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SHORT COMMUNICATION

# Effects of cryopreservation and density-gradient washing on phospholipase C zeta concentrations in human spermatozoa

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Junaid Kashir obtained a BSc in Molecular Genetics and Biotechnology from the University of Sussex in 2007 and is currently a PhD student in the Nuffield Department of Obstetrics and Gynaecology, University of Oxford. Junaid's research adopts a multifaceted approach to identify and characterize potential links between the oocyte activation factor PLCζ and certain forms of human male factor infertility. Alongside his research, Junaid has developed a strong interest in teaching and contributes to the University of Oxford's MSc in Clinical Embryology.

**Abstract** Cryopreservation and density-gradient washing (DGW) are routinely used in infertility treatment. This study used quantitative immunofluorescence analysis to report how these techniques affect concentrations of the oocyte activation factor, phospholipase C zeta (PLCζ) in spermatozoa from fertile men. DGW significantly elevated the proportion of spermatozoa in which PLCζ could be detected (by 25–81%; $P < 0.0001$). In contrast, in four donors, cryopreservation significantly reduced PLCζ concentrations (by 20–56%; $P < 0.0001$). These findings indicate that while DGW positively selects spermatozoa with detectable PLCζ, cryopreservation has significant detrimental effects upon PLCζ concentrations. Since reduced PLCζ concentrations have been implicated in deficient oocyte activation and infertility, further study is highly warranted.



**KEYWORDS:** assisted reproductive technology (ART), cryopreservation, density-gradient washing, oocyte activation, phospholipase C zeta (PLCζ), spermatozoa

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## Introduction

Infertility affects one in seven couples globally and assisted reproductive technology now accounts for ~7% of births in some countries (Kashir et al., 2010). While advances in assisted reproductive technology have revolutionized infertility treatment, success rates vary, with pregnancy and delivery rates for IVF and intracytoplasmic sperm injection (ICSI) rarely exceeding 30% and 23%, respectively (de Mouzon et al., 2010; ICMART, 2009). Consequently, it is crucial that assisted reproduction protocols are constantly modified in line with scientific advances, so that clinics can consistently provide patients with the best chances of conception.

Sperm cryopreservation is a fundamental technique, not only for infertility treatment, but also for preserving fertility in individuals undergoing radio/chemotherapy or surgery (Zribi et al., 2010). However, cryopreservation can detrimentally affect sperm viability, motility and morphology (Ozkavukcu et al., 2008) and induce DNA fragmentation and oxidative damage (Zribi et al., 2010). Borges et al. (2007) showed that fertilization rates following ICSI were higher using fresh spermatozoa (73.8%) compared with cryopreserved spermatozoa (68.7%), and that spermatozoa from patients diagnosed with asthenozoospermia or oligoasthenozoospermia, may be more susceptible to freeze–thaw damage, resulting in lower fertilization rates. Another important technique used in fertility treatment is density-gradient washing (DGW). Following the development of classical swim-up methods, improved techniques such as DGW have been developed to improve the quality of spermatozoa recovered for assisted reproductive technology. Indeed, sperm quality has been shown to be better following DGW compared with swim-up preparation (Allamaneni et al., 2005).

Whilst cryopreservation can reduce concentrations of some sperm proteins such as glutathione (Gadea et al., 2011) in human spermatozoa, little is known of the potential effects upon the putative oocyte activation protein, phospholipase C zeta (PLCζ; Saunders et al., 2002). Released into the oocyte upon gamete fusion, PLCζ induces oscillations in intracellular oocyte calcium ($Ca^{2+}$), allowing meiotic resumption and exit, and allowing embryogenesis to proceed, a process termed oocyte activation (Dale et al., 2010; Kashir et al., 2010). The mechanisms underlying $Ca^{2+}$ release in activating oocytes have been the source of much debate, with opinion divided as to whether $Ca^{2+}$ is released via interaction between an oocyte receptor and a sperm ligand, or by a cytosolic 'sperm factor' released into the oocyte by the spermatozoa upon gamete fusion (for reviews, see Dale et al., 2010; Kashir et al., 2010; Miyazaki and Ito, 2006; Parrington et al., 2007). Compelling evidence now supports the sperm-factor theory of oocyte activation, particularly the fact that microinjection of spermatozoa and sperm-protein extracts initiate intracellular $Ca^{2+}$ oscillations characteristic of fertilization in the absence of any oocyte receptor/sperm ligand interaction (for reviews, see Dale et al., 2010; Kashir et al., 2010).

While general consensus agrees that PLCζ is the oocyte activation factor (Kashir et al., 2010), conclusive evidence in the form of a PLCζ - knockout mouse remains to be demonstrated. Other factors apart from PLCζ, which are able to induce meiotic progression or typical patterns of $Ca^{2+}$ release, have also been proposed in other species. Harada et al. (2007) identified a new 45 kDa protein, termed citrate synthase, as the major component responsible for egg activation in the newt *Cynops pyrrhogaster*, while Wu et al. (2007) reported a factor residing in the post-acrosomal sheath region of the perinuclear theca, termed post-acrosomal sheath domain-binding protein (PAWP), in bovine spermatozoa and other mammalian species. However the precise molecular mechanisms underlying citrate synthase and PAWP function are currently unknown. It is possible that multiple factors such as PAWP and PLCζ act collectively in the mammalian oocyte activation mechanism, or that factors such as PAWP act up-stream or down-stream of $Ca^{2+}$ signalling (Aarabi et al., 2010; Wu et al., 2007).

However, numerous studies provide evidence for PLCζ as the oocyte activation factor. Injection of recombinant PLCζ RNA and protein into mouse oocytes resulted in the initiation of $Ca^{2+}$ oscillations similar to those seen at fertilization and embryonic development to the blastocyst stage (Cox et al., 2002; Kouchi et al., 2005; Saunders et al., 2002). Immunodepletion of PLCζ from sperm extracts suppressed $Ca^{2+}$-releasing ability (Saunders et al., 2002), while RNA interference (RNAi) experiments produced transgenic mice with significantly reduced expression of PLCζ in the testis (Knott et al., 2005). Fertilization by spermatozoa from these animals was characterized by a premature cessation of $Ca^{2+}$ oscillations within the oocyte.

Recently, immunofluorescence and immunoblot analysis revealed that ICSI-failed spermatozoa from infertile patients were unable to elicit $Ca^{2+}$ oscillations upon mouse oocyte microinjection and exhibited abnormalities in PLCζ expression (Heytens et al., 2009; Yoon et al., 2008). Moreover, the activating ability of ICSI failed human spermatozoa could be rescued upon co-injection with mouse PLCζ mRNA (Yoon et al., 2008). Interestingly, following the use of ICSI along with a $Ca^{2+}$ ionophore (an artificial oocyte activator; AOA), high rates of fertilization and pregnancy were achieved using globozoospermic spermatozoa that were devoid of PLCζ expression (Taylor et al., 2009).

While Heytens et al. (2009) observed reduced PLCζ in human spermatozoa by immunoblot analysis following cryopreservation, the significance of this preliminary finding was not explored any further. The present study aimed to use immunofluorescence analysis to investigate how cryopreservation and DGW affect PLCζ concentrations in normal human spermatozoa.

## Materials and methods

Semen samples were obtained from healthy volunteers with informed written consent and ethical approval. All ejaculates ($n = 7$) exhibited normal semen parameters (WHO, 2010) and were divided into three fractions: unprocessed (raw); DGW; and a post-thaw DGW fraction in which raw spermatozoa were frozen, thawed and subjected to DGW (PT-DGW). For DGW, in brief, 40% PureSperm gradient media was overlayed on top of 80% media (PureSperm 40/80, Nidacon International, Sweden). Media was allowed to equilibrate to room temperature (RT) for 1 h and liquefied fresh semen was layered on top of the gradient and centrifuged at 300*g* for 20 min at RT. The supernatant was then

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discarded, leaving 0.5 ml above the pellet containing the most motile spermatozoa. This was then aspirated into tubes containing PureSperm Wash (Nidacon International, Sweden) and centrifuged at 500*g* for 5 min at RT. Pellets were then resuspended in PureSperm Wash media and centrifuged at 500*g* for 5 min at RT. Pellets were finally resuspended in a suitable quantity of PureSperm Wash according to pellet size.

Freeze–thaw fractions were treated with standard clinical protocols. 'SpermFreeze' (700 μl; FertilPro, Belgium) was added drop-wise to 1 ml of raw spermatozoa whilst swirling. The cryoprotectant was allowed to equilibrate to RT for a minimum of 1 h before use. Once the cryoprotectant was fully incorporated, the mixture was left at RT for 10 min and aspirated into labelled freezing straws (Cryo Bio System, France). Straws were subsequently sealed and suspended vertically in a holding tank containing liquid nitrogen in the vapour phase for 30 min. Straws were then plunged directly into the liquid. Samples were thawed by incubating straws at RT for 15 min and subjected to DGW as before, but with only 40% PureSperm, to yield the PT-DGW fraction.

All fractions were fixed with an equal volume of 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at RT. PLCζ was detected by immunofluorescent staining with an anti-PLCζ antibody as described by Grasa et al. (2008) and quantified using ImageJ (National Institute of Health, USA). Correlations between the proportion of raw and DGW spermatozoa in which PLCζ could be detected were determined for donors 2–7 ($n = 6$) but not donor 1 owing to insufficient volume. Proportions were arcsine - transformed prior to statistical analysis. Correlations between the relative fluorescent intensity of PLCζ for DGW and PT-DGW fractions for all donors ($n = 7$) were determined using the Student's *t*-test (Graph-Pad InStat, San Diego, USA). Data are presented as mean ± standard error of the mean (SEM) and a *P*-value of 0.05 was considered statistically significant.

## Results and discussion

Rapid growth in assisted reproductive technology has developed a range of sperm preparation methods including

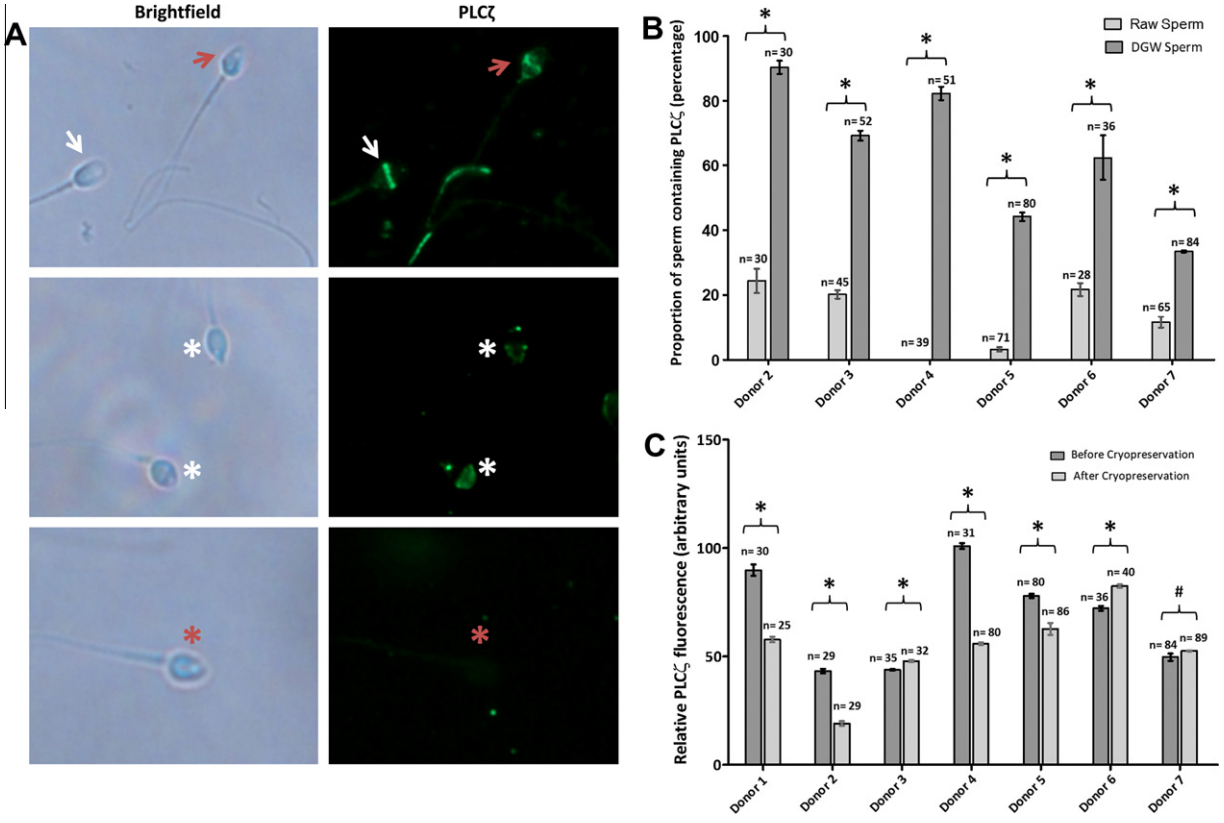


**Figure 1** (**A**) Representative bright-field (left panel) and corresponding PLCζ immunofluorescence (right panel) of human spermatozoa showing characteristic localization patterns previously identified by Grasa et al. (2008). White arrow = equatorial localization; red arrow = equatorial + post-acrosomal localization; white asterisk = equatorial + acrosomal localization; red asterisk = representative image of raw spermatozoon not exhibiting PLCζ immunofluorescence. Images were captured at 40× magnification and enlarged digitally. (**B**) Relative proportions of spermatozoa exhibiting PLCζ fluorescence in raw and DGW fractions for donors 2–6. Asterisk = statistically significant values ($P < 0.0001$, Student's *t*-test); $n$ = number of spermatozoa analyzed for each donor for both fractions. Data were arcsine-transformed prior to analysis and represent mean ± SEM. (**C**) Relative PLCζ fluorescence in spermatozoa from donors 1–7 before and after cryopreservation. Fluorescent intensity was quantified in arbitrary units using ImageJ. Asterisk = statistically significant differences ($P < 0.0001$, Student's *t*-test); # = not statistically different; $n$ = number of spermatozoa analyzed for each donor for both fractions. Data are mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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density-gradient washing and cryopreservation. The effects of these treatments upon vital sperm parameters such as motility, viability, structural integrity and DNA damage are subject to increasing scrutiny (Ozkavukcu et al., 2008; Zribi et al., 2010). This study examined how such treatments affect PLCζ concentrations in human spermatozoa. DGW is used routinely to select the most motile and morphologically normal spermatozoa from an ejaculate for IVF or ICSI (Allamaneni et al., 2005). The current analyses demonstrate that DGW resulted in a significant elevation (six donors; $P < 0.0001$) in the proportion of spermatozoa in which immunoreactive PLCζ patterns (**Figure 1A**) could be detected (representing elevations of 26–81%) compared with raw fractions (**Figure 1B**), indicating that raw samples contain a higher proportion of spermatozoa devoid of immunoreactive PLCζ. This may be due to the removal of senescent and dead spermatozoa from this fraction, along with particulate debris. Since DGW selects the best-quality spermatozoa for assisted reproductive technology, this proportional increase in PLCζ, which appears to vary between individuals, indirectly confirms the importance of PLCζ at fertilization and suggests that PLCζ concentrations in spermatozoa may represent a useful biomarker of fertility.

As cryopreservation has been reported to reduce post-thaw sperm quality (Ozkavukcu et al., 2008; Zribi et al., 2010), this study investigated whether one aspect of this reduction in quality may be due to reduced concentrations of PLCζ within the sperm head. There was a significant reduction ($P < 0.0001$) in PLCζ immunofluorescence in spermatozoa following cryopreservation compared with fresh DGW fractions in four out of seven fertile donors, in which PLCζ immunofluorescence was reduced by 20–56% (**Figure 1C**). Conversely, and for reasons that remain unclear, two donors exhibited increased concentrations of immunofluorescence following cryopreservation ($P < 0.0001$) while one donor exhibited no change. The mechanisms underlying the detrimental effects of cryopreservation upon spermatozoa may be multifactorial, involving excessive reactive oxygen species production during freeze–thaw and subsequent changes in membrane function and structure (Gadea et al., 2011), possibly resulting in disruption of PLCζ localization or leakage from the sperm head.

In conclusion, this study firstly shows that DGW yields a processed sample with a significantly greater number of spermatozoa exhibiting detectable PLCζ protein than in the more heterogeneous raw sample. Second, it demonstrates that cryopreservation appears to significantly affect concentrations of PLCζ in spermatozoa. Whilst this effect may vary between individuals following DGW, the majority of donors (57%) exhibited significantly reduced concentrations of PLCζ following cryopreservation. Given that reduced concentrations of PLCζ have been linked to male infertility (reviewed by Kashir et al., 2010), the data indicate that a more substantial study, involving a larger population of donors, is highly warranted. This study also demonstrates that PLCζ represents a powerful tool to investigate sperm quality in assisted reproductive technology, which may contribute towards improving current clinical protocols and thus chances of conception.

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