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DEBATE

Is a review of standard procedures for cryopreservation needed?

Safe and effective cryopreservation—should sperm banks and fertility centres move toward storage in nitrogen vapour?

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A recent consultation documentation by the Human Fertilisation and Embryology Authority (HFEA) which focused on the safe cryopreservation of gametes and embryos highlighted the need for a review of the way that fertility clinics in the UK store potentially infective material. The main points for consideration were to: (i) ensure containers used for cryopreservation are guaranteed by manufacturers to withstand low temperatures; (ii) use secondary containers, i.e. 'double bagging' of samples if stored in the liquid phase; and (iii) store in nitrogen vapour as a 'safer' alternative. In this article we examine a number of issues related to vapour storage which need careful consideration, including safety, cost and the effectiveness of various storage techniques in maintaining gamete and embryo viability. We also discuss the effectiveness of vapour storage in comparison with current liquid nitrogen storage techniques. In conclusion, we propose that fertility clinics should be compelled to review their cryopreservation procedures, not just because of new legislation or indeed fear of litigation but by a moral obligation.

Key words: cryopreservation/nitrogen vapour/safety/sperm banks

Introduction

A recent consultation documentation by the Human Fertilisation and Embryology Authority (HFEA) focusing on the safe cryopreservation of gametes and embryos (HFEA, 1998) highlighted the need for a review of the way that fertility clinics in the UK store potentially infective material. Publication of the document was driven largely by Department of Health guidelines (National Health Service Executive, 1997) issued after several cases of hepatitis B transmission from a leaking blood bag, in patients receiving autologous bone marrow transplantation (Tedder *et al*, 1995). As a result the Department of Health set up an advisory group to develop specific policies for the processing and storage of bone marrow stem cells, with particular emphasis on quality control and good laboratory practice. The main points for consideration were to: (i) ensure containers used for cryopreservation are guaranteed by manufacturers to withstand low temperatures; (ii) use secondary containers, i.e. 'double bagging' of samples if stored in the liquid phase; and (iii) store in nitrogen vapour as a 'safer' alternative.

Although primarily aimed at bone marrow stem cells, the document suggested that similar principles should apply to other cryopreserved cells and tissues, and indeed the HFEA working group recommended that the new code of practice address this specifically for gametes and embryos. Within the Blood Transfusion Service there has been a drive towards liquid nitrogen vapour storage as the 'safer' alternative, however, members of the assisted reproductive technology (ART) community are understandably keen to avoid a 'knee-jerk' reaction and follow suit. There are obviously a number of issues related to vapour storage, which need careful consideration:

Safety

At present there is no direct evidence of cross-contamination in a cryobank within a fertility clinic setting. There is however evidence of the presence of the hepatitis C virus in different ejaculates of the same semen donor (Mckee *et al*., 1996) and these are known to survive in the liquid phase. The safe cryopreservation of infected samples from infertile hepatitis C virus male patients has previously been highlighted (Massey *et al*., 1996).

Cost

The smallest automated nitrogen vapour vessels cost more than £5000 (UD$7660). In addition, many units with smaller storage facilities would find it difficult to make use of the relatively large capacity offered by the vapour storage systems currently available, an inefficient and expensive use of freezer space. Adaptation of current liquid nitrogen vessels would significantly affect storage capacity.

Viability of spermatozoa and embryos

With the existence of temperature gradients within vapour storage vessels, there is a general concern that viability of spermatozoa and embryos after nitrogen vapour storage would not be comparable with that of storage in the liquid phase.

The aim of this article is to discuss these issues one by one.

We use the small amount of experience we have had with vapour storage in our clinic, discussing the advantages and potential pitfalls. We discuss the reasons for changing our procedures and specifically address the issues of safety, quality control, cost and cell viability.

The sperm bank

Clinics with successful donor programmes and large numbers of oncology referrals are all aware how quickly storage space in vessels can quickly diminish. Liquid phase sperm banks based on medium sized vessels, e.g Taylor Wharton HC35, Statebourne Bio 35 soon reach full capacity and can very quickly occupy most of the floor space in the cryostore. Weekly vessel filling then becomes a mammoth task. Alarms on vessels should now be mandatory. Whether sensors are in place to detect low nitrogen levels or rising temperatures, in an age where expectations of high standards are increasing and andrology laboratory accreditation is fast approaching, every quality control measure possible must be taken. 'Retro-fitting' of low-level alarms is possible but can be technically difficult and usually involves re-modelling of vessel lids to provide access for probes.

In our own cryostore, at least four vessels were in need of replacement. Coupled to the high cost of fitting alarms to a further six, it was decided to replace the entire system with two vapour cryostores (Taylor Wharton 10K), supplied by a single 240 l vessel using hoses connected on a T-piece. The capacity of each, depending on the inventory design is 5000–8000 1.2 ml vials or 32 000 0.75 ml straws. Our inventory was designed using combinations of towers, containing vial boxes and canisters, which can take either straws in goblets or vials on canes. Although this does not necessarily make the best use of freezer space, it was necessary to accommodate 16 000 existing patient straws as well as cryovials. Moving the entire 'bank' also provides a further opportunity for audit, although this can significantly lengthen the process of transferring samples from liquid to the vapour freezers. If this is planned, then thought should be given to liquid nitrogen consumption, as the exercise increased nitrogen consumption by almost 90%. This figure has reduced since completion to around 50%, roughly 150 l per week.

Apart from the obvious increase in capacity, several integral features of an automated system soon become apparent, all of which lend themselves to quality control and help to reduce the chances of loss or damage to valuable biological material, due to human error. They include autofilling (a fill cycle commences when the liquid nitrogen level is unable to maintain the temperature under the lid at –140°C or below, or when the lid is replaced for fast temperature recovery) and alarms (generation of local and remote alarms for high temperatures, lid open, fault finding, over/underfilling). Data logging of all events, including filling activity, temperature, nitrogen levels and alarms. All logged events can be printed using a chart recorder or 'down-loaded' to a computer.

Although extremely useful, total reliance on automation would be foolish. Auto-fill systems, for example are a potential hazard. If moisture is allowed into the nitrogen supply hoses, ice can accumulate and migrate to the solenoid-valves which control the flow of nitrogen. In a worst case scenario, the valve can be 'frozen open' allowing a constant flow of nitrogen, quickly emptying the supply vessel and turning the vapour phase freezer into an 'overflowing liquid' phase freezer. Therefore, a secondary 'back-up' solenoid should be fitted to any auto-fill system. Additional operating procedures need to be in place to ensure that the freezers are performing as they should. Procedures for regular checking of the controller settings, independent temperature monitoring and regular servicing are essential.

Safety of vapour storage

Cross contamination incidents are thankfully rare and indeed have not as yet been reported from a cryobank within an infertility clinic/sperm bank. However, how can we be sure that cross contamination has not already occurred? The majority of sperm storage facilities do not routinely screen, e.g. oncology and vasectomy patients, prior to storage. Even if they did, it would be extremely difficult to provide adequate quarantine facilities or indeed cover a suitably broad spectrum of pathogens. As new pathogens are now being discovered with great regularity, it would be unwise to rely on screening of only those considered most dangerous at any given time. The incubation period of human inmmunodeficiency virus (HIV) and hepatitis present additional problems. What if sero-conversion occurs during quarantine? Are samples then disposed of? The only practical solution would be that each quarantine vessel would be limited to samples from a single patient. In our clinic, this would require in the region of 60 separate vessels to cover 6 months of quarantine, a clearly impractical solution. In addition, it has already been suggested that a considerable source of contamination of storage facilities is the liquid nitrogen itself (Fountain *et al.*, 1997). Clearly, screening of the liquid nitrogen supply is not possible.

There is an obvious difference in risk between viral transmission from blood products (which are then transfused) and transmission by insemination/ART. From a European cohort, the risk of HIV infection from a single act of unprotected intercourse has been calculated at 2 in 1000 (de Vincenzi, 1994) and it remains unclear as to whether or not sexual transmission is a major route of the spread of hepatitis C (Semprini *et al.*, 1998; Neumayr *et al.*, 1999; Wejstal, 1999). If we also bear in mind that sperm preparation for use in ART significantly lowers the viral load of the inseminate (Kim *et al.*, 1999; A.Semprini, personal communication), and that there is a relatively low uptake/usage of stored patient samples (7–10%), it could take many years and thousands of inseminations before an incident of cross-contamination in a sperm bank becomes apparent.

The fact that a cross-contamination incident has not yet occurred may well have provided us with a false sense of security. Clearly, we must assume that such an incident is possible and must take as many practical steps as we can to prevent transmission to a patient. Is vapour storage therefore inherently safer than storage in liquid? It would seem so, as a vector for viral transmission cannot be identified. The honest

answer however is that we cannot say for sure. Fountain *et al*. (1997) demonstrated growth of a number of skin and other environmental micro-organisms from both liquid and vapour vessels. Although more species were grown from liquid, potentially pathogenic *Aspergillus* spp. were commonly found in the vapour vessels. However, when swabs from blood bags were cultured, transmission in vapour could not be demonstrated, even after a further 2 weeks of culture. The suggestion that liquid is a more effective transport medium for infectious material is not incredible. Any individual who has had the misfortune of having to empty and clean a liquid nitrogen vessel will be well aware of the veritable 'biological soup' or detritus at the bottom. Storage in the gaseous phase may well eliminate much of this obvious risk and understandably is now standard practice in the Blood Transfusion Service.

Other safety measures

Assessing the risk of cross-infection in a liquid nitrogen storage vessel or indeed from a vapour vessel, is almost impossible, therefore common sense must prevail. We have to take every available practical step to reduce the risk of transmission. A recent article (Clarke, 1999) described a number of failings in some of the practices currently used in sperm cryopreservation and put forward some simple yet sensible suggestions to improve on them. The areas highlighted in his paper should be viewed as 'good practice' and considered alongside storage in the gaseous phase. For example: straws should be manufactured from material which will not shatter after being subjected to ultra-low temperatures; filling and sealing protocols should be reviewed, in particular the use of polyvinyl alcohol powder, which is both an ineffective sealant and a potential source of contamination; vials should not be used in liquid nitrogen unless used with a second skin e.g. cryoflex or used with lids which contract and expand at the same rate. New straws are now available with filling and sealing protocols that should reduce the risk of contamination, for example the I.M.V. Cryobiosystem. To add to these recommendations, we could add that treatments performed using stored spermatozoa should involve a preparation step using density gradients and sperm washing techniques to reduce a potential viral load of the sample (Kim *et al.*, 1999; Levy *et al*., 2000). Simple intracervical inseminations, which therefore potentially carry a higher risk of transmission, should perhaps be avoided. A similar precaution could be taken prior to freezing, aimed at reducing the viral load of the sperm tank.

Is sperm and embryo viability maintained in vapour storage?

Undoubtedly, one major consideration for laboratories considering a move towards vapour storage might be sperm survival. Depending on exactly what system is in place, a rise in temperature to anywhere between –160°C and –190°C may be expected. Although short term storage is possible at relatively low temperatures, e.g. –79°C, theoretically, long-term storage viability will only be maintained providing the temperature remains below –130°C, that of the so-called 'glassy transformation temperature' (Meryman, 1963; Clarke, 1999). Maintenance of ultra-low temperatures within the vapour freezer appears to relate to the design and material used in the storage inventory (Rowley and Byrne, 1992; M.J.Tomlinson, unpublished observations). If the inventory is made from materials of high conductance, packed tightly together, then temperature near the top of the inventory remains extremely cool. Indeed temperature probing of our aluminium canisters and towers using a thermocouple has shown excellent conductivity, with temperatures between –160 to –180°C recorded at the top. Temperature sensors of the automated freezer are placed below the lid and rarely show temperatures higher than –160°C. Newer vapour freezers appearing on the market have a 'nitrogen liquid jacket' which extends to the top of and completely surrounds a dry vessel (CBS vapour freezer; phiTec International, Milton Keynes, UK) thereby giving liquid temperatures throughout a vapour vessel. Similarly low temperatures seem to be achievable with relatively low-tech/low cost equipment. Clarke (1999) recently described similarly achievable temperatures using existing liquid vessels with only a 15 cm depth of nitrogen (Clarke, 1999). Their system has been used for some 3 years, and clinical results have been comparable to those using sperm storage in liquid.

Table I. Pregnancies from insemination using donor spermatozoa stored in either liquid nitrogen or in nitrogen vapour

	Liquid nitrogen	Nitrogen vapour
Treatment cycles	89	57
No. (%) pregnancies per cycle	19 (21)	12 (21)

Vapour storage has been in use in our clinic for >2 years and we have attempted to monitor performance in that time using a number of simple parameters. Firstly, to examine very short term storage, post-thaw results from donor ejaculates (n = 40) split between liquid and vapour storage were examined. Perhaps, understandably, 'short-term' viability of spermatozoa was unaffected with no differences observed between groups with respect to percentage reduction on motility (36% liquid compared with 39% vapour). This is not perhaps surprising since samples can be adequately stored for up to 3 weeks in a dry shipping vessel. Examination of the clinic's donor insemination results as yet has not revealed any adverse affects of storage in vapour (Table I). We have also examined the viability of mouse embryos in vapour storage. 1-cell mouse embryos (n = 30) were kept in storage for 1 year; 25 survived thawing and they were all cultured to the blastocyst stage. Out of the 25 that survived, 23 formed normal blastocysts (92%), comparable with any results obtained from liquid nitrogen storage (Shaw *et al*., 1991).

Discussion

The 'fertility world' should adopt a common sense approach, pre-empting the kind of problems faced by the Blood Transfusion Service with the above incident. A review of

current standard operating procedures to gradually incorporate at least some of the suggestions put forward by the Department of Health would be a sensible preventative measure. Technical advances, for example in straw/vial material, filling and sealing, and inventory design are all relatively low cost and simple to implement. Whether patient screening and quarantine will ever provide adequate security is a contentious issue. The dilemma we are faced with is the following: Is the lack of concrete clinical evidence to show that vapour storage is inherently safer than liquid, sufficient reason not to move toward it? As suggested by Clarke (1999), the risk of cross-infection in either is probably unquantifiable. Clinical results so far in our clinic, as well as in others (Clarke, 1999), are promising, and theoretically, sperm and embryo survival should be no different to that of liquid phase storage. We owe it to our patients to optimize all of our procedures. To the individuals concerned, stored gametes and embryos are priceless, therefore centres, not just in the UK but across the world, should be compelled to review their cryopreservation procedures, not just because of new legislation or indeed fear of litigation but by a moral obligation.

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