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ORIGINAL ARTICLE

Sperm Biology

Advancing male age differentially alters levels and localization patterns of PLCzeta in sperm and testes from different mouse strains

Junaid Kashir[1,2,*], Bhavesh V Mistry[2,*], Maha Adel Gumssani[1,2], Muhammad Rajab[2], Reema Abu-Dawas[1,2], Falah AlMohanna[2], Michail Nomikos[3], Celine Jones[4], Raed Abu-Dawud[2], Nadya Al-Yacoub[2], Kevin Coward[4], F Anthony Lai[3,5], Abdullah M Assiri[1,2]

Sperm-specific phospholipase C zeta (PLCζ) initiates intracellular calcium (Ca^{2+}) transients which drive a series of concurrent events collectively termed oocyte activation. Numerous investigations have linked abrogation and absence/reduction of PLCζ with forms of male infertility in humans where oocyte activation fails. However, very few studies have examined potential relationships between PLCζ and advancing male age, both of which are increasingly considered to be major effectors of male fertility. Initial efforts in humans may be hindered by inherent PLCζ variability within the human population, alongside a lack of sufficient controllable repeats. Herein, utilizing immunoblotting, immunofluorescence, and quantitative reverse transcription PCR (qRT-PCR) we examined for the first time PLCζ protein levels and localization patterns in sperm, and *PLCζ* mRNA levels within testes, from mice at 8 weeks, 12 weeks, 24 weeks, and 36 weeks of age, from two separate strains of mice, C57BL/6 (B6; inbred) and CD1 (outbred). Collectively, advancing male age generally diminished levels and variability of PLCζ protein and mRNA in sperm and testes, respectively, when both strains were examined. Furthermore, advancing male age altered the predominant pattern of PLCζ localization in mouse sperm, with younger mice exhibiting predominantly post-acrosomal, and older mice exhibiting both post-acrosomal and acrosomal populations of PLCζ. However, the specific pattern of such decline in levels of protein and mRNA was strain-specific. Collectively, our results demonstrate a negative relationship between advancing male age and PLCζ levels and localization patterns, indicating that aging male mice from different strains may serve as useful models to investigate PLCζ in cases of male infertility and subfertility in humans.

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INTRODUCTION

Oocyte activation encompasses a series of events driven by intracellular calcium (Ca^{2+}) oscillations, initiated by testis-specific phospholipase C zeta (PLCζ).[1,2] Immunodepleting PLCζ from sperm extracts diminished Ca^{2+}-release within oocytes,[2] while PLCζ was confirmed to be present in extract fractions capable of inducing Ca^{2+} oscillations.[3,4] Recombinant PLCζ cRNA and protein initiated Ca^{2+} oscillations and embryogenesis to the blastocyst stage upon microinjection into mouse oocytes, without any sperm.[2,5] RNA interference (RNAi) disrupted testicular *PLCζ* expression in mice, with sperm inducing abnormal Ca^{2+} oscillations and a reduced litter size.[6] Finally, sperm from knockout mouse models of *PLCζ* were unable to elicit Ca^{2+} oscillations following intracytoplasmic sperm injection (ICSI; whereby a single sperm is microinjected directly into the oocyte). *In vitro* and *in vivo* fertilization experiments exhibited abnormally high levels of polyspermy, and a severely reduced profile of Ca^{2+} release and litter size.[7,8]

Human sperm unable to activate human and mouse oocytes, even following ICSI (oocyte activation deficiency; OAD), either fail to elicit Ca^{2+} oscillations, or demonstrate a reduced capacity to do so.[9] Mutations in the *PLCζ* gene of such patients result in predicted modifications of the enzyme fold, and abrogation of activity and/or levels within the sperm. Furthermore, abnormal patterns of PLCζ localization in human and mouse sperm have been correlated with abnormal sperm morphology and reduced fertility outcomes.[9–13] A particular influencer of male fertility is advancing male age, with older males exhibiting poorer sperm parameters and reproductive outcomes, particularly post-40 years of age.[14] However, few studies have investigated the relationship between advancing male age and PLCζ. Yeste *et al.*[15] indicated that increasing age in human males did not significantly alter sperm PLCζ, although progressive motility negatively correlated with increasing male age. However, Kashir *et al.*[16] showed that significant variability in total levels of human PLCζ could prevent

[1]College of Medicine, Alfaisal University, Riyadh 11533, Saudi Arabia; [2]Department of Comparative Medicine, King Faisal Specialist Hospital and Research Centre, Riyadh 11211, Saudi Arabia; [3]College of Medicine, QU Health, Qatar University, Doha PO Box 2713, Qatar; [4]Nuffield Department of Women's and Reproductive Health, University of Oxford, Level 3, Women's Centre, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom; [5]Biomedical Research Centre, Qatar University, Doha PO Box 2713, Qatar.
*These authors contributed equally to this work.
Correspondence: Dr. J Kashir (jkashir@alfaisal.edu)
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indicative quantification of PLCζ. Factors such as lifestyle differences, diet, and genetic/epigenetic influencers, make it difficult to associate age with altered sperm proteins such as PLCζ in humans. Thus, such studies require the use of controllable and reliable animal models to fully ascertain such potential relationships.

Haverfield *et al.*[17] examined the impact of female age upon Ca^{2+} oscillatory ability in oocytes, indicating that Ca^{2+} release at fertilization remained largely unchanged with advancing maternal age, but exhibited subtle changes in Ca^{2+} processing, potentially leading to more of an impact in response to even slightly altered levels of activation stimuli (*i.e.*, PLCζ). However, the impact of advancing age upon PLCζ in male mice has yet to be ascertained. Significant differences were observed in fertility and sperm quality, particularly between inbred and outbred strains,[18,19] where generally inbred strains are regarded as less fertile compared to outbred counterparts.

Herein, we utilized immunoblotting and immunofluorescence to semi-quantitatively ascertain the effect of advancing male age upon PLCζ protein levels and localization patterns in mouse sperm, while also quantitatively examining levels of *PLCζ* mRNA levels within testes. We examined mice at 8, 12, 24, and 36 weeks of age to ensure a wide age distribution. To account for potential inter-strain variability, we also performed our analyses in two separate strains of mice, the C57BL/6 (B6; inbred) and CD1 (outbred) strains. Finally, we also examined whether mouse sperm exhibited the same extent of PLCζ variability as in human sperm, and whether advancing male age and mouse strain exerted any significant effect upon this.

MATERIALS AND METHODS

Animal tissue processing

Ethical use of animals for this study was approved by the Research Advisory Council of the King Faisal Specialist Hospital and Research Center (Riyadh, Saudi Arabia; RAC # 2160 014), allowing use of C57Bl/6J (B6) and CD1 strains of male mice. Animals were raised under controlled environmental conditions (temperature: 21°C ± 1°C; humidity; 12 h light/12 h dark cycles) in the animal housing facility. Animals were provided with a standard pelleted chow and tap water *ad libitum*. We examined mice of 8 weeks, 12 weeks, 24 weeks, and 36 weeks of age, where 3 mice per age group were used for each strain (a total of 6 mice per age group were examined). Mouse testes and cauda epididymi were excised and washed in pre-warmed phosphate buffered saline (PBS; Sigma Aldrich, Dorset, UK). Testes were snap-frozen in liquid nitrogen and stored at −80°C until subsequent RNA extraction. Testes from 4-week-old mice of both strains were also used to extract RNA for quantitative reverse transcription PCR (qRT-PCR) to serve as baseline measurements for the average-fold change in *PLCζ* RNA with advancing age (as discussed in subsequent sections).

Isolated cauda epididymi were immersed in pre-warmed M2 media (Sigma Aldrich) at 37°C. Cauda tissue were pierced and incubated at 37°C for approximately 30 min to allow motile sperm to swim out. Cauda were discarded, and the sperm suspension centrifuged (5427R table top centrifuge; Eppendorf, Stevenage, UK) at 500*g* for 10 min at room temperature (RT). The sperm pellet was re-suspended in PBS with protease inhibitors (PI; complete ULTRA Tablets, Roche, Mannheim, Germany), and again centrifuged at 500*g* for 10 min at RT, and the sperm pellet resuspended in PBS + PI. Sperm counts were performed using a hemocytometer chamber (Hausser Scientific, Horsham, PA, USA), and single use aliquots made consisting of 1 million sperm per aliquot in ×2 reducing Laemelli sample loading buffer (BIO-RAD, Watford, UK). These were snap-frozen in liquid nitrogen and stored at −80°C until required. The remaining sperm suspension was centrifuged at 500*g* for 10 min at RT, and the sperm pellet resuspended in 4% paraformaldehyde, and fixed at room temperature for 15 min. Sperm were washed again twice with PBS and resuspended in 0.2 mol l^{-1} sucrose (Sigma Aldrich) with protease inhibitors. Sperm were then smeared onto slides pre-coated with 0.01% poly-L-lysine (Sigma Aldrich), and stored for later use.

SDS-PAGE and Immunoblotting

Immunoblotting was performed as previously described.[16,20,21] Briefly, single-use sperm aliquots were thawed and heated at 101°C for 5 min, followed by immediate vortexing, cooling on ice, and brief centrifugation at RT. Protein samples were separated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Hybond, GE Healthcare Life Sciences, Marlborough, MA, USA) using the wet-transfer method at 100 V for 1 h. Transfer and protein separation efficacy were determined by staining membranes with 0.1% (*w/v*) ponceau stain (in 5% [*v/v*] acetic acid). Membranes were incubated overnight at 4°C with primary antibodies diluted at 1:1000 (PLCζ EF polyclonal raised in rabbit as described previously[21]) or 1:5000 (alpha [α]-tubulin monoclonal raised in mouse; Sigma Aldrich), followed by incubation with appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h at RT diluted at 1:1000 (anti-rabbit; Sigma Aldrich) or 1:5000 (anti-mouse; Sigma Aldrich). HRP detection was achieved using the ECL select kit, following the manufacturers recommended protocol (GE Healthcare Life Sciences). Chemiluminescence was detected using the ImageQuant LAS4000 (GE Healthcare Life Sciences) imaging system.

Membranes were also subject to a modified mild stripping protocol (Abcam, Cambridge, UK) to enable probing with different antibodies following mild membrane stripping (stripping buffer: 15 g glycine, 10 g SDS, 10 ml Tween 20, 1 ml β-mercaptoethanol, upto 1 l in double-distilled water [ddH_2O], pH 2.2). Following stripping, membranes were again blocked and re-probed with appropriate primary and secondary antibodies. Stripping efficacy was confirmed by reprobing with appropriate secondary antibody only, which confirmed removal of previous antibody.

Immunofluorescence microscopy

Immunofluorescence was performed as previously described.[16,20,21] Hydrophobic molds were drawn using a Peroxidase-Antiperoxidase (PAP) pen (Vector laboratories, Peterborough, UK) around cauda sperm which had previously been smeared onto pre-coated poly-L-lysine slides. Sperm were permeabilized by addition of PBS-1% Triton X-100 (*v/v*) to PAP molds for 1 h at RT. Antigen unmasking/retrieval (AUM) was performed by immersion in sodium citrate buffer (10 mmol l^{-1} Sodium Citrate, 0.05% Tween 20, pH 6.0) and heating at full power in a microwave as previously described.[20] Following resting at room temperature for 15 min, PAP molds were washed three times with PBS. Blocking was performed using PBS-10% bovine serum albumin (BSA; Sigma Aldrich). Cells were incubated with primary antibodies at appropriate dilutions with PBS-5% BSA overnight at 4°C, after which cells were incubated with AlexaFluor-488 conjugated goat anti-rabbit secondary antibody (1:100; Life Technologies, Glasgow, UK), diluted in PBS-5% BSA for 1 h at RT. Finally, cells were mounted with Vectashield mounting

medium containing 4'-6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and slides stored at 4°C in the dark until imaging (not more than 3 days).

Images were captured at ×40 and ×100 magnifications (with oil-immersion, type FF, Cat. No. 16916-04, Electron Microscopy Sciences, Hatfield, PA, USA), using an OLYMPUS BX53 fluorescence microscope (Olympus, Waltham, MA, USA). An OLYMPUS DP73 camera (Olympus) was used to capture images using OLYMPUS cellSens Entry software (Olympus). Brightfield images were captured alongside the corresponding fluorescence images obtained using a fluorescein isothiocyanate (FITC) filter. Care was taken to ensure images were captured at the same exposure time throughout.

Sperm PLCζ analysis

Sperm PLCζ fluorescence quantification was performed on images obtained at 40× magnification as previously described.[20,21] Only sperm in the same plane of view were analyzed for each image, while sperm obscured by overlying debris or by tails/heads of other sperm were excluded. Fluorescent quantification was limited to the sperm head only, consisting of quantification of total PLCζ immunofluorescence and the proportion of PLCζ localization patterns observed. Such analyses were performed with the ImageJ software package (National Institutes of Health, Bethesda, MD, USA) using the regions of interest tool.[20,21] One hundred cells per animal per age group were analyzed (three hundred cells per age group for each strain; a total of six hundred cells per age group). Total fluorescence for each cell was subtracted with background fluorescence (relative fluorescence). PLCζ immunoblotting quantification was achieved through relative density quantification using the ImageJ software package.[20,21] Pixel density of PLCζ bands identified by the EF (PLCζ) antibody was normalized with the pixel density of bands identified by the α-tubulin antibody to give the relative density for PLCζ.

RNA extraction and quantitative reverse transcription PCR

Total RNA was extracted from testis using the TRIzol reagent (Thermo Fisher Scientific, Glasgow, UK) according to the recommended protocol following tissue homogenization (Polytron PT-10-35, Kinematica AG, Eschbach, Germany). Extracted RNA quality was examined by gel electrophoresis on 2% denaturing Tris-acetate-EDTA (TAE) agarose gels pre-stained with ethidium bromide. Only RNA with an OD260/OD280 ratio between 1.8 and 2.1 and intact 28S and 18S RNA bands were used for first strand complementary DNA (cDNA) synthesis using the Superscript III First Strand Synthesis system (Invitrogen, Life Technologies, Waltham, MA, USA) and a BIO-RAD C1000 Touch™ thermal cycler (BIO-RAD, Watford, UK) as per the manufacturer's protocol using Oligo(dT) primers. RNA from testes of both strains were processed for cDNA synthesis simultaneously to avoid experimental variation. Synthesized cDNA was stored at −20°C until required.

Quantitative reverse transcription PCR (qRT-PCR) was performed on 7500 Fast real-time PCR system (Applied Biosystems, Los Angeles, CA, USA) using PowerUp™ SYBR® Green Master Mix (Applied Biosystems) and cDNA synthesized from the total RNA samples of testes at different age groups of B6 and CD1 mice strains, following the recommended protocol. Specific primer sets were used for *PLCζ* (designed against the X-domain; forward primer: 5'-CCAGTAGTGCTGTCCTTAGA-3', reverse primer: 5'-TCTGGAGACGGTAGTGTATC-3'; amplicon size: 137 bp) and ribosomal protein S2 (*Rps2*; forward primer: 5'-CTGACTCCCGACCTCTGGAAA-3', reverse primer: 5'-GAGCCTGGGTCCTCTGAACA-3'; amplicon size: 112 bp) as a positive control and normalization factor. Amplification without cDNA was used as a negative control, alongside a separate reaction using genomic DNA to test for potential contamination. Amplification conditions were 95°C for 15 min, 40 cycles of 95°C at 30 s and 61°C for 30 s, and primer extension at 72°C for 30 s, followed by melting curve analysis (95°C for 15 s, 60°C for 15 s, and 95°C for 15 s) to verify amplicon specificity.

All reactions were performed as 3 technical replicates with three biological samples. Relative expression levels of target genes were analyzed by the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen.[22] Cycle threshold values (Ct) for the genes of interest and *Rps2*, a housekeeping gene, were determined and collected using the 7500 Software version 2.3 (Applied Biosystems). Ct values for *PLCζ* were normalized to the corresponding Ct values of *Rps2* values in each sample, and then the average fold change of *PLCζ* mRNA in 8–36 weeks of age testes was calculated relative to the level of *PLCζ* mRNA in 4 weeks of age testis that was used as a reference.

Statistical analyses

Mean values and the standard error of the mean (s.e.m.) were subject to statistical tests as appropriate to determine statistical significance using the Prism 7.0 software package (GraphPad, San Diego, CA, USA). Data are presented as mean ± s.e.m. with the exception of measurements for variability which was measured by comparisons between the average standard deviations (s.d.). To evaluate differences between two variables, the *t*-test was employed using Welch's correction for unequal standard deviations. Statistical comparisons between multiple variables were performed (including multiple comparisons tests) using either the one-way or two-way analysis of variance (ANOVA) as appropriate. ANOVA analysis was followed by the two-stage step-up method of Benjamini *et al.*,[23] to control the false discovery rate following multiple comparisons. Data distribution were normal, and variances were homogenous prior to running ANOVA. All such analyses were performed on relative fluorescence values obtained after antigen unmasking. $P \leq 0.05$ was considered to be statistically significant. Linear or polynomial regression models were constructed as appropriate and fitness of models determined by r^2 values. Pearson correlation coefficients (r) were calculated to determine significance of correlations, and $P \leq 0.05$ was considered to represent a statistically significant correlation. Positive/negative values represented positive/negative correlations, respectively.

RESULTS

PLCζ profiles in mouse sperm

As previously reported,[20,21,24] the EF polyclonal antibody against PLCζ specifically recognized a single band corresponding to the predicted molecular weight of mouse PLCζ (approximately 74 kDa) within sperm (**Figure 1a**). This corresponded to PLCζ localization predominantly at the post-acrosomal regions of mouse sperm, with a secondary, less intense acrosomal population (**Figure 1b**). PLCζ quantification did not reveal any significant differences between levels of PLCζ and mouse strain examined, regardless of age (**Figure 1c** and **1d**).

PLCζ localization in mouse sperm in relation to advancing male age

Postacrosomal PLCζ was predominantly present in younger mice (8 and 12 weeks of age), which increased in intensity upon progression from 8 weeks to 12 weeks of age. At 8 weeks of age, sperm exhibited a predominantly postacrosomal pattern of localization. However, by 12 weeks of age, the acrosomal pattern became more intense. Progressing from 12 weeks to 24 weeks of age, localization patterns of PLCζ became increased in the acrosomal populations compared to the postacrosomal population. By 36 weeks of age, sperm exhibited a

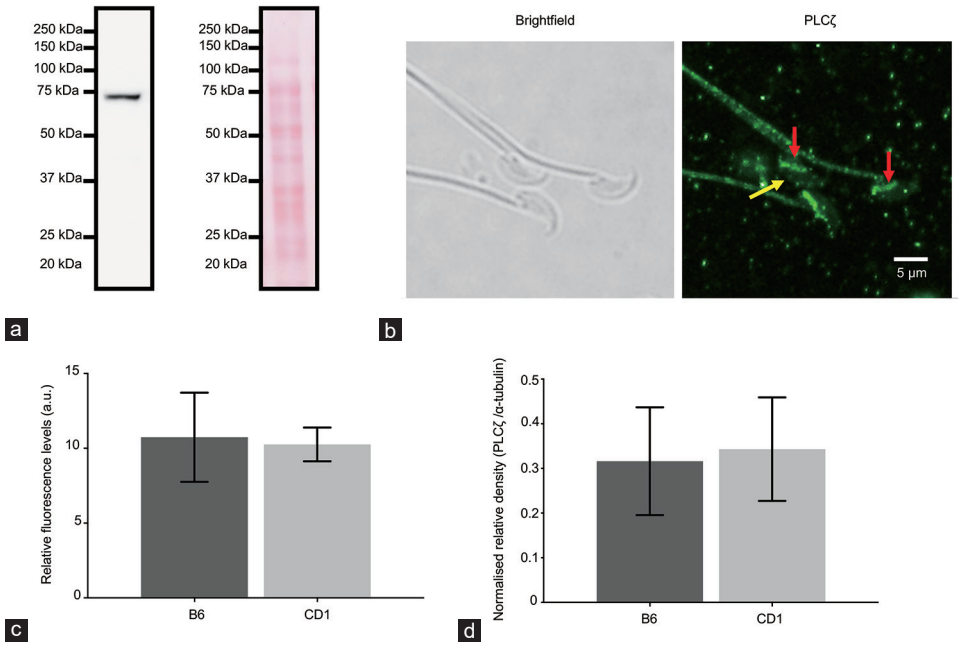

Figure 1: (**a**) Representative immunoblotting analysis and corresponding ponceau-stained image indicating recognition of a single band corresponding to the molecular weight of PLCζ (approximately 74 kDa) in mouse sperm. (**b**) Representative immunofluorescence images of observed PLCζ localization patterns in mouse sperm. Brightfield (left panel) and PLCζ (green fluorescence; right panel) images were obtained. PLCζ localization was observed at the postacrosomal (red arrows) and acrosomal (yellow arrow) regions of the mouse sperm head. Fluorescence was also observed in the sperm tail. Images obtained were captured at ×100, scale bar = 5 μm. Quantification of PLCζ using (**c**) immunofluorescence and (**d**) immunoblotting did not indicate any significant differences in PLCζ from B6 or CD1 sperm, regardless of age. B6: C57BL/6 inbred mouse strain; CD1: outbred mouse strain; PLCζ: phospholipase C zeta; a.u.: arbitrary units.

diminished intensity at both acrosomal and postacrosomal regions of the sperm head (**Figure 2**). A consistent observation throughout all ages and strains examined was the presence of PLCζ fluorescence in the tail, which did not seemingly change in fluorescence intensity, at least to an observable degree. However, as we were unable to quantify the tail fluorescence observed, the extent of any fluctuations in levels remains unknown.

Sperm PLCζ quantification using immunofluorescence and immunoblotting

Levels of PLCζ fluorescence in mouse sperm, regardless of strain, altered significantly with advancing male age (**Figure 3a**) as indicated by one-way ANOVA analysis. Collectively, levels of PLCζ fluorescence increased by approximately 58% when progression from 8 weeks (relative fluorescence of 9.5 arbitrary units [a.u.]) to 12 weeks of age (relative fluorescence of 15 a.u.). However, progression from 12 weeks to 24 weeks (relative fluorescence of 9.1 a.u.), then 24 weeks to 36 weeks (relative fluorescence of 2.2 a.u.) of age led to successive reductions in PLCζ fluorescence by approximately 58% and then approximately 78% (**Figure 3a**). A similar significant distribution (as indicated by one-way ANOVA analysis) was observed with increasing age in sperm from the B6 strain of mice, with maximal levels of sperm PLCζ fluorescence observed at 12 weeks of age (relative fluorescence of 17.5 a.u.). This followed an increase of approximately 79% from 8 weeks of age (relative fluorescence of 9.8 a.u.), but then declining rapidly with increasing age to 24 weeks (relative fluorescence of 6.5 a.u.) and then 36 weeks (relative fluorescence of 1.2 a.u.) of age (reductions of approximately 63% and approximately 86%, respectively; **Figure 3b**).

Sperm from CD1 mice revealed a slightly different dynamic, with PLCζ fluorescence increasing by approximately 35% upon progression from 8 weeks (relative fluorescence of 8.5 a.u.) to 12 weeks of age (relative fluorescence of 11.5 a.u.). PLCζ fluorescence remained constant upon progression from 12 weeks to 24 weeks of age (relative fluorescence of 11 a.u.), followed by a significant decline of approximately 68% upon progression from 24 weeks to 36 weeks of age (relative fluorescence of 3.5 a.u.; **Figure 3c**). Regression models suggested that levels of PLCζ exhibited a very dynamic relationship with advancing male age, with sperm from separate strains exhibiting a differential relationship (**Figure 3d**). Two-way ANOVA analysis further indicated that both age and mouse strain exerted a statistically significant effect upon levels of fluorescence, indicating that both factors determined levels of PLCζ within the sperm head.

Immunoblotting of sperm from all ages identified specific bands for both PLCζ, and α-tubulin, at the expected molecular weights (approximately 74 kDa and approximately 50 kDa, respectively; **Figure 4a**). Quantification of PLCζ relative density (normalized with α-tubulin) indicated that inclusive of both strains, total levels of sperm PLCζ exhibited a linear, negative correlation with advancing male age (r =-0.9995; r^2 = 0.9991; **Figure 4b**).

However, each separate strain indicated a differential dynamic relationship between total sperm PLCζ levels and advancing age. Advancing from 8 weeks (normalised relative density of 0.65 a.u.) to 12 weeks of age (normalized relative density of 1.4 a.u.) resulted in a significant increase of PLCζ in B6 mice by approximately 58%, but alternatively in a decrease of approximately 39% in sperm from CD1 mice between 8 weeks and 12 weeks of age (normalized relative densities of 1.6 a.u. and 0.98 a.u., respectively). However, from 12 weeks to 36 weeks of age PLCζ did not significantly change as indicated by one-way ANOVA. However, two-way ANOVA indicated that the observed distributions were statistically significant, with both strain and age exerting a significant effect (**Figure 4c**) as was observed for the results obtain for fluorescence quantification.

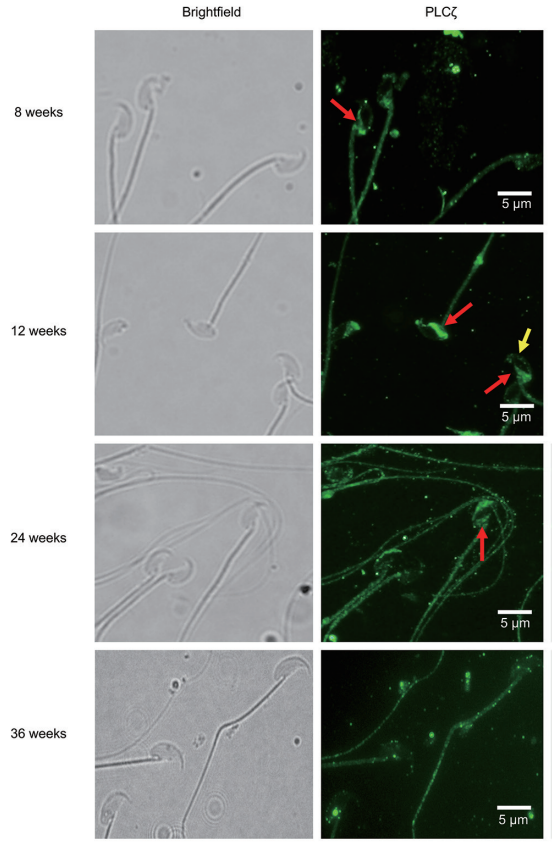

Figure 2: Representative immunofluorescence images of observed PLCζ localization patterns in mouse sperm with relation to advancing male age, regardless of strain, at 8 weeks, 12 weeks, 24 weeks, and 36 weeks of age. Brightfield (left panel) and PLCζ (green fluorescence; right panel) images were obtained. PLCζ localization at the postacrosomal is indicated by red arrows, while acrosomal PLCζ is indicated by a yellow arrow. Fluorescence was also observed in the sperm tail throughout all ages examined. Images obtained were captured at ×100, scale bars = 5 μm. Images are representative of sperm from 6 animals from each age group, with a total of at least 600 cells examined per age group. Images are representative of each age group (*i.e.*, the majority of sperm exhibited the depicted pattern(s) of localisaton. PLCζ: phospholipase C zeta.

Average fold change in testicular PLCζ mRNA levels

Levels of *PLCζ* mRNA in testes, regardless of strain were generally higher in younger (8- and 12-week-old) mice compared to older (24- and 36-week-old) mice. *PLCζ* mRNA levels did not differ significantly between younger mice (8 weeks and 12 weeks of age; average fold change of 1.2 and 1.4, respectively), and older mice (24- and 36-week-of age; average fold change of 0.6 and 0.57, respectively), as indicated by one-way ANOVA (**Figure 5a**). However, *PLCζ* mRNA from B6 mice testes exhibited a similar relationship with age as with sperm PLCζ protein. Specifically, an increase of approximately 50% was observed upon progression from 8 weeks to 12 weeks of age (average fold change of 0.6 and 1.2, respectively), followed by a significant successive reduction of approximately 75% and approximately 50% upon progression to 24 weeks and 36 weeks of age, respectively (average fold change of 0.3 and 0.15, respectively), as indicated by one-way ANOVA (**Figure 5b**). However, no significant decrease in levels of *PLCζ* mRNA were observed in CD1 testes with advancing male age (**Figure 5c**).

Variance in levels of sperm PLCζ with relation to strain and advancing age

PLCζ variance in both strains did not differ from one another when examined regardless of age (data not shown). However, when examined in relation with age, variance exhibited a statistically significant negative correlation as indicated by both immunofluorescence ($r = -0.9789$; $r^2 = 0.9582$) and immunoblotting ($r = -0.9019$; $r^2 = 0.8135$; **Figure 6a** and **6b**). Both the B6 and CD1 strains exhibited unique relationships between PLCζ variance and age. Generally, B6 sperm exhibited decreasing variance with increasing age, albeit with slightly different dynamics when examining relative fluorescence (**Figure 6c**) or relative density (**Figure 6d**). However, CD1 sperm only exhibited a significant distribution of variation in relation to age following fluorescence quantification (**Figure 6e**), with relative density quantification not exhibiting any significant change with increasing age (**Figure 6f**). Increasing levels of PLCζ were strongly associated with increasing variance in PLCζ inclusive of both strains. The same was observed upon individual strain examination, with the B6 and CD1 strains also exhibiting statistically significant positive correlations between PLCζ variance and levels (**Supplementary Table 1**).

DISCUSSION

Oocyte activation at mammalian fertilization mediated via intracellular Ca^{2+} oscillations within the oocyte are predominantly thought to be induced by sperm-specific protein PLCζ.[2] Numerous studies have linked abrogation/absence/reduction of sperm PLCζ with various conditions of human male infertility.[9–13] Increasing male age is also accepted to negatively affect human sperm function and pregnancy outcomes.[25] However, while there are clear indications between age-dependent fluctuations in gene expression between multiple tissue types,[26] the same has not specifically been investigated in relation to sperm or fertility related proteins. Furthermore, non-human animals are less clear regarding links between advancing male age and sperm quality (*i.e.*, motility, viability, and velocity) and quantity (*i.e.*, volume and concentration). While such parameters seem to decline in some species such as rodents or humans, others such as fish remain unchanged. Advancing male age can be associated with reduced fertilization rates in some, but not all, species studied.[27,28] A further complication is strain varieties, potentially compounded by inbreeding, which in turn may downregulate genes such as PLCζ which are required to ensure the efficacy of developmental processes such as oocyte activation and pre-implantation embryogenesis.

Herein, we investigated for the first time the effect of advancing male age in two different strains of mice, to ascertain whether mouse strain (inbred or outbred) also affected PLCζ profiles in mouse sperm and testes. Collectively, we report that advancing male age resulted in an overall reduction of PLCζ levels in sperm and testes, while also dynamically altering localization patterns of PLCζ. We ascertained that advancing male age affected both strains uniquely. Finally, levels of PLCζ in mouse sperm exhibited significant levels of variability, similar to human sperm. However, such variability was influenced by age, strain, and total levels of PLCζ within mouse sperm.

PLCζ localization in mouse sperm is dynamically associated with advancing male age

Our findings are consistent with most previous studies, which report PLCζ within the postacrosomal and acrosomal regions of the mouse sperm head.[2,3,29] However, we further observed that sperm from younger mice (8–12 weeks of age) exhibited a predominantly postacrosomal pattern of localization, while sperm from older mice (>24 weeks) exhibited higher levels of acrosomal PLCζ compared to their younger counterparts, alongside decreasing levels of PLCζ within sperm, although this requires specific quantification in future.

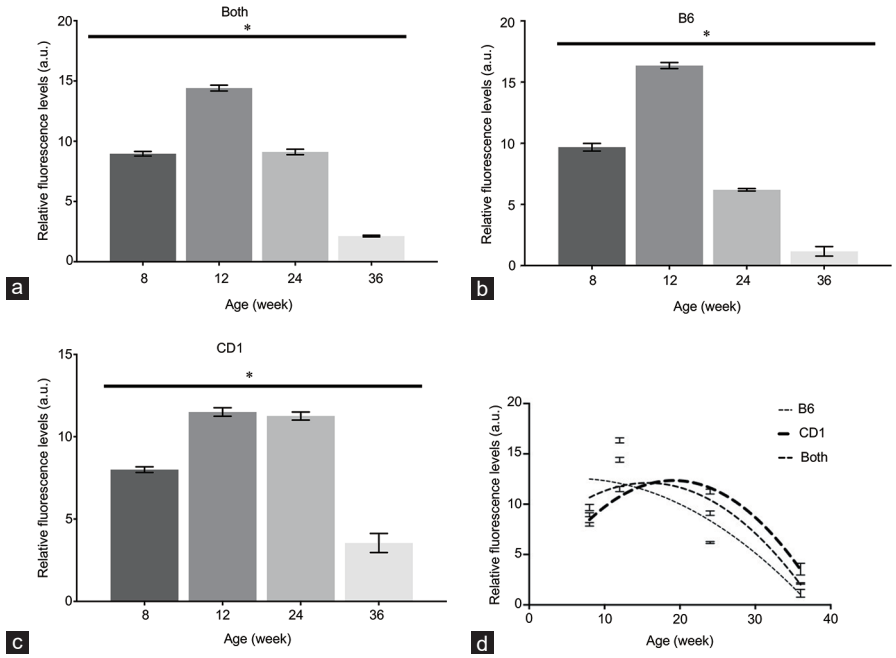

Figure 3: Quantification of sperm head PLCζ fluorescence with relation to advancing age in sperm from (**a**) both strains of mice collectively, (**b**) B6 mice, and (**c**) CD1 mice. (**d**) Polynomial regression models indicating the dynamic relationship between PLCζ fluorescence and advancing male age in both B6 and CD1 strains of mice. r^2 values placed adjacent to each model indicate the strength of the constructed models. Data are represented as mean ± s.e.m. *Statistically significant differences ($P \leq 0.05$). Comparisons are made between groups overlapped by the upper line. a.u.: arbitrary units; B6: C57BL/6 inbred mouse strain; CD1: outbred mouse strain; s.e.m.: standard error of the mean; PLCζ: phospholipase C zeta.

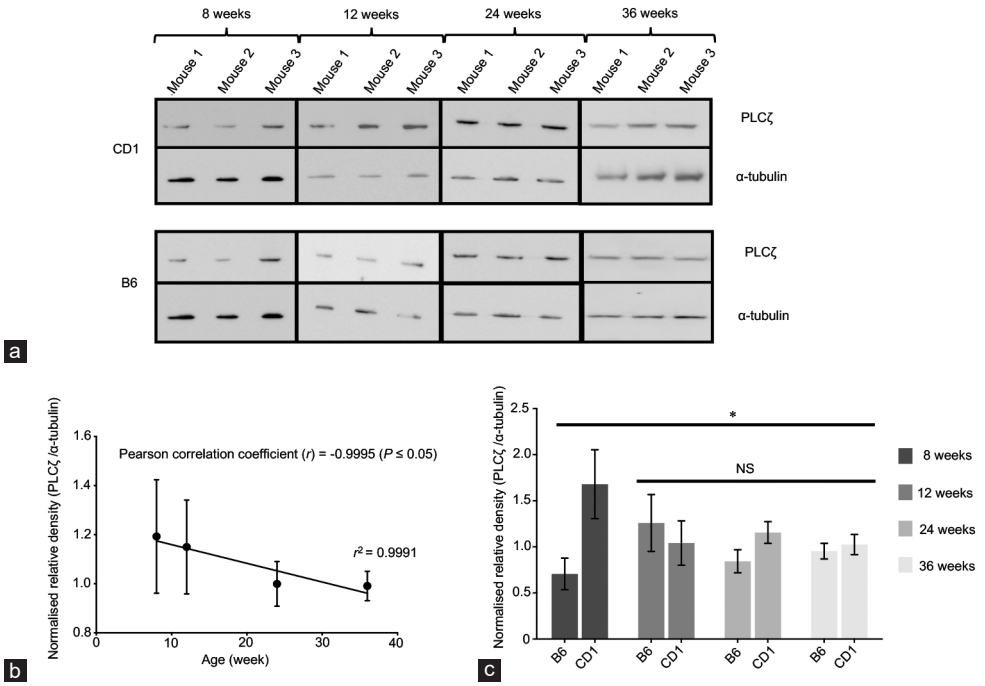

Figure 4: (**a**) Representative immunoblotting analysis of sperm from CD1 (top panel) and B6 (bottom panel) strain of mice, indicating band intensity obtained for PLCζ and α-tubulin. Images are representative of 3 × 3 repeats from each strain of mouse, for each age group. (**b**) Linear regression model constructed using normalized relatively density of PLCζ in relation to age, regardless of strain. A statistically significant negative correlation can be observed (Pearson's correlation coefficient [r] = –0.9995; r^2 = 0.9991) with advancing male age. (**c**) Representative histogram indicating the relative density observed in sperm from B6 and CD1 mice in relation to advancing male age. *Statistically significant differences ($P \leq 0.05$). NS: not significant. Comparisons made are indicated by the respective line above the bars. B6: C57BL/6 inbred mouse strain; CD1: outbred mouse strain; PLCζ: phospholipase C zeta.

Each PLCζ population has been suggested to represent differential functional roles,[29,30] with the postacrosomal population in mice suggested to be the most physiologically relevant. Bi *et al.*[31] suggested that acrosomal PLCζ perhaps could play a role in sperm capacitation

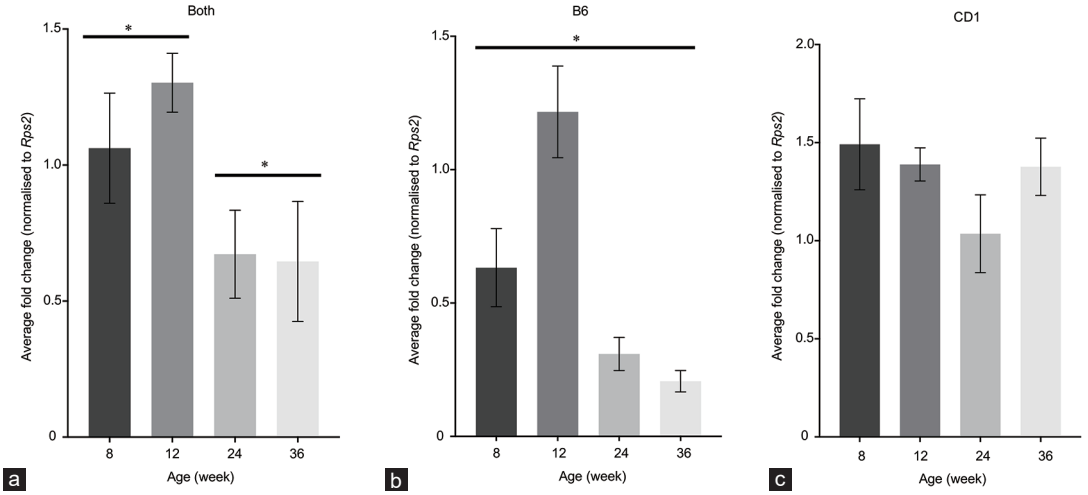

Figure 5: Illustrative histograms representing changes in *PLCζ* RNA (arbitrary units) in 8-, 12-, 24-, and 36-week-old testes from (**a**) both strains collectively, (**b**) B6 mice, and (**c**) CD1 mice. Data are represented as the average fold change in *PLCζ* RNA (normalized with *Rps2*) ± s.e.m. *Statistically significant differences ($P \leq 0.05$). Comparisons made are indicated by the respective line above the bars. B6: C57BL/6 inbred mouse strain; CD1: outbred mouse strain; *PLCζ*: phospholipase C zeta; *Rps2*: ribosomal protein S2; s.e.m: standard error of the mean.

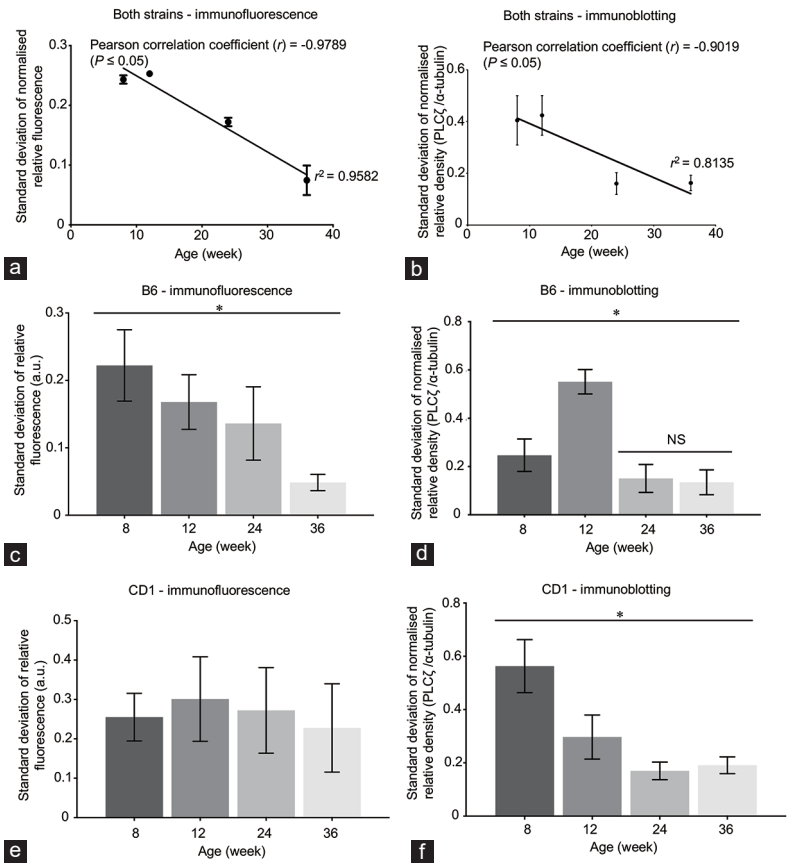

Figure 6: Representative linear regression models indicating the negative correlation between sperm PLCζ variance from mice at 8 weeks, 12 weeks, 24 weeks, and 36 weeks of age, inclusive of both B6 and CD1 strains as indicated by (**a**) immunofluorescence and (**b**) immunoblotting. Representative histograms showing the relationship between sperm PLCζ variance from mice at 8 weeks, 12 weeks, 24 weeks, and 36 weeks of age from the B6 strain of mice individually as indicated by (**c**) immunofluorescence and (**d**) immunoblotting; and from the CD1 strain of mice individually as indicated by (**e**) immunofluorescence and (**f**) immunoblotting. Data are represented as the average standard deviation (s.d.) in relative fluorescence or normalized relative density (both in arbitrary units) ± s.e.m. *Statistically significant differences ($P \leq 0.05$). Comparisons made are indicated by the respective line above the bars. NS: not significant; B6: C57BL/6 inbred mouse strain; CD1: outbred mouse strain; PLCζ: phospholipase C zeta; s.e.m: standard error of the mean.

or the acrosome reaction. It is not clear from our results whether the acrosomal populations we observed were contained within the acrosomal vesicle, or directly beneath this compartment. Our observed dynamic modulation of the acrosomal population between ages seems particularly significant as Young *et al.*[29] demonstrated that the acrosomal population diminished following capacitation of mouse and human sperm, with the postacrosomal population gaining dominance. Considering that capacitation can be viewed as a "biochemical maturation" of sperm, perhaps the acrosomal population represents an "immature" state of PLCζ, while the postacrosomal population represents a more "mature," and physiologically relevant population. Thus, sperm from older males perhaps contains PLCζ with an altered activation capacity compared to sperm from their younger counterparts.

Sperm from patients with globozoospermia (morphologically abnormal round-headed sperm lacking acrosomes) exhibit low success in oocyte activation without clinical intervention.[18] Such sperm are not thought to contain PLCζ, but those that do exhibit an abnormal dispersed pattern and rarely results in successful fertilization without clinical intervention.[32] When sperm from the same population exhibiting a small acrosomal bud was used for fertility treatment, pregnancy without artificial oocyte activation protocols could be achieved.[33] PLCζ in such sperm localized to and around this acrosomal structure.[34] Furthermore, testicular immunohistochemistry indicated that PLCζ first becomes detectable at the spermatid stage,[29,35,36] within the forming acrosomal vesicle of mouse, equine, and human round and elongating spermatids.[37,38] Thus, the biochemical and functional significance of acrosomal PLCζ requires further focused investigations, particularly in light of its structural association with sperm and potential clinical significance.

Advancing male age correlates to decreasing levels of PLCζ in sperm and testes

Although levels of PLCζ did not differ between B6 and CD1 strains when examined independent of age, advancing male age corresponded to a significant collective decrease in PLCζ protein and mRNA levels in sperm and testes, respectively. Considering that reduced/absent levels of PLCζ correspond to abnormal sperm morphology, sperm DNA fragmentation and oxidation, and abnormal pregnancies,[9–13] this potentially indicates that male mice of differential ages may represent useful models with which to study male factor conditions where PLCζ is significantly reduced, as well as to study variability in PLCζ as discussed subsequently.

Generally, mice reach sexual maturity at 6–8 weeks of age for breeding purposes in the laboratory.[39] Generally, however, our data suggested that at least with regards to PLCζ, peak levels were observed at 12 weeks of age. Conversely, if the 8 weeks of age group is to be regarded as minimally fertile (*i.e.*, containing minimal levels of PLCζ required for normal fertility), then levels of PLCζ observed in 36-week-old males were significantly lower, indicating lowest fertility in relation to PLCζ. While we were unable to ascertain corresponding profiles of Ca^{2+} release for sperm from each distinct age group of mice due to lack of the required infrastructure, it is still possible to assert that the level of decrease in PLCζ (particularly between 12- and 36-week-old males) could be enough to assert a significant physiological effect. The effective range of PLCζ levels in fertile human sperm is approximately 4-fold, and 2-fold in mouse sperm, which yields successful activation and healthy development to blastocyst stage. Considering that on average we observed an approximately 2–3-fold difference between peak levels of PLCζ at approximately 12 weeks of age and lowest levels at 36 weeks of age, perhaps such drastic reductions in levels of sperm PLCζ could result in at least altered profiles of Ca^{2+} release.[40]

While we consider that the level of differences we observed in levels and localization patterns between the different ages and strains of mice were significant enough to imply a major functional significance, we also appreciate that this would only be fully possible following an in-depth comparative investigation of the resultant profiles of Ca^{2+} release from sperm of such mice. While it could be argued that such in depth investigations were beyond the scope of the current study, it is imperative that future investigations examine not only the resultant profiles of Ca^{2+} oscillations from sperm of such differentially aged mice from disparate strains, but also perhaps to examine profiles of pre-implantation embryogenesis to obtain a holistic view of the functional significance of the discrepancies in PLCζ that we identify.

Advancing male age differentially decreases levels of PLCζ in sperm and testes amongst different mouse strains

Both strains we examined were differentially affected by increasing age. Such fluctuations may not necessarily be limited to just mice, as a similar dynamic was observed in zebrafish by Johnson *et al.*[29] who reported that sperm concentration increased with male age initially up to approximately 24 months of age, but then declined in groups >25 months of age. A major difference between inbred (B6 in this case) and outbred (CD1 in this case) strains are that of fertility. Generally, inbred mice are usually preferred over outbred mice, as inbred strains are assumed to exhibit a homogenous genetic background. This, however, may not necessarily be true as inbred strains did not exhibit any difference in trait variance compared to outbred strains.[41] Furthermore, inbreeding leads to poor fecundity, with inbred mice generally producing 3–9 pups per litter, while outbred strains such as CD1 produce 12 pups per litter.[41] Such variability is thought to arise due to selection of specific genetic traits in homozygosis, leading to a relaxation of selection pressure for otherwise fitness-related genes.[42]

Such assertions could be given credence from our current results, as we show that generally, the CD1 strain of mice were able to sustain peak levels of *PLCζ* for longer compared to B6 mice, with B6 mice exhibiting a rapid decline in levels of *PLCζ* sooner than CD1 mice at both the sperm protein and testicular mRNA levels. Generally, levels of *PLCζ* mRNA remained constant between 8 and 12 weeks of age, dropping significantly upon progression of age from 12 weeks to 24 week of age. However, B6 mice exhibited the same fluctuation in levels of *PLCζ* mRNA as was observed in sperm, while levels of CD1 *PLCζ* testicular mRNA remained constant throughout. Perhaps the reason underlying declines in levels of *PLCζ* may differ between B6 and CD1 mice, with sperm PLCζ decline in B6 mice attributable to a drop in levels of *PLCζ* mRNA, while sperm PLCζ decline in CD1 mice perhaps due to a decline in protein translation following constant mRNA production.

Considering the consistent demonstrations of the specificity of this antibody in both human and mouse sperm,[12,13,20,21,24,40] we believe that our current results along with numerous others, consistently suggest that the tail fluorescence observed in both mouse and human sperm may represent potential functional populations of PLCζ.[9–11,13,15,16,20,40] Indeed, this appears conserved across mammalian species,[37,43] while microinjection of equine tails into mouse oocytes resulted in high frequency Ca^{2+} oscillations, suggesting presence of functional PLCζ.[37] Perhaps such populations serve as additional 'reservoirs' of PLCζ to ensure adequate profiles of Ca^{2+} release are initiated at oocyte activation, particularly considering that the entirety of the sperm, including the tail, is fully incorporated into the oocyte at fertilization.[44]

Tail populations of PLCζ could also perhaps explain the differences observed in levels of PLCζ as quantified by immunofluorescence compared to immunoblotting. Even though both methods exhibited a consistent overall decrease in levels of PLCζ with increasing age when levels were examined regardless of strain, immunofluorescence indicated an initial increase in both strains from 8 weeks to 12 weeks of age, followed by a decline in levels up to 36 weeks of age. However, this was not reflected in the immunoblotting data, which showed more consistency in levels of PLCζ, particularly after 12 weeks of age.

This is perhaps attributable to the tail population, as our immunofluorescent quantification represents sperm head PLCζ fluorescence as the method of quantification did not allow quantification of tail fluorescence. However, as the entirety of the sperm were loaded onto gels for immunoblotting, an additional tail population of PLCζ would perhaps have compensated for decreasing levels of PLCζ in the sperm head, explaining the discrepancy between the two methods of quantification. Indeed, such an assertion would be in line with suggestions that any tail populations of PLCζ would serve as additional 'reservoirs' to ensure adequate Ca^{2+} release at oocyte activation. However, such assertions are of course purely speculative, and it is imperative the future studies perform a more in-depth examination behind the physical and functional veracity of these observations.

PLCζ variability in mouse sperm is associated with increasing levels and advancing male age

Kashir *et al*.[16] demonstrated significant inherent variability within human sperm, an observation confirmed by subsequent investigations.[11,12,15] However, such examinations have been limited to human sperm. We show herein for the first time that levels of mouse PLCζ also exhibit significant variability, as quantified by both immunofluorescence and immunoblotting. However, as age increased, levels of PLCζ variability decreased. Conversely, variability in levels of PLCζ positively correlated with increasing levels of PLCζ, *i.e.*, as levels of sperm PLCζ increased, so did variability. However, as with levels of PLCζ protein and mRNA, variability in PLCζ levels also affected both B6 and CD1 strains in different ways. Both immunofluorescent and immunoblotting quantification indicated a declining trend of PLCζ variability in B6 sperm. However, variability declined only in the immunofluorescent quantification of CD1 sperm, not significantly changing when utilizing immunoblotting.

CONCLUSION

To conclude, our current results collectively establish for the first time that advancing male age generally decreases levels of PLCζ protein and mRNA in sperm and testes, respectively. However, the pattern of such decline seemed specific to the type of strain examined, with advancing male age affecting each type of strain differentially. Furthermore, we also showed advancing male age significantly altered the observed pattern of PLCζ localization in mouse sperm, with younger mice (8–12 weeks of age) exhibiting an increasing amount of post-acrosomal PLCζ, and older mice (24–36 weeks of age) exhibiting both post-acrosomal and acrosomal populations in equal amounts. Regardless of the specific mechanisms underlying the decrease in PLCζ observed in both strains, it is clear that levels of PLCζ in mouse sperm are affected by the genetic makeup of the particular strain observed. Considering that the human population is considerably more complex than the laboratory animals used in most studies including this one, perhaps any potential affect that age may exert upon PLCζ in human sperm may have been masked. It is essential that similar in-depth analyses are performed with a larger patient population, taking into account potential factors that could skew results including genetic background.

AUTHOR CONTRIBUTIONS

JK, BVM, FAL, AMA, CJ, and KC were responsible for study and experiment design, and overall scope of the study. JK. BVM, MN, MAG, MR, CJ, and R AbuDawas performed the majority of experimental procedures, with further contributions by R Abu-Dawud and NAY. JK wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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Supplementary Table 1: Analysis of correlations between the variance observed in sperm phospholipase C zeta as indicated by the standard deviation of relative fluorescence and normalized relative density with total levels of phospholipase C zeta (values in arbitrary units)

	Both	*B6*	*CD1*
Relative fluorescence	r=0.9743 (P<0.05)	r=0.9034 (P<0.05)	r=0.9286 (P<0.05)
Normalised relative density	r=0.9031 (P<0.05)	r=0.8843 (P<0.05)	r=0.9034 (P<0.05)

Statistically significant (P≤0.05) differences are indicated, along with the corresponding Pearson's correlation coefficient (r; positive values indicate a positive correlation)