Human Reproduction Vol.17, No.3 pp. 704–709, 2002

The effects of cryopreservation on sperm morphology, motility and mitochondrial function

M.O'Connell[1], N.McClure[1,2] and S.E.M.Lewis[1,3]

[1]Department of Obstetrics and Gynaecology, The Queen's University of Belfast, Institute of Clinical Science, Grosvenor Road and [2]Regional Fertility Centre, Royal Maternity Hospital, Belfast BT12 6BJ, Northern Ireland

[3]To whom correspondence should be addressed. E-mail: s.e.lewis@qub.ac.uk

BACKGROUND: The effects of cryoinjury were determined simultaneously on the mitochondrial function, motility, morphology and viability of ejaculated human sperm. METHOD: Rhodamine 123 (R123) uptake (% of sperm) and stain intensity were used to determine sperm mitochondrial activity before and after cryopreservation from the semen of 50 men attending for infertility investigation. Morphology was assessed using Tygerberg's strict criteria and viability was assessed by eosin Y. Sperm motility was measured using computer-assisted semen analysis (CASA). RESULTS: Freeze–thawing caused a 37% ($P = 0.001$) reduction in normal morphological forms of sperm. All CASA sperm motility parameters except amplitude of lateral head displacement were similarly reduced. R123 uptake and intensity within sperm mitochondria decreased by 36 and 47% respectively (both $P = 0.001$). In addition, there was a similar significant decrease (31%, $P = 0.001$) in the viability of the sperm. CONCLUSIONS: Sperm morphology, motility, mitochondrial activities and viability are equally susceptible to cryopreservation-induced damage. R123 intensity is a novel and robust indicator of mitochondrial function before and after such trauma.

Key words: cryopreservation/mitochondria/rhodamine 123 uptake/sperm

Introduction

Cryopreservation of semen is routinely used in a variety of circumstances including assisted reproduction, pre-radiation or chemotherapy treatment, as 'fertility insurance' for men undergoing vasectomy and for storage of donor semen until seronegativity for HIV and hepatitis is confirmed. It is also used for storage of sperm retrieved from azoospermic patients who have undergone testicular sperm extraction or percutaneous epididymal sperm aspiration. Cryostorage of testicular and/or epididymal sperm ensures sperm are available for multiple ICSI treatment from a single biopsy (Tournaye, 1999).

Cryopreservation has been reported to cause changes in sperm morphology, including damage to mitochondria, the acrosome and the sperm tail (Wooley and Richardson, 1978). Therefore, the proportion of fully functional sperm that retain intact membranes, tail and mitochondrial activity after freeze–thawing is low (Holt, 1997). Sperm motility is particularly sensitive to such damage (Henry *et al*., 1993). While it is generally accepted that sperm motility is reduced by cryopreservation, the mechanism by which this occurs is, as yet, unclear. Despite many advances in cryobiology, the salvage rate has changed little (Centola *et al*., 1992; Sharma and Agarwal, 1996).

Sperm are made up of several compartments enclosed within plasma and mitochondrial membranes. These membranes must remain intact and functional to permit cell competence. Energy is also necessary both for sperm motility and fertilization. This energy is supplied in the form of ATP synthesized either by glycolysis in the cytoplasm (Ford and Rees, 1990) or through oxidative phosphorylation (OXPHOS) in the mitochondria (Mahadevan *et al*., 1997). The relative contributions of the two processes to ATP generation are as yet unclear. However, the ATP generated by OXPHOS in the inner mitochondrial membrane is transferred to the microtubules to drive motility (Zamboni, 1982). Hence reduced motility in sperm may be associated with mitochondrial damage. Although numerous studies have measured these parameters individually, none has measured all the parameters within the same sperm population and under the same conditions. Therefore, it has not been possible to ascertain if the decrease in sperm motility can be accounted for entirely by a loss of mitochondrial function. Rhodamine 123 (R123) uptake (percentage of sperm) and stain intensity (AU) were used to determine mitochondrial function before and after cryopreservation (Windsor and White, 1993).

The present study focuses on the relationship between mitochondrial activity, sperm motility parameters, morphology and viability pre and post-cryopreservation by measuring all these parameters simultaneously.

Materials and methods

Subject selection

Semen samples were obtained from 50 subjects attending the Andrology Laboratory for infertility investigation at the Regional

Fertility Centre, Belfast. All men had given informed consent for this study according to the regulations of the Research Ethical Committee of our University. They were asked to abstain from ejaculation for 48 h but not more than 72 h before the sample was produced, at the laboratory, by masturbation.

Sperm preparation and determination of motility parameters

All semen samples had normal concentrations ($\geq 20 \times 10^6$ ml), below normal morphology (see below, Morphological assessment of sperm) (Table III). Percentage progressive motility was measured by conventional light microscopy. Semen samples were allowed to liquefy at 37°C for 20 min. Routine semen analysis was performed under light microscopy according to World Health Organization criteria (World Health Organization, 1999). The sample was then divided into two aliquots. Sperm motility parameters of aliquot one were measured at 37°C using a Hamilton Thorne Integrated Visual Optical System sperm analyser (Version 10.7; Hamilton Thorne Research, Beverly, MA, USA) and 20 µm depth Microcell counting chambers (Conception Technologies Inc., La Jolla, CA, USA). The settings employed for analysis were from acquisition rate (Hz), 50; minimum contrast, 7; minimum size, 6; low-size gate, 0.4; high-gate size, 1.6; low-intensity gate, 0.4; high intensity gate, 1.6; magnification factor, 2.04. The following motility parameters were recorded for each sample before and after cryopreservation: number of sperm exhibiting motility and progressive motility (those sperm which exhibit an actual space-gain motility); straight line velocity (VSL; the straight line distance from beginning to end of a sperm track divided by the time taken); average path velocity (VAP; the average path velocity of sperm); curvilinear velocity (VCL; a measure of the total distance travelled by a given sperm divided by the time elapsed); the amplitude of lateral head displacement (ALH; the mean width of sperm head oscillation); and beat cross frequency (BCF; the frequency of the sperm head crossing the sperm average path).

Cryopreservation of sperm

Aliquot two was diluted 1.0:0.7 with SpermFreeze™, a 15% glycerol based cryoprotectant in HEPES buffer (FertiPro N.V., 8730 Beernem, Belgium) and subjected to static vapour phase cooling (Mortimer, 1994) for 15 min before being plunged into liquid nitrogen. Samples were subsequently thawed at 37°C. Once totally thawed, an equal volume of Biggers–Whitten–Whittingham (BWW) (Biggers *et al*., 1971) medium was added to each cryovial and the cells were centrifuged at 200 *g* for 6 min to remove any traces of Spermfreeze cryoprotectant as required for motility and fluorometric assessment.

Viability of sperm

A total of 20 µl of each aliquot was mixed with 20 µl of 0.5% eosin Y stain on a glass microscope slide and viewed using light microscopy to determine the percentage of viable sperm. Live sperm remained white while dead sperm stained red, since the integrity of their plasma membranes had been compromized causing an increase in membrane permeability that led to uptake of the dye.

Fluorometric assessment and measurement

Sperm from both aliquots were incubated for 30 min at 37°C, 5% CO_2 with R123 (Sigma-Aldrich, Poole, Dorset, UK) at a resuspending final concentration of 10 µg/ml. Excess R123 was removed from the sperm by washing in BWW (Biggers *et al*., 1971) medium and centrifuged at 300 *g*, three times. Slides were viewed using a Nikon (Eclipse E600) epi-fluorescence microscope, equipped with an excitation filter of 515–560 nm from a 100 W mercury lamp and a barrier filter of 590 nm. Within each field the total number of sperm was counted using light microscopy. The proportion of sperm that acquired R123 staining was then evaluated using fluorescence microscopy. Fifty images were captured and analysed by an image analysis system to determine R123 sperm intensity using the computer programme, Fenestra (Kinetic Imaging Ltd, Liverpool, UK). All fresh samples were analysed within 60 min of production.

Morphological assessment of sperm

Slides were prepared by the method of Hall *et al.* (Hall *et al*., 1995). After preparation, sperm were stained using the Diff-Quik staining kit (Baxter Dale Diagnostics AG, Dubinger, Switzerland). A total of 100 sperm were assessed by microscopy with oil immersion at ×1000 magnification. To be classified as normal by the Tygerberg strict criteria (Kruger *et al*., 1986) a sperm cell must have a smooth oval configuration with a well defined acrosome involving 40–70% of the sperm head, no defects of neck, midpiece or tail and no cytoplasmic droplets >50% the size of the sperm head. Borderline forms were counted as abnormal, in contrast to the conventional World Health Organization method (World Health Organization, 1992). By these criteria, the cut-off point for normality is ⩽14%. Defects were subdivided into head, midpiece or tail abnormalities.

Statistical analysis

Due to the possible non-Gaussian distribution of the data, the non-parametric Wilcoxon matched pairs test was used to assess differences between fresh and freeze–thawed sperm within each parameter. Values are therefore expressed as median ± interquartile range. Stepwise linear regression analysis was applied to determine any correlations between the different parameters. The relationships between variables were evaluated using multiple regression analysis. Statistical differences were considered to be significant if $P < 0.05$. All analyses were performed using the Statistica 5.0 package (Statsoft Version 5.1, Hamburg, Germany).

Results

The numbers of viable sperm and those with functional mitochondria (percentage R123 uptake and R123 intensity) decreased significantly after cryopreservation (Table I). Cryopreservation resulted in a highly significant reduction of all motility parameters (except ALH) as assessed by computer assisted semen analysis (CASA; Table II). The number of sperm with normal morphology was also significantly reduced after freeze–thawing. There was a marked increase in amorphous, megalo and elongated heads and in midpiece abnormalities. The number of loose heads and tails was also increased. There was a significant decrease in the proportion of sperm that had cytoplasmic droplets after freeze–thawing (Table III).

There was a positive correlation between R123 uptake and R123 intensity and the numbers of progressively motile sperm in fresh and frozen sperm (Table IV). There was also a close relationship between the numbers of sperm taking up R123 and their CASA motility parameters before freezing. This relationship was only maintained after freeze–thawing between R123 uptake and R123 intensity with the numbers of progressively motile sperm but not with their velocities (Table IV). Before freezing, R123 uptake showed a positive correlation with normal morphology, but this relationship was lost in post-thaw sperm (Table V). However, its negative correlation with midpiece abnormalities was maintained after cryopreservation. In contrast, the positive relationship between R123 intensity

Table I. The effects of cryopreservation on mitochondrial activity and viability

Parameter	Fresh semen	Freeze–thawed semen	% Change	*P*-value
R123 uptake (%)	62.3 (29.0–89.0)	39.8 (17.0–59.0)[a]	–36	0.0001
R123 activity (AU) (Fluorescence activity)	25.3 (13.6–39.4)	13.2 (4.8–24.2)[a]	–47	0.0001
Viability	81.2 (81.0–95.0)	55.8 (41.0–72.0)[a]	–31	0.0001

Values are medians (± IQR), $n = 50$.
[a]Significance $P < 0.05$, Wilcoxon matched pairs test.
AU = stain intensity.

Table II. The effects of cryopreservation on sperm motility

Parameter	Fresh semen	Freeze–thawed semen	% Change	*P*-value
Motile (%)	40.2 (12.0–66.0)	24.8 (7.0–42.0)[a]	–33	0.0001
Progressively motile	22.9 (7.0–37.0)	13.6 (2.0–22.0)[a]	–41	0.0001
VAP (μm/s)	51.2 (46.9–58.4)	35.3 (27.8–46.4)[a]	–31	0.0001
VSL (μm/s)	42.1 (39.4–45.6)	29.8 (22.4–35.1)[a]	–29	0.0001
VCL (μm/s)	75.5 (67.9–84.1)	51.5 (42.1–64.3)[a]	–32	0.0001
ALH (μm)	3.8 (3.2–4.6)	3.9 (2.4–4.0)[a]	0.1	0.05
BCF (Hz)	19.1 (17.1–21.2)	15.6 (12.5–18.4)[a]	–18	0.001
LIN (%)	57.3 (52.0–62.0)	47.7 (47.0–54.0)[a]	–16	0.01

Values are medians (± IQR), $n = 50$.
[a]Significance $P < 0.05$, Wilcoxon matched pairs test.
VAP = average path velocity; VSL = straight line velocity; VCL = curvilinear velocity; ALH = amplitude of lateral head displacement; BCF = beat cross frequency; LIN = linearity.

Table III. The effects of cryopreservation on sperm morphology

Parameter	Fresh semen	Freeze–thawed semen	% Change	*P*-value
Normal morphology (%)	8.2 (4.0–13.0)	5.2. (2.0–8.0)[a]	–37	0.001
Amorphous (%)	12.0 (7.0–18.0)	18.0 (13.0–22.0)[a]	+50	0.001
Megalo (%)	7.5 (4.0–11.0)	10.5 (9.0–13.00)[a]	+28	0.001
Elongated (%)	6.5 (2.0–9.0)	9.0 (6.0–12.0)[a]	+27	0.001
Midpiece abnormalities (%)	10.0 (8.0–12.0)	19.0 (15.0–22.0)[a]	+46	0.001
Cytoplasmic droplet (%)	7.2 (2.0–13.0)	3.0 (0.0–5.0)[a]	–58	0.001
Loose heads and tails (%)	8.0 (3.0–12.0)	11.3 (6.0–19.00)[a]	+29	0.001
Tail defects (%)	6.0 (4.0–12.0)	9.3 (4.0–16.0)[a]	+35	0.001

Values are medians (± IQR), $n = 50$.
[a]Significance $P < 0.05$, Wilcoxon matched pairs test.

and normal morphology (Table V) and the negative relationship with midpiece abnormalities (Table VI) was maintained in post-thaw sperm. Sperm midpiece abnormalities also showed a strong inverse relationship to motility parameters; however, few of these relationships remained in post-thaw sperm (Table VI).

Discussion

Cryopreserved semen is used routinely in assisted conception. However, detailed examination suggests that the proportion of fully functional sperm in a freeze–thawed sample is considerably reduced (Holt, 1997). Motility is one of the parameters most seriously affected by freezing (Watson, 1995). It is also a strong predictor of the ability of a given sample to achieve fertilization *in vitro* (Donnelly *et al*., 1998). Despite its importance, the mechanism by which motility is reduced has not been elucidated. Sperm motility is partially dependent on mitochondrial function (Kao *et al*., 1998). Mitochondria are strategically wrapped around the midpiece to provide accessible energy to the tail filaments, thus facilitating efficient propulsion for the sperm both to reach the oocyte and to penetrate its zona pellucida. Mitochondria are the cell's major source of oxidative energy through their production of ATP via the electron transport chain (ETC). The ETC itself is made up of subunits that have been synthesized by the mitochondrial DNA (except complex II). It is the communication that exists between these complexes and ETC that produces ATP.

Sperm possess both plasma and mitochondrial membranes and susceptibility to freeze–thawing damage may differ depending on accessibility to cryoprotectants. It is well documented that experiments using evaluations of a single parameter are, therefore, of limited use. In this study, we measured motility, normal and abnormal morphology and mitochondrial

Table IV. The relationship between mitochondrial function and motility before and after freeze–thawing

	Before freeze–thawing				After freeze–thawing			
	R123 Uptake (%)		R123 Intensity (AU)		R123 Uptake (%)		R123 Intensity (AU)	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
P.motile (%)	0.62	0.005	0.46	0.05	0.57	0.05	0.54	0.05
VAP (μm/s)	0.84	0.0001	0.52	0.05	0.33	NS	0.39	NS
VSL (μm/s)	0.79	0.0001	0.51	0.05	0.38	NS	0.37	NS
VCL (μm/s)	0.69	0.002	0.47	0.05	0.41	0.06	0.34	NS
ALH (μm)	0.43	0.03	0.39	NS	0.31	NS	0.53	0.01
BCF (Hz)	0.37	NS	0.40	NS	0.40	NS	0.33	NS
LIN (%)	0.88	0.01	0.34	NS	0.72	0.001	0.32	NS
R123 uptake (%)	0.77	0.001	0.40	NS				

Significance $P < 0.05$, $n = 50$, r = correlation coefficient (stepwise linear regression).
P.motile = progressively motile; VAP = average path velocity; VSL = straight line velocity; VCL = curvilinear velocity; ALH = amplitude of lateral head displacement; BCF = beat cross frequency; LIN = linearity; NS = not significant.

Table V. The relationship between mitochondrial function, motility and normal morphology before and after cryopreservation

	Before freezing Normal morphology		After freeze–thawing Normal morphology	
	r	*P*	*r*	*P*
R123 uptake (%)	0.49	0.05	0.36	NS
R123 intensity (AU)	0.61	0.05	0.70	0.001
Motile (%)	0.54	0.01	0.50	0.05
Progressively motile (%)	0.40	NS	0.37	NS
VAP (μm/s)	0.81	0.0001	0.53	0.01
VSL (μm/s)	0.70	0.001	0.49	0.05
VCL (μm/s)	0.49	0.02	0.56	0.01
ALH (μm)	0.32	NS	0.31	NS
BCF (Hz)	0.32	NS	0.34	NS
LIN (%)	0.37	NS	0.36	NS

Significance $P < 0.05$, $n = 50$, r = correlation coefficient (stepwise linear regression).
VAP = average path velocity; VSL = straight line velocity; VCL = curvilinear velocity; ALH = amplitude of lateral head displacement; BCF = beat cross frequency; LIN = linearity; NS = not significant.

Table VI. The relationship between midpiece abnormalities and motility before and after cryopreservation

	Before freezing Midpiece abnormalities		After freeze–thawing Midpiece abnormalities	
	r	*p*	*r*	*p*
Motile (%)	–0.49	0.05	–0.39	NS
Progressively motile (%)	–0.48	0.05	–0.38	NS
VAP (μm/s)	–0.49	0.05	–0.53	0.01
VSL (μm/s)	–0.49	0.05	–0.53	0.01
VCL (μm/s)	–0.51	0.001	–0.45	0.05
ALH (μm)	–0.53	0.001	–0.30	NS
BCF (Hz)	–0.51	0.001	–0.35	NS
LIN (%)	–0.54	0.01	–0.43	0.05
R123 Uptake	–0.51	0.001	–0.52	0.001
R123 Intensity	–0.41	0.05	–0.43	0.05

Significance $P < 0.05$, $n = 50$, r = correlation coefficient (stepwise linear regression).
VAP = average path velocity; VSL = straight line velocity; VCL = curvilinear velocity; ALH = amplitude of lateral head displacement; BCF = beat cross frequency; LIN = linearity; NS = not significant.

function within each sample to compare the sensitivity of each as a marker of post-thaw survival.

Various mitochondrial stains are available to measure mitochondrial membrane potential (MMP) (Garner *et al*., 1997). For example, 5,5′,6,6’-tetrachloro-1,1’,3,3′ tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), Rhodamine 123, MitoTracker Green FM (MITO) and 3,3′-dihexiloxocarbocyanine iodide ($DiOC_6$) (Salvioli *et al*., 1997). Using these stains, mitochondrial function can be measured in two ways. The percentage of sperm exhibiting stain uptake indicates those sperm within the total population with functional mitochondria. Secondly, this can be refined to measure activity within the mitochondria by quantifying the fluorescence intensity of the stain in these individual sperm. The sensitivity of each stain has been assessed and the results correlate well (Garner *et al*., 1997; Salvioli *et al*., 1997) both with mitochondrial function and with each other. Since R123 is accumulated by mitochondria in response to the electrochemical gradient set up by the mitochondrial membrane potential, R123 uptake is sensitive to factors, such as potassium or hydrogen ion levels, which directly reduce the mitochondrial membrane potential (Windsor and White, 1993). Therefore, it is useful in the evaluation of membrane-mediated injuries. However, in previous studies there has been difficulty in preventing photo bleaching while allowing sufficient time for functional mitochondria to accumulate R123 (Windsor and White, 1993). We avoided this problem by incubating the sperm in R123 for a shorter time and subsequently removing excess R123 by washing the sperm three times in BWW. This also resolved the difficulty of contaminant staining in the sperm head, which is known to give inaccurate midpiece measurements (Tucker *et al*., 1986).

It has been suggested (Henry *et al*., 1993) that motility, membrane integrity and mitochondrial function are similarly affected by cryopreservation. In agreement, we found that the plasma and mitochondrial membranes were equally vulnerable.

The extent of damage caused by freeze–thaw to plasma membranes (viability –31%) was nearly identical to the reduction in the number of sperm with functional mitochondria (R123 uptake –36%). This suggests that the reduction in motility may be explained by an impairment of mitochondrial activity. This is also supported by the actual intensity of R123 staining, showing that not only have less sperm maintained functional mitochondria after freeze–thawing, but also activity within the mitochondria is similarly damaged.

It is becoming increasingly apparent that mitochondria are initiators of cell death by apoptosis (Dinsdale *et al*., 1998; Green and Reed, 1998; Sun *et al*., 1999). Our study suggests that the parameters measured here are interdependent and that functional plasma and mitochondrial membranes are necessary to maintain motility. However, it may also be that cryoinjury to mitochondria sets an apoptosis-like mechanism in motion. After thawing, this could lead to further damage to plasma membranes and loss of function, as observed in decreased motility. In this study, cryopreservation led to a decrease in all the motility parameters (except ALH), the values being reduced to half their pre-freeze values with a similar reduction in functional mitochondria activity. Earlier studies have shown a similar trend, with cryopreservation resulting in a comparable reduction in motility parameters (Critser *et al*., 1987; Holt *et al.*, 1988; Leffler and Walters, 1996).

One mechanism suggested for the reduction in motility is an irreversible looping of the flagellum that is known to occur in rat sperm (Holt *et al*., 1988). In our study, we found an increase in tail abnormalities in human sperm after freezing. However, decreases in the numbers of progressively motile sperm and their respective velocities were similar to the reduction in mitochondrial function, showing the close relationship between these parameters. Previous work has also demonstrated a correlation between R123 fluorescence and sperm motility (Evenson *et al*., 1982; Auger *et al*., 1993).

Our results also demonstrate that both the numbers of progressively motile sperm and their morphologies were closely associated with R123 uptake and intensity, both before and after cryopreservation. However, the relationship between their velocity profiles and the level of mitochondrial activity was not as apparent as might have been anticipated. One explanation for this may be that the major source of energy for motility is glycolysis and not OXPHOS, so the decrease in post-thaw motility was not simply due to an impairment of mitochondrial metabolism. The relative contributions of glycolysis and oxidative phosphorylation (OXPHOS) to the generation of ATP in human sperm are still unclear. It has been has suggested that glycolysis is the dominant producer of ATP in human sperm (Peterson and Freund, 1970; Ford and Rees, 1990), whereas other studies (Storey and Kayne, 1975; Ford and Harrison, 1981) have shown that OXPHOS is also a common pathway.

Another explanation may be the variation in mitochondrial activity within the population of sperm that increased in the post-thaw sample (Table I). Our results are in agreement with earlier studies that examined the mitochondrial status of sperm and demonstrated that alterations in energy metabolism can be affected without a change in membrane integrity (Vetter *et al*., 1998). Similarly, Holt found that a loss of progressive motility in ram sperm caused by cooling was not due to mitochondrial inactivity (Holt, 1997).

Before and after freezing, a strong positive correlation was observed between R123 uptake and intensity, motility and the numbers of progressively motile sperm. Previous studies have also demonstrated a correlation between R123 fluorescence and fresh sperm motility (Evenson *et al*., 1982; Auger *et al*., 1993) although comparisons have not been made with post-thaw sperm. After freezing, the relationship between the conventional measure of R123 uptake and morphology was lost, although a strong relationship was preserved with R123 intensity, suggesting that it is a more robust indicator of mitochondrial function after trauma such as cryopreservation. Before and after freezing, normal morphology and motility had a strong positive correlation, while abnormal morphology in the sperm midpiece correlated negatively with velocity parameters; VAP, VSL VCL and linearity confirming the sensitivity and reliability of morphology assessment by Tygerberg criteria and motility by CASA. The associations between motility parameters and mitochondrial function were diminished after freeze–thawing. This suggests that not all sperm within a sample are uniformly damaged by freeze–thawing.

Perhaps the concept of 'good and bad freezers', as postulated by Watson, extends beyond variations in semen from different individuals to the ability of individual sperm from within one sample to survive cryopreservation (Watson, 1995). This is an extremely important issue, as many assisted conception centres and sperm banks routinely cryopreserve whole semen rather than freeze those subpopulations of sperm with the best pre-freeze motilities and morphologies. It is possible that the presence of protective seminal plasma in a whole semen sample may not compensate for the deleterious effects of subnormal sperm.

Further work is ongoing to determine whether the post-thaw survival of sperm can be improved by freezing selected subpopulations rather than whole semen.

Acknowledgements

The authors thank Mrs M.Kennedy for her technical assistance. M.O'C. received financial support from the Fertility Research Trust, Northern Ireland.

References

Auger, J., Leonce, S., Jouannet, P. and Ronot, X. (1993) Flow cytometric sorting of living, highly motile human spermatozoa based on evaluation of their mitochondrial activity. *J. Histo. Cytochem*., **41**, 1247–1251.

Biggers, J.D., Whitten, W.K. and Whittingham, D.G. (1971) The culture of mouse embryos *in vitro*. In Daniel, J.C. (ed.) *Methods in Mammalian Embryology.* Freeman, San Francisco, pp. 86–116.

Centola, G.M., Raubertas, R.F and Mattox, J.H (1992) Cryopreservation of human semen—comparison of cryopreservatives, sources of variability, and prediction of postthaw survival. *J. Androl.*, **13**, 283–288.

Critser, J.K., Arneson, B.W., Aaker, D.V., Huse-Benda, A.R. and Ball, G.D. (1987) Cryopreservation of human spermatozoa. II. Post thaw chronology of motility and of zona-free hamster ova penetration. *Fertil. Steril*., **47**, 980–987.

Dinsdale, D., Zhuang, J. and Cohen, G.M. (1998) Redistribution of cytochrome c precedes the caspase-dependent formation of ultracondensed mitochondria,

with a reduced inner membrane potential, in apoptotic monocytes. *Am. J. Path*., **155**, 607–618.

Donnelly, E.T, Lewis, S.E.M., McNally, J. and Thompson, W. *(*1998) *In vitro* fertilization and pregnancy rates: the influence of sperm motility and morphology on IVF outcome. *Fertil. Steril*., **70**, 305–314.

Evenson, D.P., Darzynkiewicz, Z., and Melamed, M.R (1982) Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motolity. *J. Histochem. Cytochem*., **30**, 279–280.

Ford, W.C.L. and Harrison, A. (1981) The role of oxidative phosphorylation in the generation of ATP in human spermatozoa. *J. Reprod. Fert.*, **63**, 271–278.

Ford, W.C.L. and Rees, J.M. (1990) The bioenergetics of mammalian sperm motility. In Gagnon, C. (ed.) *Controls of Sperm Motility: Biological and Clinical Aspects*. CRC Press, Boca Raton, FL, pp. 175–202.

Garner, D.L., Thomas, C.A., Joerg, H.W., DeJarnette, J.M. and Marshall, C.E. (1997) Flurometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biol. Reprod*., **57**, 1401–1406.

Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science*, **281**, 1309–1312.

Hall., J.A., Fishel, S.B., Timson, JA., Dowell, K. and Klentzeris, LD. (1995) Human sperm morphology evaluation pre- and post-Percoll gradient centrifugation. *Hum. Reprod*., **10**, 342–346.

Henry, M.A., Noiles, E.E., Gao, D., Mazur, P. and Critser, J.K. (1993) Cryopreservation of human spermatozoa. IV. The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity, and mitochondrial function. *Fertil Steril*., **60**, 911–918.

Holt, W.V. (1997) Alternative strategies for the long-term preservation of spermatozoa. *Reprod. Fertil. Dev*., **9**, 309–319.

Holt, V.W., Morris, G.J., Coulson, G. and North RD (1988) Direct observation of cold-shock effects in ram spermatozoa with the use of a programmable cryomicroscope. *J. Exp. Zool*., **246**, 305–314.

Kao, S.H., Chao, H.T. and Wei, Y.H. (1998) Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa. *Mol. Hum. Reprod*., **4**, 657–666.

Kruger, T.F., Menkveld, R., Stander, F.S.H. and Lomband, C.J. (1986) Sperm morphological features as a prognostic factor in IVF. *Fertil. Steril*., **46**, 1118–1123.

Leffler, K.S. and Walters, C.A. (1996) A comparison of time, temperature, and freezing variables on frozen sperm motility recovery. *Fertil. Steril*., **65**, 282–284.

Mahadevan, M.M., Miller, M.M., and Moutos, D.M. (1997) Absence of glucose decreases human fertilization and sperm movement characteristic *in vitro. Hum. Reprod*., **12**, 119–123.

Mortimer, D. (1994) *Practical Laboratory Andrology.* Oxford University Press, Oxford.

Petersson, R.N. and Freund, M. (1970) ATP synthesis and oxidative phosphorylation metabolism in human spermatozoa. *Biol. Reprod.*, **3**, 47–54.

Salvioli, S., Ardizzuni, A., Franceschi, C. and Cossarizza, A. (1997) JC-1, but not $DIOC_6$ or rhodamine 123 is a reliable fluorescent probe to assess mitochondrial membrane potential changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett*., **411**, 77–82.

Sharma, R.K and Agarwal, A. (1996) Sperm quality improvement in cryopreserved human semen. *J. Urol.*, **156**, 1008–1012.

Storey, B.T. and Kayne, F.J. (1975) Energy metabolism of spermatozoa. The Embden–Myerhof pathway of glycolysis. *Fertil. Steril.*, **26**, 1257–1265.

Sun, X.M., MacFarlane, M., Zhuang, J., Wolf, B.B., Green, D.R. and Cohen, G.M. (1999) Distinct caspase cascades are initiated in receptors-mediated and chemical-induced apoptosis. *J. Biol. Chem*., **274**, 5053–5060.

Tournaye, H. (1999) Surgical sperm recovery for intracytoplasmic sperm injection: which method is to be preferred? *Hum. Reprod*., **14**, 71–81.

Tucker, M.J., Ahula, K., Stevens, P.A. and Craft, I.L. (1986) Cryopreservation of human spermatozoa: an assessment of methodology using rhodamine 123. *Arch. Androl*., **17**, 179–187.

Vetter, C.M., Miller, J.E., Crawford, L.M., Armstrong, M.J., Clair, J.H., Conner, M.W., Wise, D.L. and Skopek, T.R. (1998) Comparison of motility and membrane integrity to assess rat sperm viability. *Reprod. Toxicol*., **12**, 105–114.

Watson, P.F. (1995) Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod. Fertil. Dev*., **7**, 871–891.

Windsor, D.P. and White, I.G. (1993) Assessment of ram sperm mitochondrial function by quantitative determination of sperm rhodamine 123 accumulation. *Mol. Reprod. Develop*., **36**, 354–360.

Wooley, D.M. and Richardson, D.W. (1978) Ultrastructural injury to human spermatozoa after freezing and thawing. *J. Reprod. Fert*., **53**, 389–394.

World Health Organization (1992) *WHO Laboratory Manual for the Evaluation of Human Semen and Semen–Cervical Mucus Interaction*, 3rd edn, Cambridge University Press, Cambridge.

World Health Organization (1999) *WHO Laboratory Manual for the Evaluation of Human Semen and Semen–Cervical Mucus Interaction*, 4th edn, Cambridge University Press, Cambridge.

Zamboni, L. (1982) The ultrastructure pathology of the spermatozoon as a cause of infertility—the role of the electron-microscopy in the evaluation of semen quality. *Fertil. Steril.*, **48**, 711–734.

Submitted on October 10, 2000; resubmitted on March 14, 2001; accepted on November 6, 2001