First recorded pregnancy and normal birth after ICSI using electrophoretically isolated spermatozoa

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BACKGROUND: DNA damage in the male germ line is associated with poor fertilization and cleavage rates, impaired embryo quality and early pregnancy loss. Given these associations, embryologists are keen to develop techniques that will allow the selection of viable spermatozoa exhibiting low levels of DNA damage for assisted conception purposes. METHODS: In this article, we describe a novel electrophoretic approach for the rapid isolation of cells possessing little DNA damage. The limits of the method were examined using cryostored and snap-frozen semen samples as well as testicular biopsy material. In addition, clinical utility was demonstrated in a case study involving treatment of a patient exhibiting persistently high levels of DNA damage in his spermatozoa. RESULTS: From a range of difficult starting materials (biopsies, cryostored semen and snap-frozen sperm suspensions), the electrophoretic system rapidly isolated populations of motile, viable, morphologically normal spermatozoa exhibiting high levels of DNA integrity. Clinical application in a couple suffering from long-term infertility associated with extensive DNA damage in the male germ line led to the first human pregnancy following such electrophoretic sperm isolation. CONCLUSIONS: The electrophoretic procedure holds promise as a convenient method for the rapid preparation of high-quality spermatozoa for assisted conception purposes.

Key words: assisted conception/DNA damage/electrophoretic method/human spermatozoa/sperm isolation

Introduction

Assisted conception techniques are now responsible for up to 7% of births in some developed countries (Skakkebaek et al., 2006). Although this technology has revolutionized the treatment of infertile couples, apprehension about the incidence of birth defects in such children has not been fully allayed, especially when clinical circumstances are extreme (Jansen, 2005). The possibility of introducing DNA damage into the embryo through the use of defective spermatozoa in assisted conception protocols has been a particular source of concern (Aitken, 1999). DNA damage in human spermatozoa is negatively correlated with pregnancy rates in both natural and assisted conception cycles and has been linked with both increased rates of miscarriage and diseases in the offspring, including childhood cancer (Aitken, 1999; Loft et al., 2003; Lewis and Aitken, 2005).

In the light of these considerations, we have developed a rapid, effective technique for the isolation of human spermatozoa exhibiting minimal DNA damage (Ainsworth et al., 2005). This system is based on two principles: (i) the highest quality spermatozoa in the ejaculate are the most electronegative (Kirchhoff and Schroter, 2001; Giuliani et al., 2004; Ainsworth et al., 2005) and (ii) spermatozoa can be separated from other contaminating electronegative cells (such as leucocytes and precursor germ cells) by virtue of their small cross-sectional size (Ainsworth et al., 2005). In this study, we have investigated the limits of this sperm isolation system by assessing its ability to isolate spermatozoa from a range of difficult starting materials including cryostored semen, snap-frozen sperm suspensions and testicular biopsies. We also confirm the clinical utility of this system by reporting the world’s first pregnancy and term delivery using electrophoretically isolated spermatozoa in a couple suffering from long-term infertility associated with persistently high levels of DNA damage in the male germ line.

Materials and methods

Semen samples

Semen samples were allowed to liquefy at room temperature and were then subjected to a routine semen analysis using guidelines set out by the World Health Organization (1999). At least 100 cells were analysed for each parameter assessed (vitality, morphology and motility). All procedures were approved by the University of Newcastle and Sydney IVF Human Research Ethics Committees.
Electrophoretic sperm isolation

The prototype electrophoretic system has previously been described (Ainsworth et al., 2005) and consisted of a configurable cartridge comprising two 400-µl chambers separated by a 5-µm polycarbonate filter and bounded by 15-kDa polyacrylamide membranes that allowed the free transit of electrolytes. The electrophoresis buffer contained 10 mM HEPES, 30 mM NaCl and 0.2 M sucrose, pH 7.4, with an osmolarity of 310 mOsmol/kg. The samples were run at 23°C for 5 min with a constant applied current of 75 mA and a variable voltage of between 18 and 21 V. The motility and morphology of the isolated and residual sperm populations were assessed using the World Health Organization criteria (1999), whereas DNA damage was assessed using the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) and sperm chromatin stability assays (SCSA), as previously described (Virro et al., 2004; Ainsworth et al., 2005).

To determine whether this electrophoretic system could be used to prepare spermatozoa from the seriously compromised samples that might be encountered in assisted conception clinics, we assessed its performance using cryostored human semen samples, testicular biopsies and specimens snap frozen in liquid nitrogen from infertile patients attending Sydney IVF. Cryostorage was performed using the traditional buffered oocyte yolk–glycerol system at a 1:1 dilution (Richardson, 1976; Weidel and Prins, 1987). A controlled cooling cycle was employed beginning at +20°C and then decreasing at –6°C per minute until a temperature of –80°C was reached. The straws were then plunged directly into liquid nitrogen.

Clinical case

We examined the clinical utility of the device in the treatment of a couple suffering from long-standing infertility as a consequence of severe oligozoospermia, eventually shown to be associated with high levels of DNA damage in the spermatozoa. The couple (both 31 years) had first presented with 2.5 years’ primary infertility and had conceived in the third oocyte-retrieval cycle (the eighth embryo-transfer cycle), and subsequently, a normal female was delivered at term. Before this, one cryostored embryo had resulted in a transiently positive pregnancy test, and in subsequent stimulated cycles, cleavage rates had become poor. A further seven oocyte-retrieval cycles with different stimulation strategies (average number of oocytes 7.5, range 6–13) was persistently attended by good fertilization rates but poor embryo cleavage. Standard IVF with ICSI procedures were performed as described elsewhere (Jansen, 2003).

Statistics

Statistical comparisons between groups were conducted with the paired t-test and differences with a probability of \( P < 0.05 \) considered significant.

Results

Cryostored human spermatozoa are commonly used for assisted conception, particularly in cases where the male partner has elected to preserve his gametes before treatments that affect fertility, such as vasectomy or chemotherapy. Within 5 min, the electrophoretic method generated cell populations containing 10.8 ± 3.8 (SEM) \( \times 10^6 \) spermatozoa/ml from cryostored semen samples originally containing 39.6 ± 11.1 \( \times 10^6 \) ml. The sperm populations isolated with this procedure contained no detectable contaminating cells and were significantly more viable (\( P < 0.01 \)) and motile (\( P < 0.05 \)) than the residual seminal population (Figure 1A).

Even when presented with the highly immature spermatozoa found in testicular biopsies, the electrophoretic system successfully isolated a subpopulation of cells that exhibited more residual motility (twitching) and significantly more vitality than the unselected population (Figure 1A). This isolation was rapid (5 min), efficient (28.4 ± 7.1% of spermatozoa recovered) and selective, with no contaminating cells being detected despite the complex cellular composition of the original biopsy material. The spermatozoa selected from testicular biopsies and cryostored semen samples were also significantly enriched for normal sperm morphology (Figure 1B). The electrophoretic separation method was also effective for snap-frozen spermatozoa which, although
lacking both vitality and motility, can be suitable for ICSI (Lacham-Kaplan et al., 2003). In this case, 5-min electrophoresis generated sperm populations containing $4.1 \pm 1.7 \times 10^6$ purified spermatozoa per millilitre that were significantly enriched for normal morphology (Figure 1B; $P < 0.001$) from 200-μl aliquots of sperm suspensions containing $56.2 \pm 20 \times 10^6$ spermatozoa/ml. The enhanced vitality and morphological normality of the electrophoretically separated spermatozoa were also reflected in significantly reduced levels of DNA damage (Figure 1C).

In the light of these encouraging preliminary data, we sought to assess the value of this novel sperm separation system clinically. The couple, as described above, exhibited long-term infertility associated with high levels of DNA damage in the male germ line, as assessed by a SCSA level of 30% before recommencing IVF with ICSI. Despite changes in medication for psoriasis, the use of antioxidants and repeated attempts to improve semen parameters by frequent ejaculation (Greening, 2004), the DNA fragmentation index (DFI) for this patient’s spermatozoa remained at levels between 30 and 50% during the further six stimulation cycles for conventional IVF with ICSI. After consideration of the empirical data reported in this article and after the demonstration of improved SCSA scores in vitro, ethical approval for individual clinical application and informed consent were obtained in an attempt to improve semen quality by electrophoresis before further IVF treatment involving ICSI.

Electrophoretic sperm isolation was used on two separate occasions. On the first occasion, fertilization was successfully achieved by ICSI, but no pregnancy resulted. During the second successful cycle, the patient produced an oligozoospermic ejaculate on the day of oocyte retrieval containing $3.2 \times 10^6$ spermatozoa/ml and an equivalent number ($2.1 \times 10^6/μl$) of contaminating round cells. Of the spermatozoa that were present, 30% were vital, 18% were motile and 26% were TUNEL positive. Following 5 min of electrophoretic separation, a purified sperm population was generated that was 62% vital, 24% motile and 14% TUNEL positive. Assessment of these samples by SCSA revealed a DFI that fell from 41% in the ejaculated sample to 15% in the electrophoretically separated population. Because DFI values of $>30\%$ have been associated with poor blastulation and pregnancy rates (Virro et al., 2004), this reduction in DNA damage was considered to be clinically important. ICSI was then conducted using the electrophoretically isolated spermatozoa. Five of seven oocytes were fertilized, and two of the embryos were transferred on day 3. Embryo transfer was followed by pregnancy and, later, the birth of a second healthy girl with no complications.

Discussion

This brief report extends our original study conducted using semen samples obtained from unselected donors (Ainsworth et al., 2004) to include the kind of seriously compromised material that might be encountered in an assisted conception setting. The results of this analysis indicate that the electrophoretic approach is suitable for the selective isolation of viable, motile, morphologically normal spermatozoa from cryostored semen and confirm our previous analysis in revealing that the isolated cells exhibit low levels of DNA damage. In large-scale studies with freshly ejaculated spermatozoa, we have found that the number of spermatozoa recovered using the electrophoretic sperm isolation method is a linear function of sperm concentration in the original ejaculate ($R^2 = 0.84$) over a range of starting concentrations from 17 to $161 \times 10^6/ml$ (data not shown). We have not yet conducted a detailed analysis of the performance of the system with severely oligozoospermic samples and so cannot comment on the minimal concentration of spermatozoa required for the method to be successful. However, the method appears to be generally capable of recovering $\sim 20\%$ of a given sperm population, even when faced with the complex cellular mixtures represented by testicular biopsies. With such samples, the electrophoretic method proved an effective means of isolating high-quality spermatozoa possessing little DNA damage that, by all laboratory criteria, would be suitable for ICSI. Most surprising of all, this method permitted the isolation of morphologically normal spermatozoa possessing low levels of DNA damage after these cells had been snap frozen for long-term storage. Thus, even though the plasma membranes of these cells have been terminally damaged by the freeze–thaw procedure, the charge differences that differentiate normal from abnormal spermatozoa were sufficiently retained to allow successful electrophoretic separation. We hypothesize that these charge differences are associated with the relative presence of sialated proteins on the sperm surface, such as CD52 (Schorer et al., 1999; Giuliani et al., 2004; Ainsworth et al., 2005). The latter is a highly charged glycosylphosphatidylinositol (GPI)-anchored protein, the expression of which appears to be significantly correlated with normal sperm morphology (Giuliani et al., 2004). The fact that neuraminidase treatment prevents the electrophoretic isolation of spermatozoa (C. Ainsworth, unpublished results) is in keeping with the proposed importance of sialic acid expression in the separation procedure.

The confirmation that the electrophoretic technique described in this article is of clinical value has been presented in the form of a case report involving a couple experiencing infertility associated with persistently high levels of DNA damage in the spermatozoa, as measured by both the TUNEL and the SCSA assays. Relationships between DNA damage in human spermatozoa and male infertility have been previously described in the literature (Lewis and Aitken, 2005). In this study, we demonstrate that this clinical condition can be addressed by selecting undamaged spermatozoa from the ejaculate using cell size and electronegative charge as the selection criteria. Using these principles, we have generated the world’s first term pregnancy using electrophoretically isolated spermatozoa, in a couple for whom all other management strategies had failed. Clearly, more extensive clinical trials will have to be conducted to examine the detailed therapeutic value of this sperm isolation procedure for a range of male reproductive pathologies.

Although spermatozoa exhibiting large amounts of DNA damage can still be capable of fertilizing the oocyte (Twigg et al., 1998) and could generate embryos with the potential to develop to term (Gandini et al., 2004), this does not mean that the use of DNA-damaged spermatozoa in assisted conception is acceptable practice. In general, damaged DNA carried into the zygote by the fertilizing spermatozoon will be repaired by
the oocyte (Aitken et al., 2004). However, pathology can result when this DNA damage is converted into a genetic or epigenetic mutation as a consequence of aberrant DNA repair before the S phase of the first mitotic division. (Aitken and Krausz, 2001; Shimura et al., 2002; Aitken et al., 2004). The highly significant impact of paternal age on the incidence of dominant genetic diseases such as achondroplasia (Crow, 2000) could be mediated by such an aberrant repair mechanism, given the high levels of DNA damage seen in the spermatozoa of ageing males (Singh et al., 2003; Aitken et al., 2004). This would explain why the incidence of the achondroplasia mutation in human spermatozoa does not rise at the same rate as the incidence of the condition in their offspring (Hurst and Ellegren, 2002). According to our hypothesis, the mutation is commonly being generated in the oocyte rather than the spermatozoa, as a consequence of the defective repair of sperm DNA following fertilization (Aitken et al., 2004). It is important to note that dominant genetic mutations such as achondroplasia, although paternally mediated, are extremely rare (2–4 in every 100 000 births). Thus, even if the DNA-damaged spermatozoa are being used in assisted techniques such as ICSI, several hundred thousand births would have to occur before we would see a statistically significant change in overt childhood disease. However, this does not mean that such practices are not increasing the mutational load carried by the offspring; rather, it is just that the mutations do not have the dominance needed to generate a phenotypic change.

In the light of such considerations, assisted conception protocols should, whenever possible, avoid the use of DNA-damaged spermatozoa. In this context, the electrophoretic sperm isolation procedure could make a significant contribution to good clinical practice in this area.

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Conflicts of interest
R.J.A. is a consultant for Life Therapeutics, the manufacturer of the electrophoretic sperm isolation device used in this report.

References