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Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation

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Cryopreservation of human spermatozoa is extensively used in artificial insemination and IVF programmes. Despite various advances in cryopreservation methodology, the recovery rate of functional post-thaw spermatozoa remains mediocre, with sperm motility being significantly decreased after freezing. This aim of this study was to investigate the effects of cryopreservation on both DNA integrity and morphology of spermatozoa from fertile and infertile men. Semen samples were obtained from 17 fertile and 40 infertile men. All samples were prepared by discontinuous Percoll density centrifugation (95.0:47.5). Samples were divided into aliquots to allow direct comparison of fresh and frozen spermatozoa from the same ejaculate. Aliquots for cryopreservation were mixed with a commercial cryoprotectant and frozen by static phase vapour cooling before plunging into liquid nitrogen. Thawing was carried out slowly at room temperature. Sperm DNA integrity was determined using a modified alkaline single cell gel electrophoresis (comet) assay and sperm morphology analysed using the Tygerberg criteria. DNA of semen and prepared spermatozoa from fertile men was found to be unaffected by cryopreservation. In marked contrast, spermatozoa from infertile men were significantly damaged by freeze–thawing. Cryopreservation had a detrimental effect on morphology of semen and prepared samples from fertile and infertile men.

Key words: comet assay/cryopreservation/human spermatozoa/male infertility/morphology

Introduction

Cryopreservation of human spermatozoa has been possible for many years (Sherman, 1954). It is widely used in assisted conception units to preserve male gametes and provide the opportunity for future fertility, for example in the treatment of malignancy (Sanger *et al.*, 1992). Cytotoxic chemotherapy, radiotherapy and some kinds of surgical treatments may lead to testicular failure or ejaculatory dysfunction. Freezing of spermatozoa before initiation of treatment provides patients with fertility insurance. However, due to damage associated with freezing, it may be necessary to utilize IVF or intracytoplasmic sperm injection (ICSI). Individuals with extremely poor sperm samples may also be helped with innovative techniques which allow freezing and post-thaw recovery of single or a few spermatozoa by cryopreservation in evacuated, empty human or animal zonae pellucidae (Cohen *et al.*, 1997).

In donor insemination programmes, the use of frozen semen allows detailed screening of donors for infections such as human immunodeficiency virus (HIV) and hepatitis B prior to release of spermatozoa for insemination (Sherman, 1987). It can take 3–6 months for a man to become seropositive and all semen samples are routinely stored for at least 6 months before use.

The most commonly reported detrimental effect of cryopreservation on human spermatozoa is a marked reduction in motility (Critser *et al.*, 1988; Englert *et al.*, 1989; Yoshida *et al.*, 1990). This is despite many advances in cryopreservation methodology (Centola *et al.*, 1992; Agarwal *et al.*, 1995). The primary cause of cellular damage during cryopreservation is the formation of intracellular ice (Muldrew and McGann, 1990; Watson, 1995). Whenever cells, or culture media, are cooled below their freezing point, water is removed from the solution in the form of ice. The concentration of solutes remaining in the unfrozen fraction increases, thereby both depressing the freezing point (Brotherton, 1990) and increasing the osmotic pressure of the remaining solution. Hence, biological systems freeze progressively over a wide temperature range, during which the solute becomes gradually more concentrated as the temperature falls (Brotherton, 1990). This leads to irreversible rupturing of plasma and nuclear membranes and disturbance of cellular organelles. The nucleus has generally been considered to be a stable constituent of the cell. However, recent studies have suggested that this is not the case and that inappropriate chromatin condensation can occur (Royere *et al.*, 1988, 1991) with freezing.

Cryoprotectants such as glycerol or propanediol can be

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added to cells to reduce freezing damage by lowering the salt concentrations and increasing the unfrozen water fraction, thereby reducing osmotic stress. They can also insert into phospholipid membranes to reduce the likelihood of fracture (Watson, 1995).

Further cellular damage may be caused during the thawing process as the ice melts or re-crystallizes. Slow thawing is most likely to induce injury, as it allows time for consolidation of microscopic ice crystals into larger forms which are known to be damaging (Mazur *et al.*, 1981). The production and dissolution of ice is associated with the actual rate of freezing and thawing. Slow freezing and gradual dehydration may accommodate cell survival whereas rapid freezing and thawing is more likely to result in cell death (Muldrew and McGann, 1990).

The aim of the current study was to determine the DNA integrity and morphology of spermatozoa from fertile and infertile men before and after cryopreservation to determine if damage is induced in sperm DNA by freeze–thawing and to establish if any differences exist between the two groups.

Materials and methods

Collection of semen samples

Semen samples were obtained from a total of 57 subjects following a recommended minimum of 3 days and a maximum of 7 days sexual abstinence. Men of proven fertility ($n = 17$) were recruited from patients attending the Day Procedure Unit at the Royal Victoria Hospital, Belfast for vasectomy. Infertile men ($n = 40$) were recruited from patients attending the Regional Fertility Centre, Belfast for either IVF ($n = 33$) or ICSI ($n = 7$). Infertile subjects were the partners of women who had failed to conceive after 2 years of unprotected intercourse. Informed consent for participation was obtained and the project was approved by the Queen's University of Belfast Research and Ethics Committee.

Semen analysis

Within 1 h of production, a routine semen analysis was performed using light microscopy to determine concentration and motility according to World Health Organization guidelines (WHO, 1999). Sperm morphology was determined using the strict criteria laid down by Kruger *et al.* (Kruger *et al.*, 1987). An aliquot of semen was retained and the remainder of the sample was prepared by Percoll density centrifugation.

Preparation of samples

Samples were prepared using a two-step discontinuous Percoll gradient (95.0–47.5%; Pharmacia Biotech AB, Uppsala, Sweden). Each aliquot of liquefied semen was layered on top of the gradient and centrifuged at 450 *g* for 12 min. The resulting sperm pellet was concentrated by centrifugation at 200 *g* for 6 min. The final sperm preparation was suspended in a suitable volume of Biggers, Whitten and Whittingham medium (BWW; Biggers *et al.*, 1971) supplemented with 600 mg albutein (Alpha Therapeutic UK Ltd, Norfolk, UK).

Evaluation of sperm morphology

Sperm morphology was assessed in semen using the strict criteria laid down by Kruger *et al.* (Kruger *et al.*, 1987). The sample (5 µl) was evenly spread along the length of a microscope slide which had been thoroughly cleaned with 95% v/v industrial methylated spirit (Adams Healthcare, Leeds, UK) prior to use. The resulting thin smear was allowed to air dry for 20 min before staining which was carried out using a Diff-Quik staining kit (Baxter Dade Diagnostics AG, Dubingen, Switzerland). Stained slides were air-dried for 30 min and large (22×50 mm) coverslips were applied in a fume cupboard using synthetic toluene-based mounting medium (Shandon Inc., Pittsburgh, PA, USA). Morphological assessment was performed at ×1000 magnification under oil-immersion and at least 100 spermatozoa were counted on each slide. Results were expressed as the percentage of normal spermatozoa observed on each slide.

In order to be classified as normal by strict criteria, a spermatozoon must have a smooth, oval configuration with a well-defined acrosome incorporating 40–70% of the sperm head, no neck, midpiece or tail defects, and no cytoplasmic droplets of more than one-half the size of the sperm head. Head defects were subdivided into amorphous, megalo, small, loose head or duplicated. Midpiece defects included all midpiece defects and cytoplasmic droplets. Tail defects included coiled and duplicated tails. Spermatozoa with borderline morphologies were counted as abnormal.

Division of sperm samples

Each sample was divided into four aliquots as follows: fresh semen, cryopreserved semen, freshly prepared spermatozoa and cryopreserved prepared spermatozoa.

Cryopreservation of spermatozoa

Spermatozoa were pipetted into cryovials (Nalge Company, Rochester, NY, USA) and mixed 1:0.7 with Spermfreeze™ cryoprotectant (FertiPro NV, Sint-Martens-Latem, Belgium) which was added dropwise with gentle swirling. Spermfreeze™ was stored at 4°C but was allowed to equilibrate to room temperature before use. The mixture was left at room temperature for 10 min and then frozen by static phase vapour cooling. Aliquots were suspended in liquid nitrogen vapour [10 cm above the level of liquid nitrogen (–80°C)] for 15 min. The samples were then plunged into liquid nitrogen (–196°C) and stored until required.

Thawing of spermatozoa

Spermatozoa were removed from liquid nitrogen and the caps of the cryovials were loosened to prevent them from exploding. The samples were left to thaw at room temperature for 15–20 min. When samples were totally thawed, an equal volume of BWW buffer was added to each cryovial and the cells were centrifuged at 200 *g* for 6 min to remove any traces of Spermfreeze™ cryoprotectant. The supernatant was removed and the pellet resuspended in a suitable volume of BWW (~400 µl) determined by the concentration of spermatozoa obtained.

Determination of DNA integrity using a modified alkaline single cell gel electrophoresis (comet) assay

The DNA status of individual spermatozoa was determined using a modified alkaline single cell gel electrophoresis (comet) assay (Hughes *et al.*, 1998; Donnelly *et al.*, 1999, 2000). This involved embedding spermatozoa in agarose, lysing the membranes and breaking down the protein matrices. Fragmented strands of DNA are drawn out by electrophoresis to form a comet 'tail' leaving a 'head' of intact DNA. Intact and damaged DNA is quantified using epifluorescence microscopy and image analysis. The following procedure was carried out under yellow light to prevent further induced damage to DNA.

Embedding of spermatozoa in agarose gel

Fully frosted microscope slides (Richardsons Supply Co. Ltd, London, UK) were gently heated, covered with 100 µl of 0.5% normal melting point agarose in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS; Sigma-Aldrich Company Ltd, Poole, Dorset, UK) at <45°C and

immediately covered with a large (22×50 mm) coverslip. The slides were placed in a chilled metal tray and left at 4°C for at least 30 min to allow the agarose to solidify. The coverslips were then removed and 1×10^5 spermatozoa in 10 µl BWW were mixed with 75 µl of 0.5% low melting point agarose at 37°C. This cell suspension was rapidly pipetted on top of the first agarose layer, covered with a coverslip and allowed to solidify at room temperature.

Lysing of cells and decondensation of DNA

The coverslips were removed and the slides were immersed in a Coplin jar containing freshly prepared cold lysing solution [2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris; pH 10, with 1% Triton X-100 (Sigma-Aldrich Company Ltd) added just before use] for 1 h at 4°C. Slides were then incubated for 30 min at 4°C with 10 mmol/l dithiothreitol (DTT; Sigma-Aldrich) followed by 90 min incubation at 20°C with 4 mmol/l lithium diiodosalicyclate (LIS; Sigma-Aldrich) (Robbins *et al.*, 1993).

Unwinding of DNA

The slides were removed from the lysis solution + DTT + LIS and carefully drained of any remaining liquid. A horizontal gel electrophoresis tank was filled with fresh alkaline electrophoresis solution (300 mmol/l NaOH, 1 mmol/l EDTA, pH 13.0: Sigma-Aldrich) at 12–15°C. The slides were placed into this tank side by side with the agarose end facing the anode and with the electrophoresis buffer at a level of ~0.25 cm above the slide surface. The slides were left in this high pH buffer for 20 min to allow DNA in the cells to unwind.

Separation of DNA fragments by electrophoresis

Electrophoresis was conducted for 10 min at 25 V (0.714 V/cm) adjusted to 300 mA by raising or lowering the buffer level in the tank. After electrophoresis the slides were drained, placed on a tray and flooded with three changes of neutralization buffer (0.4 mol/l Tris; pH 7.5; Sigma-Aldrich) for 5 min each. This removes any remaining alkali and detergents that would interfere with ethidium bromide staining. The slides were then drained before being stained with 50 µl of 20 µg/ml ethidium bromide (Sigma-Aldrich) and covered with a large coverslip.

Image analysis

The slides were viewed using a Nikon E600 epifluorescence microscope, equipped with an excitation filter of 515–560 nm from a 100 W mercury lamp and a barrier filter of 590 nm. Fifty images were captured and analysed by an image analysis system using the programme Komet 3.1 (Kinetic Imaging Ltd, Liverpool, UK).

Statistical analysis

Results were analysed using Statistica 5.0 (Statsoft of Europe, Hamburg, Germany). In view of the non-Gaussian distribution of data, the non-parametric Mann–Whitney *U*-test was used to compare differences between the fertile and infertile groups. The Wilcoxon matched pairs test was employed to determine differences in fresh and frozen samples within the two groups.

Results

Semen analysis parameters for samples from fertile and infertile men

Fresh semen samples from fertile men were found to have significantly greater concentration ($P = 0.022$), total count ($P = 0.007$), progressive motility ($P = 0.001$) and total motile count ($P = 0.036$) than samples from infertile men (Table I). After cryopreservation, all semen analysis parameters were reduced in both fertile and infertile samples, but samples from fertile men were still significantly better than those from infertile individuals (Table I).

Effect of cryopreservation on DNA integrity of spermatozoa from fertile men

There was no significant difference in DNA integrity of fresh and frozen–thawed semen (61.45 and 60.38% undamaged DNA respectively) or fresh and frozen–thawed prepared spermatozoa (84.21 and 83.17% undamaged DNA respectively) from fertile men (Figure 1).

Effect of cryopreservation on DNA integrity of spermatozoa from infertile men

Cryopreservation caused a significant decrease of 24% in DNA integrity of semen from infertile men ($P < 0.005$; Wilcoxon matched pairs test; Figure 2). A significant decrease of 40% in DNA integrity was observed in prepared spermatozoa from infertile men following freeze–thawing ($P < 0.005$; Wilcoxon matched pairs test; Figure 2).

Effect of cryopreservation on morphology of spermatozoa in semen from fertile men

Cryopreservation of semen from fertile men resulted in a significant decrease from 9 to 4% normal forms (55% decrease; $P < 0.05$; Wilcoxon matched pairs test; Table II). A significant increase from 3.5 to 5% megalo head defects (30% increase) was observed following cryopreservation ($P < 0.05$), and the percentage of spermatozoa with midpiece defects was significantly reduced from 16.5 to 5% (70% reduction) in frozen thawed semen ($P < 0.05$; Wilcoxon matched pairs test; Table II).

Effect of cryopreservation on morphology of prepared spermatozoa from fertile men

Cryopreservation of prepared spermatozoa from fertile men resulted in a significant decrease from 20.5 to 9.0% normal forms (64% decrease; $P < 0.005$; Wilcoxon matched pairs test; Table III). Significant increases from 10.5 to 35.5% amorphous heads (70% increase; $P < 0.005$), and from 3.0 to 10.0% megalo heads (70% increase; $P < 0.005$) were observed in post-thaw spermatozoa. The percentage of spermatozoa with midpiece defects was significantly decreased from 10 to 8% in frozen–thawed prepared spermatozoa (20% increase; $P < 0.005$; Wilcoxon matched pairs test) following cryopreservation (Table III).

Effect of cryopreservation on morphology of spermatozoa in semen from infertile men

Cryopreservation of semen from infertile men resulted in a significant decrease from 8.0 to 3.0% normal forms (62.5% decrease; $P < 0.005$; Wilcoxon matched pairs test; Table II). Significant increases from 13.0 to 21.5% amorphous heads (40% increase; $P < 0.005$) and from 14.0 to 17.5% megalo heads (20% increase; $P < 0.005$) were observed in post-thaw semen compared to the corresponding fresh samples.

Table I. Comparison of semen analysis data for semen samples from fertile and infertile men

Semen analysis parameter	Fresh semen			Frozen–thawed semen		
	Fertile	Infertile	*P*-value	Fertile	Infertile	P value
Concentration ($\times 10^6$/ml)	71.00 (31.00–96.00)	36.00 (20.00–56.00)	0.022 (10.00–45.00)	26.00 (2.00–23.00)	11.00	0.032
Total count ($\times 10^6$)	297.68 (80.10–364.80)	111.27 (28.50–151.20)	0.007 (12.38–24.9)	16.24 (1.23–10.11)	5.91	0.021
Progressive motility (%)	44.00 (40.00–48.00)	33.00 (23.00–40.00)	0.001 (13.00–28.00)	21.00 (5.00–24.00)	12.00	0.026
Total motile count ($\times 10^6$)	116.16 (32.00–152.70)	54.53 (12.54–92.50)	0.036 (2.18–6.87)	5.26 (1.46–3.54)	2.59	0.039

Values are medians with interquartile ranges.

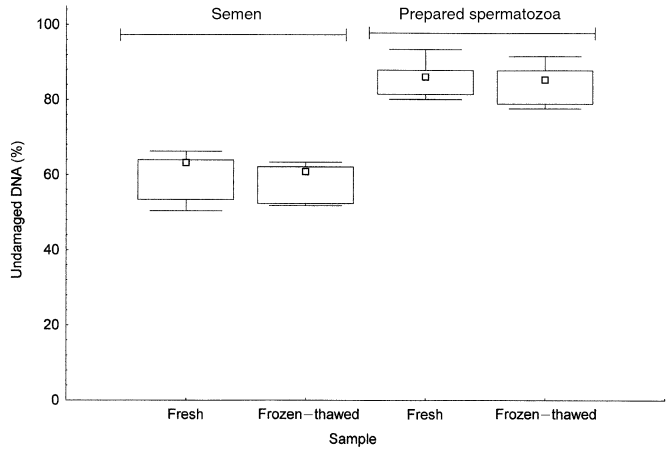

Figure 1. The effect of cryopreservation on DNA integrity of sperm from fertile men. Values are medians (small squares) with interquartile ranges (large rectangles 25–75%) and minimum–maximum values (T-bars).

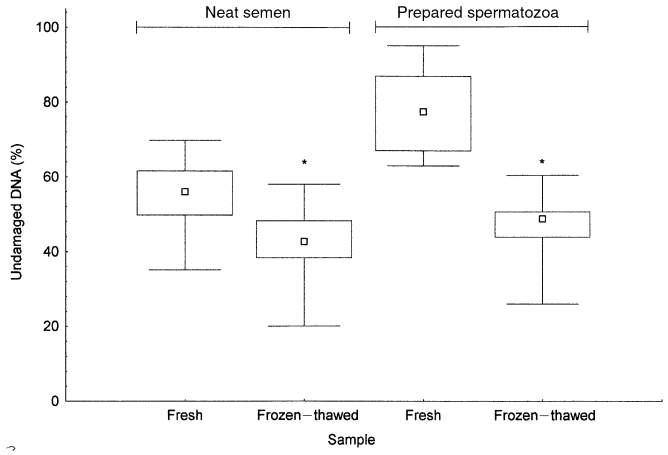

Figure 2. The effect of cryopreservation on DNA integrity of sperm from infertile men. Values are medians (small squares) with interquartile ranges (large rectangles 25–75%) and minimum–maximum values (T-bars). *Significantly lower than value for fresh aliquot ($P < 0.005$; Wilcoxon matched pairs test).

The percentage of spermatozoa with midpiece defects was significantly increased from 19.0 to 22.5% in frozen–thawed semen (16% increase; $P < 0.05$; Wilcoxon matched pairs test) (Table II). There was a significant reduction from 9.5 to 1.0% in spermatozoa with cytoplasmic droplets (90% decrease; $P < 0.005$) following cryopreservation (Wilcoxon matched pairs test; Table II).

Effect of cryopreservation on morphology of prepared spermatozoa from infertile men

Cryopreservation of prepared spermatozoa from infertile men resulted in a significant decrease from 15.5 to 5.0% normal forms (68% decrease; $P < 0.005$; Wilcoxon matched pairs test; Table III). Significant increases from 13 to 16.5% amorphous heads (36% increase; $P < 0.005$), and from 11.0 to 16.0% megalo heads (31% increase; $P < 0.005$) were observed in post-thaw spermatozoa. The percentage of spermatozoa with midpiece defects was significantly increased from 17.0 to 21.5% frozen–thawed prepared spermatozoa (21% increase; $P < 0.005$; Wilcoxon matched pairs test. There was a significant decrease from 8.0 to 2.0 in spermatozoa with cytoplasmic droplets (75% decrease; $P < 0.005$) following cryopreservation (Wilcoxon matched pairs test; Table III).

Correlation of sperm morphology with DNA integrity

There was no significant correlation between overall sperm morphology and DNA integrity in either semen or prepared sperm samples from either fertile ($r = 0.01$, $r = 0.21$ respectively) or infertile men ($r = 0.356$, $r = 0.231$ respectively). Similarly, there were no significant correlations between individual head defects and DNA integrity in either semen or prepared spermatozoa from both fertile and infertile men (Table IV).

Discussion

Despite the wide-ranging clinical applications of cryopreservation, current techniques used for human spermatozoa are still imperfect. Cryopreservation causes extensive damage to sperm membranes and decreases the percentage of motile spermatozoa and the velocity of their movement (Critser *et al.*, 1987a; Critser *et al.*, 1987b). Membrane disruption may be a consequence of liquid phase transition changes and increased lipid peroxidation (Alvarez and Storey, 1992; Alvarez and Storey, 1993; Mossad *et al.*, 1994). It has also been shown that freeze–thawing of spermatozoa results in a reduction in sperm metabolism which reduces the numbers of functional spermatozoa available for

Table II. Comparison of morphology of semen from fertile and infertile men before and after cryopreservation

Morphology (%)	Fresh semen				Frozen semen			
	Fertile	Infertile	Difference (%)	*P*-value	Fertile	Infertile	Difference (%)	*P*-value
Normal	9.00 (6.00–13.00)	8.00 (6.00–10.00)	12.00	0.669	4.00 (1.00–7.00)	3.00	25.00 (2.00–6.00)	0.664
Head defects								
Amorphous	41.50 (17.00–48.00)	13.00 (10.00–27.00)	68.70	0.014	49.00 (34.00–56.00)	21.5	56.20 (15.00–31.00)	0.039
Megalo	3.50 (1.00–13.00)	14.00 (9.00–21.00)	75.00	0.007	5.00 (3.00–8.00)	17.5	71.50 (12.00–25.00)	0.005
Other defects								
Midpiece defects	16.50 (10.00–21.00)	19.00 (16.00–22.00)	13.20	NS	5.00 (3.00–10.00)	22.5	77.80 (12.00–27.00)	0.001
Cytoplasmic droplets	1.00 (1.00–3.00)	9.50 (4.00–14.00)	90.00	0.002	0 (0)	1.00 (0–6.00)	100	0.02
Multiple defects	8.50 (4.00–14.00)	0 (0–5.00)	100	0.007	10.00 (8.00–19.00)	1.00	90.00 (0–8.00)	0.003

Values are medians with interquartile ranges.

Table III. Comparison of morphology of prepared spermatozoa from fertile and infertile men before and after cryopreservation

Morphology (%)	Fresh prepared spermatozoa				Frozen prepared spermatozoa			
	Fertile	Infertile	Difference (%)	*P*-value	Fertile	Infertile	Difference (%)	*P*-value
Normal	20.50 (15.00–23.00)	15.50 (12.00–17.00)	25.00	0.044	9.00 (6.00–14.00)	5.00	45.00 (4.00–7.00)	0.042
Head defects								
Amorphous	10.50 (6.00–14.00)	13.00 (10.00–20.00)	20.00	0.043	35.50 (34.00–56.00)	16.50	54.50 (15.00–31.00)	0.026
Megalo	3.00 (1.00–7.00)	11.00 (8.00–17.00)	70.00	0.005	10.00 (6.00–14.00)	16.00	37.50 (11.00–21.00)	0.038
Other defects								
Midpiece defects	10.00 (6.00–15.00)	17.00 (12.00–21.00)	42.00	0.028	8.00 (3.00–12.00)	21.50	62.80 (16.00–26.00)	0.001
Cytoplasmic droplets	1.00 (0–3.00)	8.00 (5.00–11.00)	88.50	0.002	0 (0–2.00)	2.00	100 (5.00–11.00)	0.002
Multiple defects	10.50 (5.00–16.00)	8.00 (4.00–11.00)	24.50	0.039	15.00 (9.00–19.00)	10.00	33.00 (4.00–15.00)	0.03

Values are medians with interquartile ranges.

assisted conception techniques (Hammerstedt *et al.*, 1990; Alvarez and Storey, 1992; Hammadeh *et al.*, 1999).

Recent research has also shown that cryopreservation reduces the ability of human spermatozoa to bind to an extract of chicken perivitelline membrane coated on microwell plates (Amann *et al.*, 1999; sperm binding assay). In a direct comparison, the percentage of spermatozoa bound was significantly lower in frozen–thawed spermatozoa compared with fresh aliquots from the same sample and the same result was observed for fresh and frozen–thawed samples obtained from different donors (Amann *et al.*, 1999). Cryopreservation has also been found to decrease the average velocity of progressively motile spermatozoa by ~30% (Keel *et al.*, 1987) in both fertile and infertile patients (Mossad *et al.*, 1994), with a greater decrease observed in infertile men compared with fertile donors. Cellular damage during freezing is usually attributed to membrane rupture caused by the formation of intracellular ice crystals during rapid cooling, by osmotic effects or by mechanical force from extracellular ice during slow cooling (Mossad *et al.*, 1994). In the current study, the aim was to determine if damage was also induced in sperm DNA.

A variety of cryoprotective media have been designed in an attempt to overcome the cellular damage caused by cryopreservation (Mahadevan and Trounson, 1983; Weidel and Prins, 1987; Jedrzejczak *et al.*, 1996). In this study, Spermfreeze™, a commercial cryoprotectant consisting of 15% glycerol in HEPES buffer, was selected. This is the cryoprotectant that is routinely used in our Regional Fertility Centre.

The temperature at which spermatozoa are stored in their frozen state is known to be of great importance. Cryopreservation of human semen in liquid nitrogen at –196°C is known to be superior to freezing in a mechanical freezer at –70°C (Trummer *et al.*, 1998) After 7 days of storage there was a

Table IV. Correlation of sperm DNA integrity with morphological head defects

Correlation of sperm DNA integrity with head defects	Samples from fertile men		Samples from infertile men	
	Semen *r*	Prepared spermatozoa *r*	Semen *r*	Prepared spermatozoa *r*
Amorphous heads	–0.148	0.060	0.036	–0.124
Round heads	–0.390	–0.287	–0.026	–0.289
Megalo heads	–0.612	0.090	–0.082	–0.160
Small heads	–0.526	–0.550	–0.097	–0.130
Elongated heads	–0.481	0.080	–0.004	–0.258
Duplicated heads	–0.303	–0.502	–0.378	–0.197

A *P*-value <0.05 was considered to be significant.
There were no significant correlations between sperm DNA integrity and any of the specific head defects indicated above.

greater decrease in motility in specimens maintained at –70°C compared to those stored at –196°C and the difference was even more significant following 3 months of storage, although no differences in sperm morphology were observed between the two groups (Trummer *et al.*, 1998). Here we stored the spermatozoa at the lower temperature.

It has also been reported that computerized slow-staged freezing of semen from men with testicular tumours or Hodgkin's disease preserves spermatozoa significantly better than standard vapour freezing in terms of motility, viability and swelling after thawing (Ragni *et al.*, 1990). However, in our Regional Fertility Centre and in numerous other units, spermatozoa continue to be frozen using the standard vapour freezing technique used in this study. This may be due to lack of access to a computerized slow-staged freezer or to the added expense that the use of such equipment may involve. A standard vapour freezing technique will usually result in sufficient spermatozoa for use in cycles of IVF or ICSI and therefore such a method was used in this investigation to reflect the practices employed at many Fertility Centres.

The present study has shown that spermatozoa from infertile men are more susceptible to freezing damage than those from fertile men. It has previously been shown that freeze–thawing causes significant damage to sperm chromatin, morphology and membrane integrity in both fertile and infertile men (Hammadeh *et al.*, 1999). Nonetheless, chromatin condensation in the infertile group was significantly greater than in the fertile group (Hammadeh *et al.*, 1999). In addition, men suffering from leukaemia are known to have significantly lower pre-freeze and post-thaw motile sperm count and curvilinear velocity compared with healthy donors (Hallak *et al.*, 1999).

The sperm chromatin structure assay (SCSA) (Evenson *et al.*, 1980) has been employed to analyse chromatin structure variations of human spermatozoa in semen (Spano *et al.*, 1999) and has shown that overall sperm quality deteriorates after cryopreservation. When thawed spermatozoa were prepared using a swim-up technique, a general improvement in nuclear maturity was seen in post-swim-up cells (Spano *et al.*, 1999).

It has been observed that spermatozoa from infertile men have a greater incidence of irregular chromatin organization and show a significant decrease in chromatin resistance to thermal denaturation compared with spermatozoa from fertile men (Evenson *et al.*, 1980; Evenson and Melamed, 1983). This points towards a possible correlation between variations in chromatin condensation and fertility (Clausen *et al.*, 1982). Defects in chromatin structure of infertile men have been shown to lead to increased DNA instability and sensitivity to denaturing stress (Balhorn *et al.*, 1988; Manicardi *et al.*, 1995). This supports data from previous studies from our own laboratory using the comet assay, where it was found that spermatozoa DNA from infertile men is more sensitive to X-ray radiation than those from fertile men (Hughes *et al.*, 1996). Other research has shown that the freeze–thawing process results in variation in the compactness of mammalian spermatozoa nuclei (Hunter, 1976; Royere *et al.*, 1988) which may account for the decreased conception rates following insemination using frozen–thawed semen or for failure of conception despite good post-thaw sperm motility. Increased sensitivity to freezing damage appears to be another feature of sperm DNA from men with fertility problems.

Poor quality spermatozoa contain partially decondensed chromatin where a functional immaturity is reflected in a lack of protamination, i.e. decondensed chromatin which allows assault by nucleases and polymerases (Bianchi *et al.*, 1993) or DNA strand breakages (Gorczyca *et al.*, 1993). Chromatin condensation is vital for spermatozoa due to the fact that spermiogenesis results in the discarding of cytoplasm causing cessation of transcription and leaving the spermatozoa incapable of undertaking DNA repair (Poccia, 1986). Hence any damage caused to sperm DNA by the freeze–thawing process will remain throughout the insemination procedure. However, it has been reported that embryonic development is very much dependent on the degree of DNA damage (Ahmadi and Ng, 1999) as the oocyte has the ability to repair small amounts of sperm DNA damage (>8%) (Ahmadi and Ng, 1999). Higher amounts of damage are likely to result in poor rates of embryo development and early pregnancy loss. Since good quality sperm DNA is of paramount importance for the correct conveyance of genetic material from one generation to the next, the additional damage caused by freeze–thawing may have further detrimental consequences.

Semen from infertile men is known to possess a greater percentage of spermatozoa with fragmented DNA than semen from fertile men (Sun *et al.*, 1997; Lopes *et al.*, 1998). In addition, the percentage of spermatozoa containing fragmented DNA is negatively correlated with fertilization rates in IVF

(Sun *et al.*, 1997) and ICSI (Lopes *et al.*, 1998). It is also known that a significant proportion of men who have a semen profile which would be classified as normal using WHO criteria, but have been classified as unexplained infertility, possess strand breaks in their sperm DNA (Høst *et al.*, 1999).

Damage to sperm DNA is strongly correlated with mutagenic events (Fraga *et al.*, 1991). Despite this, spermatozoa with damaged genetic material are still capable of fertilization and mutations and defects may not become evident until the embryo has divided or the fetus has developed (Twigg *et al.*, 1998). DNA strand breaks lead to chromosomal damage and most sperm-derived genetic mutation occurs through chromosomal breakage rather than chromosomal rearrangement as in the oocyte (Cathcart *et al.*, 1984). In addition, studies of genetic abnormalities arising from germline mutations show a higher frequency of paternal rather than maternal origin (Vogel and Rathenberg, 1975).

Sperm morphology [assessed using the Tygerberg criteria; (Kruger *et al.*, 1987)] is known to be an important determining factor in predicting the outcome of an IVF cycle (Donnelly *et al.*, 1998; Lim *et al.*, 1998) and sperm tail defects are known to be negatively correlated with fertilization rates in IVF (Lim *et al.*, 1998). It has been recommended that only spermatozoa with fully condensed nuclei of normal shape (Yue *et al.*, 1995) should be used for ICSI (Cummins and Jequier, 1994). Semen with severe sperm head abnormalities have been found to have decreased fertilizing capacity (Marsh *et al.*, 1987) and a reduced ability to establish successful pregnancies (Oehninger *et al.*, 1988).

Results from the current investigation show that the percentage of spermatozoa with normal morphology in fertile and infertile samples is similar both before and after cryopreservation. In agreement is a recent study (Hammadeh *et al.*, 1999) that also demonstrated that the percentage decrease in morphologically normal spermatozoa after freeze–thawing of semen from fertile and infertile groups appeared to be similar.

This study has found that there was no significant correlation between sperm morphology and DNA integrity in either semen or prepared sperm samples from both fertile and infertile men. This is contrast to recent findings using bull spermatozoa where it was reported that morphometry measurements were likely to be a sensitive biomarker related to fertility potential and abnormal chromatin structure (Sailer *et al.*, 1996). In the current study, it was also found that there were no significant correlations between specific head defects such as amorphous or megalo sperm heads, and sperm DNA integrity. Therefore the physical characteristics of a given human spermatozoa do not appear to be indicative of the quality of sperm DNA, i.e. the phenotype of the spermatozoa does not reflect the genotype of the cell. Similarly, cryopreservation of samples from both fertile and infertile individuals caused significant detrimental effects to sperm morphology, whereas DNA of samples from fertile men was more resistant to freezing damage than that of infertile men. This clearly demonstrates that genotype and phenotype of human spermatozoa are not similarly affected by cryopreservation.

The ability of semen from infertile men to resist freezing damage may be due to some protective constituents in seminal plasma. Seminal plasma contains an abundance of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, which removes key reactive oxygen species (ROS) such as O_2^{o-} and H_2O_2, and scavengers such as albumin and taurine (Halliwell and Gutteridge, 1989). It also contains crucial chainbreaking antioxidants such as urates, ascorbate and thiol groups (Alvarez and Storey, 1989; Lewis *et al.*, 1997). In addition, total antioxidant capacity of semen from fertile men has been found to be significantly greater than that for samples from infertile men (Lewis *et al.*, 1995). It may be the case that only fertile samples have these antioxidants present in sufficient abundance to confer protection against the trauma of cryopreservation.

The results presented here have shown that both semen and prepared spermatozoa from fertile men appear to be more resistant to freezing damage than equivalent samples from infertile men. The DNA of prepared spermatozoa from fertile men is also more resistant to damage, even in the absence of antioxidant protection afforded by seminal plasma. Further studies are ongoing to determine if any differences exist in the structure or packaging of DNA (Bianchi *et al.*, 1993; Hughes *et al.*, 1996) in spermatozoa from fertile and infertile men.

In conclusion, cryopreservation of spermatozoa from fertile men does not appear to have any deleterious effect on sperm DNA integrity in either semen or prepared samples. However, morphology of spermatozoa in both semen and prepared spermatozoa from fertile donors is significantly impaired by cryopreservation. The protection of the DNA has important implications in the use of freeze–thawed donor spermatozoa for insemination. After cryopreservation, this sperm DNA is still suitable for use in IVF or ICSI. In contrast, freezing of spermatozoa from infertile men has a significant detrimental effect on DNA integrity in both semen and prepared samples and sperm morphology is also significantly reduced. This is extremely relevant for individuals who may have spermatozoa banked for long term storage prior to chemotherapy or radiotherapy. Further work is required to optimize a freezing protocol for sperm samples from infertile men to protect their DNA for subsequent use in IVF or ICSI.

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