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OPINION

Sperm cryopreservation: is there a significant risk of cross-contamination?

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Interest in the subject of sperm cryopreservation has a long history, with thousands of published articles in the scientific/medical literature. Clinical pregnancy rates using donor insemination can now approach natural pregnancy rates (Clarke *et al*., 1997b). However, an issue which has received very little attention in the literature is the subject of microbiological cross-contamination in sperm banks. This subject obviously concerns many sperm bank managers, who have instituted several approaches aimed at reducing the perceived risks. One approach is to screen all patients or donors for human immunodeficiency virus (HIV) status, hepatitis B and C and syphilis prior to cryopreservation of their semen. Another approach involves the use of quarantine tanks to hold samples until screening results are obtained, before placing the cleared samples into the main storage system. The samples of patients with positive screening results are then transferred to specially designated tanks. Both of these approaches have obvious drawbacks because of the incubation period of HIV and the fact that the screening procedures do not cover all potential pathogens. This issue therefore needs to be addressed from another perspective in order to estimate the likelihood of its occurrence and to determine what measures, if any, might be considered appropriate to prevent or minimize it.

The occurrence of cross-contamination during liquid nitrogen storage of biological material and subsequent cross-infection of patients has previously been demonstrated (Tedder *et al*., 1995). They reported on a cluster of hepatitis B infection which was definitively traced to cross-contamination during bone marrow storage using nucleotide sequence analysis (Hawkins *et al*. 1996). Other viruses have previously been found to survive direct exposure to liquid nitrogen, including vesicular stomatitis virus (Schafer *et al*., 1976), herpes simplex virus, adenovirus (Jones and Darville, 1989), and papillomavirus (Goodman, 1960; Charles and Sir, 1971). There is also evidence of contamination of liquid nitrogen by other microorganisms, including a wide range of bacterial and fungal species (Fountain *et al*., 1997). In the latter laboratory, one liquid nitrogen tank was found to be heavily contaminated by an *Aspergillus* species which is a potential pathogen. These authors reported that 1–2% of thawed bone-marrow samples were contaminated during the cryostorage process, probably from the contaminated liquid nitrogen. Thus, there is no doubt that a range of microbes, including hepatitis B, can survive direct exposure to liquid nitrogen, and under certain conditions result in cross-infection. Given the strength of the evidence of liquid nitrogen contamination by microbes and cross-infection in certain situations, we felt that the possibility of contamination or cross-contamination during semen cryopreservation should be taken seriously.

Traditionally, semen storage protocols have not involved sterile techniques. In particular, filling of straws by the 'dip and wipe' procedure (Russell *et al*., 1997), or cracking of overfilled straws during freezing would have significant potential to contaminate the liquid nitrogen. Straws which were inadequately sealed could absorb the contaminated nitrogen, leading to a potential cross-infection episode during subsequent clinical use of the thawed semen. There is an additional risk of cross-contamination during semen processing prior to freezing in those laboratories which use containers of polyvinylalcohol (PVA) sealing powder for multiple patients or donors. The PVA powder could accumulate microbes from a number of individuals, which are then introduced to the inside of straws of another patient or donor. The storage of semen in cryovials placed in direct contact with liquid nitrogen also presents a serious risk because a significant proportion of cryovials absorb liquid nitrogen through caps which do not maintain their seal under these conditions. Although the manufacturer strongly recommends the use of a second 'skin' called Cryoflex (Product No. 343958; Nunc Nalge International, Roskilde, Denmark) to provide an adequate seal, it is common practice to store naked vials in liquid nitrogen. Recent tests conducted in my laboratory showed that 45% of Cryovials (Product No. 340711; Nunc Nalge International) without an O-ring and 85% of Iwaki Cryovials (Iwaki, Japan) with an O-ring absorbed liquid nitrogen during 3 h immersion in liquid nitrogen. In contrast, there was no evidence of liquid nitrogen condensation inside the vials when stored in the vapour phase for 16–24 h. Thus, in some instances, cryovials stored in the liquid phase could absorb up to 1 ml of potentially contaminated liquid nitrogen. Depending on the length of storage and the exact thawing protocol, any microbes in the absorbed nitrogen may have settled onto the semen interface and be left there when the nitrogen boiled off. Clinical use of this semen, particularly when multiple vials may be inseminated during a treatment cycle, could pose an increased risk of cross-infection. Some diseases such as hepatitis B require very small amounts of virus to transmit infection.

Consideration of the evidence presented above of liquid nitrogen contamination by microbes, and the common use of cryovials which absorb liquid nitrogen, leads to the conclusion that cross-infection via the clinical use of cryopreserved semen is a realistic possibility. As in any other area of clinical medicine, a significant risk of cross-infection should be taken seriously and if possible, steps taken to minimize it. Fortunately there are a number of relatively simple details and possible changes to cryopreservation procedures which would minimize the potential for contamination or cross-contamination of stored sperm samples.

Sealing of straws or vials

Adequate sealing is obviously vital. Straws can be heat-sealed at both ends, or extra attention paid to PVA powder sealing. Thus, it is important to draw the semen–cryoprotectant mixture through the inner cotton plug so that it contacts and sets the PVA powder between the inner and outer cotton plugs. At the opposite end of the straw it is important to leave an air-gap of at least 1 cm to allow for expansion during freezing, with the PVA powder set by dipping the straw end in water for several minutes. A new straw system, Cryo Bio System (CBS; http:II cryobiol.system-IMV.com) produced by IMV Technologies (L'Aigle, France) will also minimize the possibility of contamination because it is made from a shatter-proof ionomeric resin, uses a loading manifold to prevent contact between the straw and semen and allows for heat sealing at both ends. Microbiological tests on the CBS straws suggested that they provided a highly efficient seal against entry or egress of micro-organisms. These tests were conducted or supervised by Dr Bernard Guerin (Laboratoire Pour Le Controle Des Reproducteurs, Ministere de L'Agriculture, BP65.13, rue Jouet-94703 Maisons-Alfort Cedex, France). In contrast, cryovials definitely do not maintain their seal under liquid nitrogen – they should be stored inside a second skin such as Cryoflex (Nalge Nunc International) or stored in the vapour phase only, as discussed below.

Liquid nitrogen vapour storage

Previous studies have shown that human semen can be stored at temperatures as high as –79°C for up to 1 year while retaining motility and fertilizing capacity. Thus, one author (Sawada, 1964) reported 11 pregnancies after 230 inseminations in 150 patients using semen stored for 2–315 days on dry ice. However, there was found to be a progressive decline in post-thaw motility of semen stored at –79°C, which accelerated at storage temperatures above –75°C (Ackerman, 1968). It was soon concluded that long-term storage required temperatures below –100°C, resulting in the general use of liquid nitrogen (–196°C) for virtually all human cell storage. Liquid nitrogen vapour storage was apparently not considered as a realistic alternative at that time. From a theoretical perspective though, it is likely that relatively long-term storage should be possible at temperatures below –130°C (the glassy transformation temperature), beyond which ice-crystal growth and hence recrystallization is apparently impossible (Meryman, 1956).

In order to overcome the inconvenience of using Cryoflex and the problem of leaking vials if stored in the liquid phase, we have recently developed a one-step liquid nitrogen vapour cooling / storage system which gives on average, >50% motility recovery. This is a very simple system wherein the semen–cryoprotectant mixture is divided into aliquots in cryovials which are then put into capped polypropylene test tubes (Product No. 55.510; Sarstedt, Ingle Farm, South Australia). The test tubes are placed in the upper goblet of a liquid nitrogen tank (35HC; Taylor Wharton, Theodore, Alabama, USA) containing ~15 cm depth of liquid nitrogen, for both cooling and ongoing storage. The average cooling rates from room temperature to –190°C (liquid nitrogen vapour) ranged from 10–20°C/min depending on the number of vials per storage tube. The vials are thawed in air at 37°C. Initially, we had observed that the vapour temperature in a narrow-necked tank (35HC; Taylor Wharton Theodore) was quite stable at around –190°C when measured 12 times over a period of 3 months. This suggested that medium- to long-term storage in vapour was feasible. For laboratories using wide-necked storage tanks the use of aluminium racks or conductive rods (e.g. aluminium canes) could potentially minimize the temperature gradient so that the vapour temperature is around –190°C near the top of the storage space (Rowley and Byrne, 1992). The liquid nitrogen levels in tanks used for vapour storage should be measured several times per week, or liquid level alarms fitted, preferably with a remote dialling facility.

Considering the stable, low temperatures achievable with vapour storage and the early work using dry-ice storage, there was no *a priori* reason not to use this system. We have now used this procedure to store sperm samples for backup for intracytoplasmic sperm injection (ICSI) treatment for the last 2.5 years. During this period, spermatozoa from 70 patients have been stored and 21 of these patients have subsequently had cryopreserved spermatozoa used for ICSI. Of the 21 patients, nine used either testicular or epididymal spermatozoa. Overall, a normal fertilization rate of 56% was obtained, with 91% undergoing cleavage. Embryos were transferred to 20 patients in 37 cycles (approximately two embryos/cycle), resulting in six patients with raised β-human chorionic gonadotrophin (HCG) and four with clinical pregnancies by fetal heart (FH) detection (6.1% FH implantation rate per embryo transferred; 20% of patients pregnant). These results are equivalent to our overall ICSI results (Clarke *et al*., 1997a). We are now planning to extend the use of vapour storage to normal in-vitro fertilization (IVF) back-up and donor samples.

Other recommended precautions

When using PVA sealing powder, it is important to aliquot it into tubes which can be used for one patient only. Tamping straws from different patients into the same powder could result in cross-contamination (Russell *et al*., 1997).

On thawing for clinical use, any straws which are obviously cracked or have lost their seal at either end should be discarded. In addition, the outside of straws should be sterilized (e.g. by

hypochlorite) prior to cutting with sterile scissors or scalpel blade to extract semen for assisted reproductive technologies (ART).

Prior to freezing or clinical use, labelling on straws/vials should always be checked and records countersigned by a second staff member, or the patient.

Only one semen sample should be processed for cryopreservation at a time at a workstation separated from other biological samples (e.g. semen samples for analysis or blood for sperm antibody testing).

Conclusions

Considering the significant potential for cross-contamination during semen cryostorage using current methodology, and consequently the finite (but probably unquantifiable) risk of cross-infection during ART procedures, I would urge that sperm banks critically examine all of their procedures with the aim of eliminating this risk at the outset. Why continue with suboptimal procedures until a patient contracts a serious illness from cross-infection, as has occurred with bone-marrow storage? Quarantine tanks have some merit but, on their own, they are not an adequate solution because patients may be carrying viruses which have not been tested for.

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