# Sperm preparation prior to freezing and storage: A risk reduction practice which also improves post thaw sperm motility.

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## Introduction

It is suggested that IVF and sperm storage centres take a pragmatic approach to cryopreservation/storage safety and in the absence of hard data to support any one method over another, take any sensible precaution which is likely to minimise risk. Indeed, the Human Fertilisation and Embryology Authority (HFEA) have now stipulated that all centres screen patients for Hepatitis B and C and HIV prior to storage as part of a risk-reduction strategy. Although of some benefit, the effect of this may unfortunately be quite limited, especially as full quarantine (6 months for hepatitis) is impractical and because other pathogens may well come to light in the future, which cannot be screened for. For these reasons, a more comprehensive 'risk management' strategy has been suggested by a number of authors (Clarke 1999; Tomlinson and Sakkas, 2000), including:

- Screening for viral disease;
- Storage in the vapour phase of nitrogen
- Storage in appropriate plastics
- Preparation of sperm prior to storage

The latter point in this strategy forms the focus of the present study.

Preparation of sperm prior to freezing confers following potential benefits: 1. Reduction of viral load in stored samples of seropositive patients (Kim et al, 1999); 2. Reduced embryologist workload when treating patients with frozen samples, 3. Allows storage of smaller, more concentrated (therefore higher quality) volumes and, 4. Allows manipulation of sperm function to improve post-thaw survival.

Although common in the USA with 'IUI-ready' donor sperm samples, few units in the UK currently follow this practice, partly due to concerns with post-thaw quality and the initial extra work involved. This study was designed to examine whether such concerns were justified or that perhaps sperm preparation prior to freezing may represent a significant improvement.

## Experimental Methods

### Samples

Patient samples were obtained from the Diagnostic andrology service, ACU, Birmingham Women's Hospital. All samples had undergone full semen analysis and had been produced between 2 and 4 hours before processing. All samples were split and subjected to divergent processing methods.

### Sample Processing and Evaluation

Semen quality was evaluated according to World Health Organisation (WHO, 1999), although motility was analysed using manual and computerised methods (Hamilton Thorn IVOS version 10.9). All sperm were prepared by density gradient centrifugation (DGC) using Pureperm™ (Nidacon, Gothenburg, Sweden). Initially 29 samples were compared using manual semen analysis methods followed by 14 samples analysed using CASA. Controlled rate freezing was used for all experiments. A further 26 samples were prepared by DGC and incubated in capacitating conditions for up to 2 hours prior to freezing to determine whether capacitation enable sperm to better survive the freeze-thaw process. Capacitation was confirmed by the detection of tyrosine phosphorylation and the observation of hyperactivated motility.

## Experiment 1.

1.0mL of patient sample was halved and processed as follows

**1a. Preparation post freeze**

- 0.5ml was taken and 0.5mL cryoprotectant (Cryoprotec. Nidacon,) added
- Cooled using a controlled rate freezer and immersed in liquid nitrogen
- Thawed at 37°C and loaded onto a PureSperm gradient
- Washed (600g, 5 mins) and resuspended in 0.5mL PureWash (Nidacon)

**1b. Preparation pre-freeze**

- 0.5ml was taken and loaded onto a PureSperm gradient
- After centrifugation, the pellet was resuspended in 0.5mL PureWash and 0.5mL cryoprotectant was slowly added
- Cooled using a controlled rate freezer and immersed in liquid nitrogen
- Thawed at 37°C, wash buffer diluted in slowly
- washed (600g, 5 mins) and resuspended in 0.5mL PureWash

**Cooling protocol**

- 2°C/minute to -5°C
- 8°C/minute to -90°C
- plunge in liquid nitrogen @ -196°C

Total program time = 26.6minutes

## Experiment 2.

**Incubation under capacitating conditions**

- 1.5ml of sample was loaded onto a PureSperm gradient
- After centrifugation, the pellet was resuspended in 1.5mL PureWash
- 1.5ml was divided into 3x0.5ml aliquots
- Aliquot 1. 0.5mLcryoprotectant was slowly added and the sample was frozen
- Aliquot 2. Was incubated for 1 hour (37°C 5% CO2 in air), then 0.5mLcryoprotectant was slowly added and the sample was frozen
- Aliquot 3 was incubated for 2 hours and frozen in the same way
- Each aliquot was then thawed and semen parameters assessed as described previously.

## Experiment 3.

**Staining for Phosphotyrosine Residues**

- An aliquot was removed from each sample in expt 2. And 10µl sample placed on multiwell slides (Hendley, Essex, UK) containing approximately 50,000 sperm.
- Slides were left to air-dry and fixed in 100% methanol for 30 minutes
- Sperm were stained for phosphotyrosine residues using the immunocytochemical method described by Brewis et al (1998).
- Fluorescence patterns on the sperm were assessed by epifluorescence UV microscopy (Olympus BX-40) at 492 nm wavelength at x100 magnification.

## Results

Samples prepared prior to freezing demonstrated significantly improved motility than those frozen and subsequently prepared (figure 1). Post thaw motile concentration was better in the group assessed manually (0.9 v 2.6 millions/ml, p<0.001) or by computer assisted methods (0.6 v1.6 millions/ml, p=0.019).

Surprisingly, samples prepared and then incubated for 2 hours gave significantly better post-thaw returns when compared to those not incubated (2.9 v 4 millions/ml p=0.012, figure 2.). The presence of hyperactivated sperm and phosphotyrosine residues suggests that sperm were indeed becoming capacitated (figure 3.). However, there were relatively few sperm with positive staining and no significant difference between any of the time points.

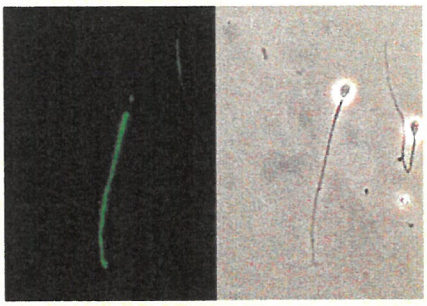
Figure 1. Motile sperm concentration (assessed manually and by CASA) in samples frozen in seminal plasma, thawed and prepared (freeze-prep) compared with those prepared prior to freezing (prep-freeze)

| | Freeze - prep | Prep - freeze | Freeze - prep | Prep - freeze |
|---|---|---|---|---|
| | Manual | | CASA | |
| N | 29 | 29 | 14 | 14 |
| Mean | .92 | 2.6 | .59 | 1.6 |
| Std. Error | .2712 | .6187 | .2297 | .4849 |
| Sig | p<0.001 | | p=0.019 | |

Figure 2. Prepared sperm thawed, and incubated for 2 hours in capacitating conditions

| Incubation time | Post prep | T0 | T1 | T2 |
|---|---|---|---|---|
| | | post thaw motile concentration | | |
| N | 29 | 26 | 26 | 26 |
| Mean | 7.99 | 2.9 | 3.3 | 4.04 |
| Std. Error | 1.06 | .57 | .54 | .79 |
| Sig | | N/A | 0.089 | 0.012 |

Figure 3. (A) Immunofluorescent localisation of tail phosphotyrosine residues in human spermatozoa (t=2) and (B) corresponding phase contrast photomicrographs of A



## Discussion

In order to make changes to existing procedures, adequate sperm survival and function (post thaw) must be demonstrated in samples pre-prepared using DGC techniques. The experiments described above suggest that freezing prepared sperm is superior to freezing in seminal plasma. Relatively low returns (in terms of motile concentration) were most probably due to the use of patient samples, which had been produced often several hours before processing. There is obvious scope for refinement of the technique. It is likely that subtle changes to cryoprotectant constituents such as glycerol, sucrose and serum albumin concentrations may confer additional benefit. Current protocols, which involve the dilution of semen (1:1) with cryoprotectant, gives us a final glycerol concentration of 7.5% and HSA 0.4%. Recent work using sperm, washed free of seminal plasma or prepared using DGC, suggests that cryosurvival improves by reducing the final glycerol concentration to 6%, raising the HSA concentration to 1% and adding 4% sucrose (Larson et al, 1997).

It appears that the manipulation of the protocol perhaps to induce sperm capacitation may further enhance our freezing process. Capacitation is known to be linked to the loss of sperm membrane cholesterol with a concomitant increase in membrane fluidity (Visconti et al, 1998). Giraud and co-workers measured membrane fluidity using fluorescence polarization techniques and demonstrated that membrane fluidity pre-freeze, is tightly correlated to post thaw survival in sperm (Giraud et al (2000). The freeze/thaw process appears to result in a rigidifying effect on the sperm membrane and suggest that sperm adaptability to this phenomenon is what ultimately governs their survival. The present study demonstrated the presence of hyperactivated sperm and phosphotyrosine residues suggesting that sperm were becoming capacitated. However with relatively low numbers of positive sperm, it was perhaps not surprising that there was no significant difference between any of the time points. Past and present dogma pushes most laboratories toward freezing the sample as soon as it is physically possible. In fact the best approach may be to remove seminal plasma and allow the sperm plasma membrane to assume a degree of flexibility before freezing. Further experiments are required to determine whether or not this is the case

Protocol manipulation of this type coupled to developments in cryoprotectant, cooling protocols and the use of plastics, which permit more efficient heat exchange, may represent real improvements in our clinical practice. There are clear advantages for all patients and in particular those with poorer sperm quality in whom cryosurvival is notoriously low e.g. sperm banking cancer patients.

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