Reproductive BioMedicine Online (2011) 23, 263–267

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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

1472-6483/$ - see front matter
doi:10.1016/j.rbmo.2011.04.006

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