrice with PBS, and the immunofluorescence protocol continued as described above following completion of AUM.

Sperm and statistical analysis

Sperm analysis was performed using Image J software (National Institutes of Health, MD, USA) utilising the regions of interest tool as previously described (Kashir *et al*., 2013). It should be noted that our analysis was limited to the sperm head, as quantitative analyses of the sperm tail was limited due to the presence of the tail in multiple planes of view. Briefly, approximately 300 cells were analysed per experiment for sperm from each species over three repeats where possible. This analysis was not possible for individual patient analysis, so care was taken to analyse at least 300 cells from each sample. Multiple fields of view were captured, and a mean level of fluorescence calculated per field of view. Fluorescence observed was relative to the

exposure rate that was utilised to capture the images in this study, and these relative fluorescence values were normalised to background fluorescence (normalized relative fluorescence). Following quantification, the mean level of normalized relative fluorescence observed from all fields of view was calculated.

The proportions of sperm exhibiting any pattern of PLCζ localisation were also quantified to assess the proportion of sperm exhibiting PLCζ fluorescence. All proportions (represented as %) were arcsine transformed prior to statistical analysis using Prism software 5.0 (Graph-Pad, CA, USA). Statistical significance was determined by one-way ANOVA followed by the Bonferroni multiple comparisons post-hoc test. The student's *t*-test was also used to determine statistical significance between two populations where appropriate. A value of $P<0.05$ was considered significant.

Results

Visual efficacy of PLCζ following immunofluorescence in human sperm is considerably reduced compared to acrosin and PAWP

Despite consistently exhibiting a single immunoreactive band (~70kDa) following immunoblotting on human sperm, native PLCζ was barely detectable following immunofluorescence using epitope-specific rabbit anti-PLCζ antibodies. This was in stark contrast to the sperm-specific markers acrosin and PAWP, antibodies against which also identified a predominant band in human sperm (~45kDa and 33kDa, respectively), (Figure 1a). In line with the published literature (Yoon *et al*., 2008; Grasa *et al*., 2008; Nomikos *et al*., 2013; Escoffier *et al*., 2015; 2016), we observed PLCζ localisation in human sperm at the equatorial and acrosomal regions of the sperm head, alongside a clear signal observed within the sperm tail. PAWP and acrosin localization was observed at the post-acrosomal and acrosomal regions of the sperm head, respectively (Figure 1b). Following the same immunofluorescence protocol and microscopic capture settings, the sperm markers PAWP and acrosin were significantly more readily visible ($p<0.05$) compared to PLCζ (relative fluorescence of 18.11, 18.14, and 1.12 respectively). There was no significant difference between relative fluorescence of PAWP and acrosin (Figure 1c), indicating optimal visualization of both markers before AUM with our protocol. It should be noted that such effects were also noted for sperm fixed with 4% paraformaldehyde (data not shown), but yielded poorer results that our current protocol.

AUM enhances visualization efficacy of PLCζ, but does not affect visualization efficacy of PAWP or acrosin in human sperm.

In an attempt to enhance visualization efficacy of PLCζ in human sperm, we employed three methods of AUM. We found that AUM resulted in a dramatic increase in visualized fluorescence in human sperm following immunofluorescent PLCζ analyses (Figure 2a), with all three methods (HCl; AT; sodium citrate solution) of AUM exhibiting a significantly increased level ($p<0.05$) of relative fluorescence observed compared to sperm without AUM (relative fluorescence of 19.03, 18.80, 26.36 respectively, compared to 11.16) (Figure 2b). In human sperm, the sodium citrate method of AUM proved most effective ($p<0.05$), with no significant difference observed between HCl and AT methods of AUM. However, for both PAWP and acrosin, AUM did not result in a significant change in observed relative fluorescence in human sperm (Figure 2c; Supplementary Figure 1). While relative fluorescence of acrosin remained unchanged following AUM (relative fluorescence of 17.65 compared to 17.68), the relative fluorescence of PAWP slightly increased following AUM (relative fluorescence of 18.11 compared to 20.82). This difference, however, was statistically non-significant ($p=0.072$).

AUM significantly enhances PLCζ visualization efficacy in porcine and mouse sperm.

The rabbit anti-PLCζ antibodies consistently identified a single predominant immunoreactive band corresponding to the predicted molecular mass of PLCζ in porcine and murine sperm (~72kDa and ~74kDa, respectively), (Figure 3a), indicating antibody specificity for native murine and porcine PLCζ. Without application of AUM, porcine PLCζ visualization efficacy was significantly enhanced compared to that in human sperm, but remained barely visible (relative fluorescence of 3.05). Application of all three AUM procedures to porcine and

murine sperm significantly (p<0.05) increased the relative fluorescence observed compared to without AUM (Figure 3b), with AT AUM exhibiting the highest increase, followed by HCl and sodium citrate methods (relative fluorescence of 13.54, 10.14, and 8.61, respectively), (Figure 3c). Similarly, in murine sperm, AUM significantly (p<0.05) enhanced visualization efficacy of PLCζ compared to no AUM (relative fluorescence of 2.88), with the exception of HCl AUM (relative fluorescence of 3.24), which did not result in a statistically significant increase (p=0.142) compared to no AUM. Sodium citrate AUM resulted in the highest increase in relative fluorescence compared to AT AUM (relative fluorescence of 5.36 and 3.84, respectively), (p<0.05) (Figure 3d). In line with previous studies, we observed PLCζ localisation in porcine sperm predominantly at the equatorial and post-acrosomal regions of the sperm head, with murine sperm exhibiting acrosomal and post-acrosomal populations. Sperm from both species also showed a clear tail localization following immunofluorescence.

Methanol fixation requires AUM for optimal visualization efficacy of PLCζ in mammalian sperm

To further investigate the nature of the AUM requirement for optimal immunofluorescence in human, porcine, and murine sperm, we examined the effect of MeOH fixation, as opposed to the aldehyde fixation performed in previous experiments. In human sperm, visualization efficacy of PLCζ was improved compared to aldehyde fixation without AUM, yet remained poor. Intriguingly, application of AT AUM on MeOH-fixed human sperm exhibited (p<0.05) improved PLCζ visualization efficacy compared to no AUM (relative fluorescence of 2.9 compared to 2.04). Localisation remained observable at the equatorial and acrosomal segments of the sperm head, but it was significantly diminished in the tail. However,

these localization patterns were more diffuse as compared to aldehyde-fixed AUM sperm (Figure 4a; Supplementary Figure 2).

In porcine sperm, MeOH fixation altered the localization of PLCζ within the sperm head compared to aldehyde-fixed sperm, exhibiting a diffuse pattern of localization throughout the acrosomal region, alongside the equatorial segment of the sperm head. The observable tail fluorescence remained unchanged. Interestingly, AT and sodium citrate AUM on MeOH-fixed porcine sperm did not significantly alter the relative fluorescence observed compared to no AUM, albeit reduced (relative fluorescence of 15.47 and 20.91 compared to 21.88, respectively; p=0.71 and p=0.62, respectively). Only HCl AUM resulted in a significant change (p<0.05), with relative fluorescence (relative fluorescence of 9.88) decreased compared to no, AT, and sodium citrate AUM (Figure 4b; Supplementary Figure 3).

Unlike porcine sperm, PLCζ localisation in murine sperm was barely observable following MeOH fixation. Furthermore, AUM did not alter the overall pattern of localization, but exhibited altered predominant distributions of PLCζ, with all methods of AUM resulting in a dominant acrosomal localization, and a diffuse pattern observed in the post-acrosomal region. However, only HCl and AT AUM resulted in a significant increase (p<0.05) in relative fluorescence compared to no AUM (5.47 and 4.77 compared to 2.35, respectively), with relative fluorescence following sodium citrate AUM (3.03) not changing significantly (p=0.16) (Figure 4c; Supplementary Figure 4).

AUM differentially affects relative fluorescence levels and proportions of sperm exhibiting PLCζ fluorescence in human sperm from different donors

Examination of AT AUM in sperm from different individual human donors revealed that AUM differentially altered the relative fluorescence observed (Figure 5a), alongside the proportion of sperm exhibiting detectable PLCζ fluorescence (Figure 5b). Of 13 patients studied, with AT AUM sperm from 10 males exhibited a statistically significant ($p<0.05$) increase in total relative fluorescence observed, and sperm from 8 males exhibited a statistically significant ($p<0.05$) increase in proportions of sperm exhibiting detectable PLCζ fluorescence. Within the population exhibiting an increase in the aforementioned parameters, the extent of the increase varied considerably, ranging from an increase of 0.34 to 2.66 a.u. (arbitrary units) for relative fluorescence, and an increase of 1.6 to 18.77% in the proportion of sperm exhibiting detectable PLCζ fluorescence. A statistically significant ($p<0.05$) decrease was also observed in sperm from 2 donors in relative fluorescence levels (patient 2: -2.59 a.u; patient 12: -0.09 a.u), and a statistically significant ($p<0.05$) decrease in proportions of sperm exhibiting PLCζ fluorescence was observed in sperm from 2 males. (patient 8: -4.53%; patient 11: -1.35%). Sperm from 1 male exhibited no significant change in normalized relative fluorescence observed (patient 13), while sperm from 2 males (patients 4 and 8) did not exhibit a statistically significant change in the proportion of sperm exhibiting detectable PLCζ fluorescence.

Discussion

Ca^{2+} oscillation-induced oocyte activation is fundamental for successful physiological fertilisation and embryogenesis in mammalian reproduction. To this extent, the sperm-borne oocyte activation factor PLCζ is critical for ensuring successful fertilisation, presenting much promise as a diagnostic measure of sperm oocyte activation capability in humans. There are potentially different approaches to assessing PLCζ in sperm.

Aghajanpour *et al*., (2011) recently attempted to quantitatively assess PLCζ RNA in globozoospermic (a sperm morphological defect with round sperm heads) men or those with low or failed fertilization in comparison with men exhibiting normal fertility, finding significant correlations between low fertilization rates and relative levels of PLCζ RNA. Alternatively, attempts to measure sperm-PLCζ deficiencies have employed microinjection of human sperm into mouse oocytes (known as the mouse oocyte activation test; MOAT) to evaluate the activation capacity of human sperm (Rybouchkin *et al*., 1996; Heindryckx *et al*., 2005; 2008). However, considering the increased relative potency of human PLCζ activity compared to mouse PLCζ when injected into mouse oocytes (Swann *et al*., 2006; Nomikos *et al*., 2014a), the MOAT may only detect extreme cases where PLCζ is completely absent from sperm, and not where a more subtle reduction in PLCζ is present, such as in a clinical setting. Furthermore, both the MOAT and RNA quantification require specialized resources and skill sets, which are not necessarily routinely available in general infertility clinics.

An alternative, and perhaps more clinically attractive approach, is immunocytological analysis of sperm-PLCζ protein. Indeed, some effort has been made with studies identifying

relationships between reduced/absent levels of sperm PLCζ and male-specific conditions of infertility (Kashir *et al*., 2010; 2012c; 2013; 2014; Nikiforaki *et al*., 2014; Escoffier *et al*., 2015; 2016; Park *et al*., 2015). However, such studies are significantly hindered by inadequate antibody specificity, at least of human PLCζ in the published literature, reflected in the significant variance of PLCζ localization patterns reported amongst these studies, an occurrence extending to studies examining porcine PLCζ. Herein, to examine such questions, we employed highly-specific polyclonal antibodies against native human, porcine, and murine PLCζ to develop a uniform protocol for immunological analysis of PLCζ in mammalian sperm.

Intriguingly, despite recognizing a single predominant band corresponding to native PLCζ with high fidelity in sperm from all three species, the antibodies we employed were unable to satisfactorily detect PLCζ via immunofluorescence on aldehyde-fixed sperm from all three species, despite our immunofluorescence protocols revealing distinctive patterns of PAWP and acrosin localization consistent with the published literature. We considered that PLCζ in sperm was somehow rendered inaccessible to the antibody following aldehyde-fixation. Indeed, epitope inaccessibility following aldehyde fixation is a common problem faced in numerous diagnostic immunohistological applications (Berod *et al*., 1981; D'Amico *et al*., 2009), an obstacle requiring a process termed AUM to overcome.

Aldehyde fixation is most commonly used for sample preservation, particularly formaldehyde and glutaraldehyde for light and electron microscopy applications, respectively (D'Amico *et al*., 2009). Aldehyde fixation, depending on the type of fixative used, results in the 'cross-linking' of protein amino acid residues by methylene bridges (French & Edsall, 1945; Fox

et al., 1985), alongside alterations in carbohydrates, nucleic acids and phospholipids (D'Amico *et al*., 2009). The extent of such cross-linking modifications depends on several factors, such as the fixation time and temperature, and fixative pH and concentration (D'Amico *et al*., 2009). Exposure of a peptide sequence to formaldehyde results in the formation of a methylol (hydroxymethyl) group on an amino acidic residue, subsequently resulting in the formation of Schiff's bases (imine groups) via condensation of methylol groups on lysine residues, which further condense with other groups including phenolic, indole, imidazole groups of arginine, asparagine, glutamine, histidine, tryptophan, cysteine and tyrosine, to form methylene bridges (Fraenkel-Conrat & Mecham, 1949; Kunkel *et al*., 2981; Metz *et al*., 2004; 2006). One line of thought proposes that intra- and inter-molecular links induced by aldehyde fixation alter the protein secondary, tertiary and quaternary structure, thereby lowering antibody accessibility to its epitope. However, other theories propose that perhaps such cross-linking may be limited to the protein primary structure (D'Amico *et al*., 2009). Regardless of the mechanisms involved, it is possible that such phenomenon may have resulted in the poor visualization efficacy of PLCζ in mammalian sperm. Indeed, application of AUM to all three species of sperm resulted in at least a 2-fold increase in observable fluorescence. However, it is unclear why this effect seemed only specific when applied to PLCζ, and not to PAWP or acrosin.

Creation of cross-links via aldehyde fixation would in theory be random, or at most driven by amino acid composition in the overall protein fold. Arginine, tyrosine and lysine residues are thought to be very reactive for creation of such cross-links, whereas asparagine, glutamine, and histidine residues show a weaker reaction (D'Amico *et al*., 2009). Distributional analysis of the human, murine, and porcine amino acid PLCζ sequence shows that the

predominant amino acids in terms of overall distribution are leucine (~9.5%), isoleucine (~7.8%), serine (~7.5%), lysine (~7%), and glutamine (~7%), indicating that apart from lysine, PLCζ does not contain amino acids that would be expected to exhibit a strong cross-linking reaction, or at least strong enough to block antibody-epitope binding to the degree that we observed. Such parameters may be influenced by the three-dimensional (3D) conformation of native protein, and in the current absence of a high-resolution PLCζ protein 3D structure, would require examination with multiple antibodies recognizing different PLCζ sequence epitopes. However, it should be noted that we employed two different antibodies against PLCζ in this study against different epitopes, albeit in different sperm species, and observed the same effect.

The mechanisms of AUM are not yet well established, with numerous theories existing to explain such phenomena, including cross-link breakage, renaturation, formation of holes and gaps, diffusible protein extraction, precipitation, stabilization, and protein epitope rehydration. Collectively though, the common proposition is that AUM treatment breaks down protein cross-linkages associated with aldehyde fixation (Leong & Leong, 2007; D'Amico *et al*., 2009), allowing antibody accessibility to epitopes. There are numerous methods by which such goals may be achieved, involving heat treatment, alkali or acid exposure, or a combination of multiple treatments (D'Amico *et al*., 2009). Indeed, our experiments indicated that application of all of these methods significantly increased the visualization efficacy of PLCζ in mammalian sperm, apart from HCl treatment in murine sperm, which did not result in a significant change.

Collectively, our results indicate that aldehyde fixation-induced cross-linking events may be limiting antibody accessibility to epitopes in mammalian sperm, requiring AUM methodology

to optimally visualize PLCζ across all three species. However, the extent of this increase varied between species examined, and the specific AUM methodology employed. All three methods had a differential effect on the extent of the increase observed in relative fluorescence, depending on species studied. It is well known that the biochemical properties of mammalian PLCζ differ, being uniquely optimized to the physiological requirements of the species in question. For example, mouse PLCζ is in a considerably more insoluble state within the sperm as compared to human or porcine PLCζ (discussed in more detail below). Thus, it is possible that each mammalian PLCζ isoform requires differential AUM treatment for optimal visualization efficacy, depending on the biochemical properties of the species of sperm PLCζ examined.

However, to investigate the extent of this phenomenon in sperm cells, we performed MeOH fixation (which fixes via precipitation of protein to the cytoskeleton) to exclude formation of aldehyde-induced cross-links. With the exception of porcine sperm, we observed AUM application again significantly improved PLCζ visualization efficacy, at least doubling the relative fluorescence observed, suggesting a more complex reason than just aldehyde-induced cross-linkages preventing antibody accessibility to epitopes. It is worth noting that we observed, at best, no significant change in relative fluorescence following AUM in MeOH-fixed porcine sperm, with most AUM methods resulting in a decrease in observable PLCζ fluorescence. However, we also observed a significantly different pattern of PLCζ localization distribution in the sperm head in porcine and murine PLCζ, with porcine PLCζ exhibiting the biggest change, predominantly being diffusely spread throughout the sperm head, as opposed to the distinct equatorial and post-acrosomal localization observed in aldehyde-fixed sperm. A shift was also observed in murine sperm, with a predominant acrosomal localization observed, as opposed to

the dominant post-acrosomal population seen in aldehyde-fixed sperm. While the overall pattern of localization did not differ in human sperm, observable patterns seemed significantly more diffuse.

Perhaps this effect may be due to disparate solubility of PLCζ, particularly in porcine sperm. Cumulative data suggest that PLCζ is present in both soluble and insoluble fractions in the sperm head, depending on species studied. Indeed, mouse PLCζ seems present largely in an insoluble state, with specific extraction protocols required to extract mouse PLCζ from the detergent–resistant perinuclear theca (in the post-acrosomal region of the sperm), contrasting with human PLCζ which is mostly readily extractable from sperm in sperm extracts (Fujimoto *et al*., 2004). However, porcine PLCζ may be somewhat intermediate between these two species, as PLCζ has been detected as both soluble and insoluble fractions in porcine sperm (Kurokawa *et al*., 2005). It is possible that specific localization patterns may be attributable to such properties, depending upon the species-specific physiological requirements of oocyte activation (Kashir *et al*., 2014). Hence, the equatorial and post-acrosomal populations may be the most physiologically relevant to oocyte activation. It is also worth noting that the sperm tail is eventually completely incorporated with the oocyte (Yanagimachi, 1994; Ramalho-Santos, 2011; Kashir *et al*., 2012d) and may thus present some physiological relevance to the tail populations of PLCζ that have been identified in both porcine and equine sperm (Bedford-Guaus *et al*., 2011; Nakai *et al*., 2011), alongside the tail populations identified in this study.

MeOH-mediated fixation is performed via precipitation of proteins to the cytoskeleton. Traditionally, this method of fixation has not been routinely used when examining soluble

proteins, due to loss of physiological localization of such proteins, hence why such protocols have previously sparsely been used for PLCζ. A further property of MeOH-fixation is that membrane permeabilisation occurs alongside fixation, potentially resulting in protein loss from the cell. Therefore, localization patterns observed in MeOH-fixed sperm are most likely non-physiological. Thus, particularly in porcine sperm, there was most likely a significant loss of protein from the sperm while being fixed via MeOH. Coupled with the multiple washing steps involved during immunofluorescence and AUM protocols, such reasoning may explain the reduced relative fluorescence observed following AUM in porcine sperm. This same principle would of course apply to human and murine sperm. It is therefore even more surprising that we observed a significant increase in relative fluorescence following the application of AUM protocols. It is worth suggesting that were it not for a shift in localization pattern and potential loss of PLCζ from the sperm head, the observable increase may have been significantly higher than currently observed in MeOH-fixed sperm.

An intriguing point of note is that we consistently observed PLCζ localisation within the tail of the all three species of sperm examined, in line with previous tail observations in the published literature (Fujimoto *et al*., 2004; Grasa *et al*., 2008; Yoon *et al*., 2008; Heytens *et al*., 2009; Young *et al*., 2009; Bedford-Guaus *et al*., 2011; Kashir *et al*., 2011a; 2011b; Nakai *et al*., 2011; Kaewmala *et al*., 2012; Kashir *et al*., 2013; Sato *et al*., 2013). Previously, only the equine and porcine tail populations seemed to tentatively correlate to the ability of the tail to initiate low frequency Ca^{2+} oscillations upon microinjection into oocytes (Nakai *et al*., 2011; Bedford-Guaus *et al*., 2011). However, particularly with human sperm, tail localisation has been attributed to non-specific binding of antibody. Herein, we show that the tail population persists in sperm from

all three species following immunofluorescent analysis with both our PLCζ-specific antibodies. Indeed, as the sperm tail is eventually incorporated with the oocyte following gamete fusion, such potential physiological relevance to tail populations of PLCζ have been suggested as potential 'reservoirs' of PLCζ that ensure a steady supply of PLCζ to the activating oocyte, maintaining the required Ca^{2+} oscillation profile (Kashir *et al*., 2014).

Examination of images before AUM indicated tail fluorescence was more readily visible compared to the sperm head, perhaps due to potentially enhanced concentration of PLCζ in this confined structure compared to the head. Indeed, this trend is repeated (at least visually) following AUM, where the tail staining also increases in intensity. However, it was not possible to effectively quantify this increase in tail fluorescence due to the presence of the tail in multiple planes of view. Thus, while our current data suggest that such tail populations may indeed be authentic in mammalian sperm, visualisation of which is also seemingly enhanced by AUM methodology, more focused experiments are required to test the biochemical and physiological validity of such assertions.

It is prudent to consider that we utilised normal fluorescence microscopy for our studies, as we felt this would provide a more physiological representation of PLCζ localisation and intensity within sperm. It has been possible to visualise PLCζ localisation in mammalian sperm via confocal microscopy in previous studies, which yielded much sharper images pertaining to localisation pattern. However, considering the increased sensitivity of confocal compared to normal fluorescence microscopy, such representations are not necessarily physiological, as even very faint signals may be amplified to obtain striking images. Indeed, we have previously shown

this using the human PLCζ antibody used in the present study (Nomikos *et al*., 2013), whereby we could effectively visualise PLCζ without AUM, but not necessarily in a manner that would be viable within an infertility clinic. Indeed, most fertility clinics are not equipped with fluorescence microscopy, and would require methods of PLCζ detection that are not as sensitive (or cost-prohibitive) as confocal microscopy. Collectively, we consider that utilisation of AUM protocols would enable increased accessibility of PLCζ as a diagnostic measure of oocyte activation capacity within the fertility clinic.

To the best of our knowledge, this is the first application of AUM protocols for the study of soluble proteins in mammalian sperm cells. We were also unable to identify any similar cytological studies upon mammalian sperm in the published literature. AUM protocols have previously been applied in studies examining nuclear proteins within oocytes. Indeed, Fulka & Langerova (2014) required employment of a similar AUM protocol to our sodium citrate method when studying nucleolar proteins in nucleolus precursor bodies within mouse oocytes and pre-implantation embryos. Li & O'Neill (2012) recently reported that application of AUM protocols to fertilizing mouse oocytes suggested overall demethylation of 5′-methylation of CpG (5meC) was perhaps not due to active demethylation of these sites as is traditionally thought, but rather due to increasing co-localisation of methyl binding domain 1 protein (MBD1) with 5meC, an observation only noticed following application of AUM. It is thus apparent, that in oocytes and embryos at least, AUM is required to resolve relationships between closely interacting proteins, particularly in highly condensed structures such as nuclei. This is reflected in our results, whereby we required application of AUM to achieve the highest fidelity of results. However, this was true for both aldehyde- and MeOH-fixed sperm (with the exclusion of porcine

sperm), indicating that this phenomenon was independent of aldehyde-induced cross-linkages. However, aldehyde-induced cross-linkages may compound the issue further.

Each method of AUM seemed to differentially affect the visualisation efficacy of PLCζ in sperm from all three species. Such an example is that sodium citrate AUM was most effective in human and mouse sperm, but was not as effective as AT AUM in porcine sperm. It is worth noting that each method of AUM utilised different principles to perform antigen unmasking, which is further complicated by the fact that PLCζ of different species possess different biochemical properties dependant on the physiology of oocyte activation of that species. A further issue to consider is that fixation method also seemed to exert an effect (aldehyde versus MeOH fixation). An example is that in aldehyde-fixed mouse sperm, the sodium citrate method yielded the best enhancement of visualisation efficacy of PLCζ, with HCL giving the lowest enhancement. However, in MeOH-fixed mouse sperm, the converse is true. Thus, presence or absence of cross-links may also exert an effect upon AUM method effectiveness.

A further complication of diagnostic evaluation of human sperm is the significant variance in terms of sperm quality amongst different males (Kashir *et al*., 2010). Indeed, this problem is reflected in recent attempts to establish correlations between PLCζ levels/localization patterns and fertility status, whereby no significant motif has yet been identified (Kashir *et al*., 2013; Yelumalai *et al*., 2015), albeit using restricted antibody specificity. Thus, to examine the effect that AUM may exert on potential diagnostic outcome, we performed AT AUM upon sperm from 13 individual human males. In the majority of cases, AUM resulted in a marked improvement of such observable parameters, the extent of which was notably variable.

Furthermore, a reduction was observed in relative fluorescence and proportions of sperm exhibiting PLCζ in sperm from two individuals each, again occurring to varying degrees.

Considering that immuno-diagnostic assays of PLCζ have been proposed to examine the fertility status of sperm with regards to OAD, such changes in parameters following AUM suggest that AUM methodology may be required to ascertain a more accurate picture of PLCζ-related fertility status in a clinical setting. Changes in relative fluorescence observed following AUM seemed minor but amounted to at least a 2-fold difference (either increase or decrease), although it was not possible to determine the clinical relevance of such difference in the current study. Kashir *et al*., (2013) previously suggested that examination of relative fluorescence may not be a valid method of examining PLCζ status in human sperm, as no correlative motifs between fertility status and observable fluorescence were found. However, the authors did not use AUM protocols to perform their analyses, which may affect such correlations. Furthermore, these authors also suggested that examining the proportion of sperm exhibiting PLCζ fluorescence may be a better way of ascertaining PLCζ-related fertility status, as such parameters seemed to correlate to sperm parameters and reproductive outcome, particularly in potential cases of sub-fertility.

In the current study, we found that the parameter most significantly affected by AUM was the proportion of sperm exhibiting PLCζ fluorescence, which in the majority of cases exhibited increases of 10-20%. In some patients, this caused values to increase from 80% to about 90%, possibly correlating to normal/high fertility, but in others (particularly Patients 2 and 5) this increment resulted in values of 50-60% increasing to above 75%. Conversely, two patients also

exhibited a decrease in proportion of sperm exhibiting PLCζ fluorescence (Patient 8 and 11; both exhibiting a drop of about 1%) following AUM. While in these two patients this proportion was relatively quite high (exceeding 80% in both cases), such observations suggest that apparently high PLCζ parameters may also fall following AUM. In both scenarios, our results suggest that AUM affects the parameters of PLCζ-related analysis, implying a risk of misdiagnosis without AUM. However, a more exhaustive study correlating fertility status, sperm parameters, and AUM is required on a much larger population before such assertions can be validated.

Conclusions

In the current study, we show that AUM methodology is required to effectively increase the visualization efficacy of PLCζ in mammalian sperm. We also show that the efficacy of such methodology is independent of fixation protocol, at least in human and mouse sperm. We propose that this phenomenon is perhaps the first indication of a strong intramolecular interaction within the PLCζ monomer, or of inter-molecular interactions either with other PLCζ units (PLCζ oligomers), or perhaps with other modulatory protein(s) in tight conformation interactions with PLCζ, thereby preventing antibody access to it's epitope. Indeed, this may have been further exacerbated by aldehyde fixation-induced cross-linkage formation. We propose that AUM sufficiently weakened such interactions, allowing increased access of antibody to it's epitope and enhancing visualization efficacy (see schematic in Figure 6). Keeping our current results in mind, we would also propose that perhaps AUM methodology potentially could greatly benefit other potential markers of gamete fertility.

However, it is worth noting that such suggestions are speculative at this stage, and require more intensive investigations to ascertain veracity. Furthermore, the exact phenomenon is most likely more complex, as the extent of AUM is seemingly species-dependent. PLCζ biochemistry and mechanism of action have been extensively examined in relation to oocyte activation. However, relatively scant attention has been paid to these parameters within sperm, largely due to the issues pertaining to antibody specificity as previously mentioned. Another question that remains unresolved pertains to how PLCζ is kept in an inactive form in the sperm, despite PLCζ being the most Ca^{2+}-sensitive and active PLC isoform identified to date. Our current results shed significant light on this, paving the way for further work to be performed. Indeed, it may

perhaps be prudent that all previous studies investigating links between PLCζ and fertility parameters are re-examined in the context of AUM.

Finally, our data suggest that AUM may be potentially applied to more effectively assess sperm PLCζ status in a clinical diagnostic setting. It is thus essential that the results reported in the current study are further expanded upon in dedicated studies examining larger patient populations, additional sperm parameters and, most important, male fertility outcome. It would be essential that such questions are resolved, before the clinical diagnostic promise of PLCζ within the infertility clinic is fully realized.

Authors' roles

JK, KS and FAL devised the project strategy. JK, MN, KS and FAL designed the experiments. PA, DS, PK and AB were involved in patient recruitment and sperm processing. The experiments were performed by JK, LB, MN, BC, and PS. JK, MN, KS and FAL prepared the manuscript, with input and approval from all authors.

Funding

JK is supported by a Health Fellowship award from the National Institute for Social Care and Health Research (NISCHR). MN is supported by a Marie Curie Intra-European Research Fellowship award. This work was also partly funded by a research grant from Cook Medical Technologies LLC.

Conflict of interest

None declared

Figure Legends

Figure 1: **Representative comparison between native PLCζ, PAWP, and acrosin in human sperm.**

(a) Coomassie staining and immunoblotting (left and right panels, respectively), **(b)** immunofluorescence, and **(c)** semi-quantification of observed immunofluorescent signal. Immunoblotting was performed on gels loaded with $5x10^5$ sperm/lane. Green immunofluorescent images indicate fluorescence using the appropriate antibody. White scale bars indicate 5μm. Relative fluorescence was quantified in arbitrary units (a.u). Asterisks denote a statistically significant difference ($p<0.05$), as determined by one way ANOVA.

Figure 2: **Comparison of PLCζ visulaisation efficacy before and after AUM in aldehyde-fixed human sperm.**

Representative comparative **(a)** fluorescence images before and after AUM and **(b)** histogram of relative fluorescence between no AUM, HCl, AT, and sodium citrate methods of AUM. Green fluorescence indicates PLCζ visualization. White scale bars indicate 5μm. Asterisks denote a statistically significant difference ($p<0.05$), while combinations of asterisks (i.e. *, **) denote significant differences between specific groups as determined by one way ANOVA. AUM: antigen unmasking; HCl: hydrochloric acid; AT: acid Tyrode's solution; DIC: differential interference contrast.

Figure 3: **Comparison of PLCζ visulaisation efficacy before and after AUM in aldehyde-fixed porcine and murine sperm.**

(a) Immunoblotting of porcine and murine native PLCζ in 4-20% gradient gels loaded with $1x10^6$ sperm/lane, showing specific identification of single immunoreactive bands corresponding to the approximate size of PLCζ. **(b)** Representative comparative fluorescence images before and after AUM in porcine (top panel) and murine (bottom panel) sperm. **(c)** Histogram indicating comparative relative fluorescence between no AUM, HCl, AT, and sodium citrate methods of AUM in porcine sperm. **(d)** Histogram indicating comparative relative fluorescence between no AUM, HCl, AT, and sodium citrate methods of AUM in murine sperm. Green fluorescence indicates PLCζ visualization. White scale bars indicate 5μm. Asterisks denote a statistically significant difference ($p<0.05$), while combinations of asterisks (i.e. *, **) denote significant differences between specific groups as determined by the students *t* test for **(a**) and one way ANOVA for **(b-d**).

Figure 4: **Comparison of PLCζ visulaisation efficacy before and after AUM in MeOH-fixed human, porcine, and murine sperm.**

Histogram indicating **(a)** comparative relative fluorescence between no AUM, and AT AUM in human sperm, and comparative relative fluorescence between no AUM, HCl, AT, and sodium citrate methods of AUM in **(b)** porcine sperm, and **(c)** murine sperm following MeOH fixation. Asterisks denote a statistically significant difference ($p<0.05$), while combinations of asterisks (i.e. *, **) denote significant differences between specific groups as determined by one way ANOVA.

Figure 5: Comparison of the effects of AUM upon relative fluorescence levels and proportions of sperm exhibiting PLCζ fluorescence in human sperm from different donors.

Histograms representing the mean change in PLCζ visualisation expressed as **(a)** relative fluorescence (a.u) and **(b)** proportions of sperm exhibiting PLCζ fluorescence (%), when AT AUM was (light grey) or was not (dark grey) applied to sperm from 13 individual patients. Asterisks denote a statistically significant difference ($p<0.05$) between patients groups for No AUM versus AUM as determined by the students *t* test.

Figure 6: **Schematic summary of proposed mechanism by which AUM increases visualization efficacy of PLCζ in mammalian sperm.**

Before AUM (left panel), PLCζ (red circle) may be in a tight conformation with either itself, other PLCζ units, or with other as yet unidentified proteins (X: light blue circle). Such strong interactions may restrict antibody binding to epitope. This could also be further exacerbated by the presence of aldehyde-induced cross-linkage formation (blue lines). Following AUM (right panel), such strong interactions may be weakened, alongside cross-linkage diminishing (dotted blue lines). Collectively, this may serve to increase antibody binding to epitope, increasing visualization efficacy of PLCζ in mammalian sperm.

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