

Safety of cryopreservation straws for human gametes or embryos: a preliminary study with human immunodeficiency virus-1

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The aim of this preliminary experimental study was to test the stability of cryopreservation straws to human immunodeficiency virus-1 (HIV-1). Three kinds of straws were tested: four polyvinyl chloride (PVC), four polyethylene terephthalate glycol (PETG) and 20 high-security ionomeric resin (IR). The PVC and PETG straws were sealed ultrasonically, and the IR straw by thermosoldering. Each sealed straw was cut in half to produce two demi-straws and then filled with 100 µl of HIV-1-containing supernatant (reverse transcriptase activity: 15 000 c.p.m./50 µl). The unsealed cotton end of PVC and PETG straws and the two halves of the IR straws (cotton and plastic plug ends) were tested. Each demi-straw was two-thirds submerged in RPMI medium at 37°C, and RPMI samples were withdrawn on days 3, 7 and 11. Viral RNA was extracted from the medium and then amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) followed by nested PCR using primers specific to HIV-1 protease. On day 7, no HIV-1 RNA was detected in any of the different samples of medium that had surrounded the unsealed PVC and PETG straws with cotton ends, but three IR specimens were positive. On day 11, PVC and PETG remained negative but HIV-1 RNA was detected in RPMI samples for two more IR demi-straws ($n = 5$). In conclusion, under these experimental conditions (at 37°C), the unsealed cotton end PVC, PETG and thermosoldered cotton end IR demi-straws appeared to be safe for HIV-1, while IR straws, sealed or unsealed with a plastic plug and with unsealed cotton ends, leaked.

Key words: assisted reproductive technology/cryopreservation/experimental study/HIV/straw

Introduction

The possibility of assisted reproductive technology for virus-carrying couples, especially those with human immuno-

deficiency virus-1 (HIV-1) (Olaitan *et al.*, 1996; Semprini *et al.*, 1997; Balet *et al.*, 1998; Marina *et al.*, 1998), led us to consider the safety of cryopreservation straws for human gametes or embryos frozen in liquid nitrogen tanks. Liquid nitrogen preserves the viruses and their potential activity (Tedder *et al.*, 1995). Indeed, Tedder *et al.* (1995) reported the transmission of hepatitis B virus from a contaminated liquid nitrogen tank. Furthermore, in practice we know that certain types of straws break in liquid nitrogen and thus release their contents. To ensure no contamination of the contents of the straws, their impermeability must be absolute and remains to be established. The possibility of cross-contamination of viral material between human semen samples during cryopreservation is thought to be very real (Bahadur and Tedder, 1997a). Only recently, a report from the Royal Veterinary College (London, UK) showed a relationship between leakage from plastic straws used for livestock semen cryopreservation and the method used to fill the straws (Russel *et al.*, 1997). However, at present, the permeability status of different types of straws is unknown, particularly those used routinely to cryopreserve human gametes or embryos (Janssens, 1997; Bahadur and Tedder, 1997b). Thus, it has been suggested that all patients' samples are quarantined in liquid nitrogen tanks for 6 months and that the entire contents of the tank are destroyed should a patient seroconvert during this period (Janssens, 1997). Should a study confirm the impermeability of all the straws, the risk of contaminating the contents of straws stored in liquid nitrogen and of their contaminating the latter would become negligible.

The aim of our experimental study was to use HIV-1 to test the impermeability of the three most common types of straws used for human gamete or embryo cryopreservation and currently stored in liquid nitrogen tanks in France.

Materials and methods

To test the impermeability of the straws, we postulated that the risk of straw leakage was similar in both directions, i.e. from outside to inside and *vice versa*. Therefore, we tested the risk of virus escape from straws. Straws (Cryobiosystem, l'Aigle, France) made of three different materials were tested: four polyvinyl chloride (PVC), four polyethylene terephthalate glycol (PETG), and 20 ionomeric resin (IR). PVC and PETG straws had an open end sealed ultrasonically and a cotton end ($n = 4$ for each) generally not sealed for current use. The IR straws ($n = 8$) also had an open end and either a cotton end ($n = 8$) or a plastic hydrophobic plug ($n = 8$). Generally both ends of IR straws were sealed by thermosoldering. In this experiment, however, the open ends of IR straws were sealed by thermosoldering, half of the other ends were sealed with cotton and half with plastic plugs. All straws were sealed empty.

Table I. Number of demi-straws used for permeability testing to HIV-1 virus

Material	Type of seal		Number	Tested
	End A	End B		
PVC	Ultrasonic	Open	4	No ^a
	Cotton	Open	4	Yes
PETG	Ultrasonic	Open	4	No ^a
	Cotton	Open	4	Yes
IR	Open	Thermosolder	4	Yes
	Open	Open	4	No ^b
	Cotton	Thermosolder	4	Yes
	Cotton	Cotton	4	Yes
	Open	Thermosolder	4	Yes
	Open	Open	4	No ^b
	Plastic	Thermosolder	4	Yes
	Plastic	Plastic	4	Yes

PVC = polyvinyl chloride; PETG = polythylene terephthalate glycol; IR = ionomeric resin.

^aNot tested because overflowed.

^bNot tested because unsealed and open.

Each sealed straw was cut into two equal halves and sealed according to the schedule shown in Table I. Each demi-straw was then filled with 100 µl of HIV-1-containing supernatant (reverse transcriptase activity: 15 000 c.p.m./50 µl). Unfortunately, because of the inability to displace air, every attempt to fill the PVC and PETG demi-straws with the open end sealed ultrasonically overflowed and contaminated the exterior of the straw; thus, only the demi-straws with the open end sealed with cotton were filled (Table I). The IR straws, because of their larger calibre, did not present any problem. The cotton end of each PVC and PETG demi-straw and both ends of the IR demi-straws were submerged two-thirds deep into a Falcon tube containing 2 ml of RPMI medium (Bio-Whittaker, Boehringer, Belgium) and placed in an incubator at 37°C under a 5% CO₂ atmosphere. A 1 ml sample of the surrounding RPMI was withdrawn on days 3, 7 and 11, each time being replaced with 1 ml of sterile RPMI medium, and frozen at -80°C until all samples were subjected to HIV-1 RNA detection simultaneously. As controls, one Falcon tube was filled with 100 µl of HIV-1-containing supernatant and 2 ml of RPMI medium, and another one was filled with 2 ml of RPMI medium. For testing, each sample was thawed and centrifuged for 1 h at 23 500 g. The pellet was resuspended in 200 µl of RPMI medium, and virus RNA was extracted, amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) followed by nested PCR (Titan RT-PCR kit, Boehringer, Mannheim, Germany), using primers specific to HIV-1 protease gene and loaded on 1% Agarose gel. Sterile water was used as the negative PCR control.

Results

No HIV-1 RNA was detected in any of the different samples drawn on day 7, from tubes containing the unsealed cotton end PVC and PETG demi-straws (Figure 1). However, HIV-1 was detected in samples from tubes containing IR demi-straws on day 7: one unsealed cotton end, one unsealed plastic plug end, and one sealed plastic plug end (Figure 2). On day 11, PVC and PETG results were the same as on day 7 (Figure 1); however, HIV-1 RNA was detected in the RPMI samples from two additional tubes, each containing the thermosoldered free end of IR demi-straws (Figure 3).

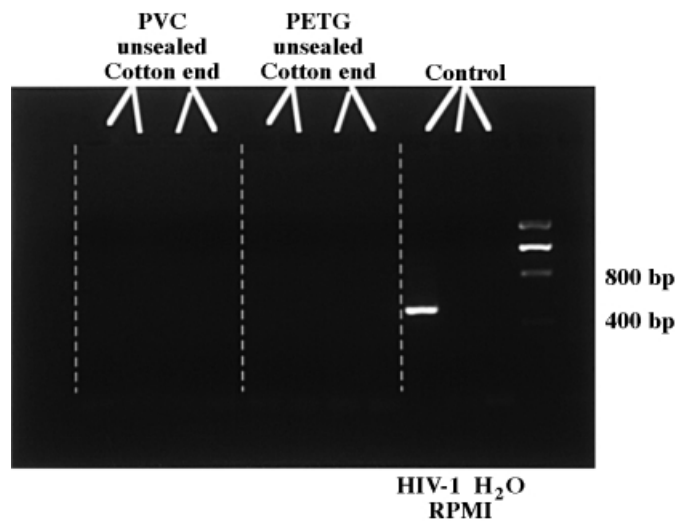


Figure 1. Viral risk and demi-straws (PVC and PETG) on days 7 and 11. White 'pointers' from left to right: four PVC demi-straws, unsealed cotton end testing; four PETG demi-straws, unsealed cotton end; three controls, HIV-1, RPMI and water.

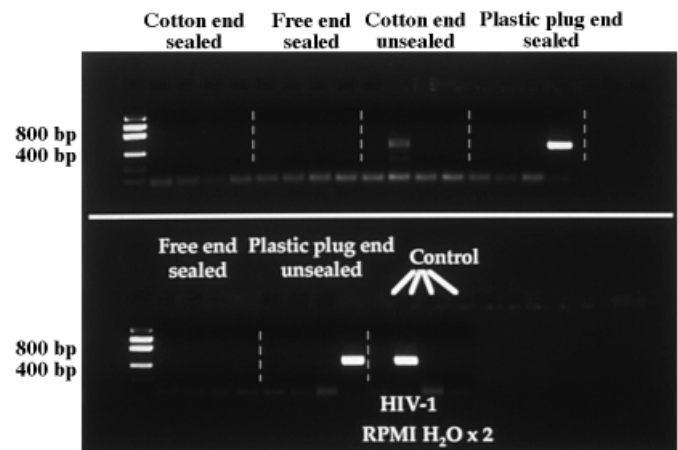


Figure 2. Viral risk and demi-straws (IR) on day 7. White 'pointers' from left to right on four controls: RPMI, HIV-1 and two water.

Discussion

Viral contamination of cryopreserved bone marrow samples from liquid nitrogen has been highlighted by the transmission of hepatitis B from virus-positive bone marrow to other negative samples (Tedder *et al.*, 1995). In other respects, the demonstration that HIV is present in seminal plasma cells (Zagury *et al.*, 1984; Stewart *et al.*, 1985; Dulioust *et al.*, 1998) indicated the possibility for HIV contamination of samples stored in cryopreservation tanks. The feasibility of separation and removal of the infective fraction of the ejaculates of HIV-positive men by gradient centrifugation and the absence of maternal seroconversion after more than 1000 intrauterine inseminations using these purified sperm fractions is reassuring (Semprini *et al.*, 1997; Marina *et al.*, 1998). However, doubt persists regarding infection of spermatozoa by HIV-1 (Bacetti *et al.*, 1994; Bagasra *et al.*, 1994; Nuovo *et al.*, 1994; Muciaccia *et al.*, 1998). Some authors also confirmed that HIV-1 remains in seminal fluid, despite antiretroviral therapy in 2–50% of

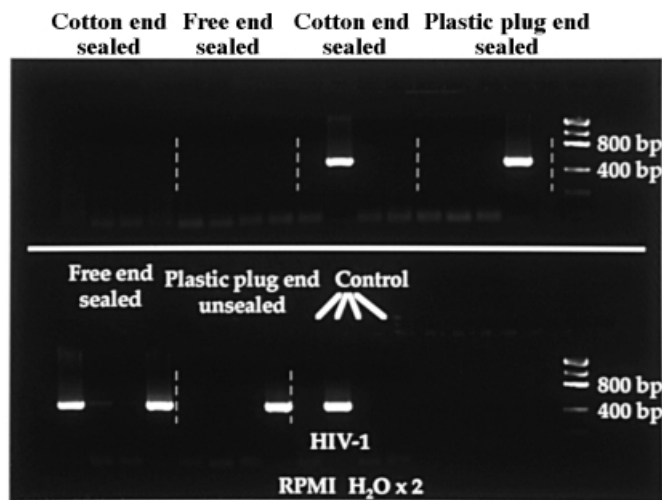


Figure 3. Viral risk and demi-straws (IR) on day 11. White 'pointers' from left to right on four controls: RPMI, HIV-1 and two water.

cases (Eron *et al.*, 1998; Luizzi *et al.*, 1998; Vernazza *et al.*, 1998; Zhang *et al.*, 1998). All these findings justified the current examination of the real safety of straws used for cryopreservation of human gametes and embryos.

Our investigation is open to several criticisms. First, we tested only HIV-1 and we have no idea what could happen with other viruses. Although HIV-1 is the biggest compared to hepatitis B and C viruses, we do not know whether molecular weight alone is a sufficiently robust criterion to test the impermeability of the straws. Second, this study was conducted over a much briefer interval compared to that over which human gametes and embryos are usually frozen in liquid nitrogen. Third, it was conducted at 37°C and in liquid nitrogen. This point is particularly relevant for the IR straws because the material from which they are made was only intended for cryopreservation and might not be safe at 37°C.

Furthermore, doubt persists as to the impermeability of the ultrasonic seals of PVC and PETG straws. Since 1998, classical PVC straws can no longer be used in France for the packaging, storage and/or in-vivo transfer of biological products of human origin because they cannot be sterilized by γ radiation. Nonetheless, many remain in liquid nitrogen tanks as do the PETG straws currently being used. Lastly, intact PVC and PETG straws should have been filled with the virus-containing solution and then sealed ultrasonically, but this procedure risks contaminating the apparatus, which might prove difficult, if not impossible, to decontaminate.

It should be noted that the increased contamination of RPMI with prolonged duration of incubation of IR straws suggests that their composition is defective at 37°C. However, this possibility does not preclude possible leakage due to defective sealing procedures. Indeed, the potential transmission of infectious agents from packaged semen during storage for artificial insemination of non-domestic livestock was recently described (Russel *et al.*, 1997). In their experimental study, leakage from the straws seemed to be dependent on the filling and sealing methods used. It has also recently been suggested that the storage of semen in cryovials placed in direct contact with

liquid nitrogen presents a risk because a proportion of cryovials absorb liquid nitrogen through caps, even when a second skin was used to provide an adequate seal (Clarke *et al.*, 1999). All these remarks justify further examination of the medical security of cryopreservation tanks, and underline the need to establish the safety of straws and good-practice guidelines for packaging human gametes or embryos for cryopreservation.

In conclusion, under these experimental conditions (37°C), the unsealed cotton end of PVC and PETG demi-straws appeared effectively to prevent HIV-1 escape, as did the thermosoldered cotton end of IR demi-straws, but doubt persists regarding sealed and unsealed plastic plug ends and for unsealed cotton ends of IR demi-straws. Additional studies are needed, especially to test the ultrasonically sealed ends of the PVC and PETG straws, and ends of IR straws under cryopreservation conditions. Should these further experiments confirm the impermeability of all the straws, the risk of contaminating the contents of other straws stored in liquid nitrogen and by the latter would become negligible.

Acknowledgement

The authors thank Janet Jacobson for translation of manuscript.

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Received on January 4, 2000; accepted on July 5, 2000