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

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BACKGROUND: The possibility of assisted reproductive technology (ART) for couples carrying viruses, especially HIV-1, necessitates consideration of the safety of cryopreserving human gametes or embryos in liquid nitrogen tanks. Following our evaluation of the safety of three kinds of straws containing HIV-1 at 37°C, we have now examined the HIV-1 imperviousness of the same straws under cryopreservation conditions. METHODS: Polyvinyl chloride (PVC), polyethylene terephthalate glycol (PETG) and high-security ionomeric resin (IR) straws (24 each) were tested. Each straw was filled with 100 µl of HIV-1-containing supernatant [reverse transcriptase (RT) activity: 15 000 c.p.m./50 µl]. Then PVC and PETG straws were sealed ultrasonically only at their free-end, and IR straws were thermosoldered at both ends. Each straw was put in a 15 ml Falcon tube which was capped and submerged in a liquid-nitrogen tank for 7 days. After bleach decontamination or not, the outside of each end of the straw was rinsed with RPMI medium (1 ml) before cryopreservation and after thawing. Viral RNA was extracted from the medium and then amplified by RT-polymerase chain reaction (PCR) followed by nested-PCR using HIV-1 protease-specific primers. RESULTS: HIV-1 RNA was detected in some PVC and PETG rinse media, probably resulting from splashing during ultrasonic sealing, but not in the rinse media of thermosoldered IR straws. CONCLUSION: Under cryopreservation conditions, IR straws would appear to be safe for HIV-1 storage in ART. For PVC and PETG straws, as highlighted in this study, the ultrasonic sealing could be the weak safety link.

Key words: assisted reproductive technology/cryopreservation/HIV-1/liquid nitrogen/straws

Introduction

The spread of viral epidemics has raised safety considerations for health professionals. In particular, concerning assisted reproductive technology (ART), French laws of good medical practice have been enacted.

The improvement and/or efficacy of treatments available for human immunodeficiency virus (HIV)-positive patients, for example, tritherapy, have prolonged life expectation and its quality, allowing these individuals to see the future differently, especially in terms of procreation.

Epidemiological studies showed a sizable risk of female seroconversion after unprotected sexual relationships within HIV serodiscordant couples (Saracco *et al*., 1993; De Vicenzi, 1994; Mandelbrot *et al*., 1997). In contrast, European studies (Semprini *et al*., 1992; 1997, 1998; Brechard *et al*., 1997b; Marina *et al*., 1998a, 1999; Tur *et al*., 1999) showed that, after more than 2000 intrauterine inseminations with sperm prepared from an HIV-seropositive partner, no mother or child seroconversion has been reported. The use of classical IVF or microinjection of a spermatozoon seems to give the same results; however, the literature on this subject is more limited and remains to be confirmed (Marina *et al*., 1988b; Tur *et al*., 1999).

All those European studies, in association with the establishment of a clear judicial framework, allows us to envisage the management of serodiscordant couples in France today. However, reluctance to undertake ART for these couples reflects the potential vertical transmission of the infection to the fetus or child, lateral infection to the healthy partner or the medical team and/or to other patients undergoing ART. A

hepatitis C seroconversion, possibly due to cross-contamination during ART, illustrates that a risk of transmission exists (Lesourd *et al*., 2000).

Since the discovery of HIV in ejaculated sperm (Zagury *et al*., 1984) and the implication of sperm as a vector of infection transmission during artificial insemination (Stewart *et al*., 1985; Centers for Disease Control, 1990), many groups have attempted to identify the ejaculate fraction responsible for the sexual transmission of this virus. The results of several studies demonstrated the presence of HIV in the seminal fluids and non-germinal cells (Zagury *et al*., 1984; Brechard *et al*., 1997a). Furthermore, it was shown that, regardless of the technique of sperm preparation used, including washing, HIV nucleic acids persisted in the fraction of purified spermatozoa (Dulioust *et al*., 1998; Tachet *et al*., 1999).

Within the framework of ART, the gametes of these patients, as well as the embryos obtained from them, are routinely cryopreserved and, other than a strategy of solitary quarantine, the potential risk to other samples stored in the same containers has been raised.

In a preliminary study (Benifla *et al*., 2000), we examined the safety in terms of HIV of three types of straws—polyvinyl chloride (PVC), polyethylene terephthalate glycol (PETG) and high-security ionomeric resin (IR)—routinely used for the cryopreservation of gametes and embryos. This experiment, conducted at 37°C, found the three types of straws tested to be unsafe. However, because the experimental conditions were not those of cryopreservation, the results must be interpreted with extreme caution and experiments must be repeated under the exact conditions of use. Thus, the objective of the present investigation was to evaluate the safety of these three types of straws in terms of HIV under cryopreservation conditions.

Materials and methods

Supplies and reagents

Twenty-four of each of the three types of straws—PVC, PETG and IR—(Cryobiosystem, l'Aigle, France) available in France were used in this study. All three have an open end and a cotton-plugged end. As in our previous study, the two ends of each type of straw were tested.

Each straw was filled aseptically in a vertical laminar flow hood with 100 µl of HIV-1-containing supernatant [reverse-transcriptase (RT) activity: 15 000 cpm/50 µl]. PVC and PETG straws were filled manually with an automatic Eppendorf pipette with the tips adapted to the straw diameter. Straws were sealed ultrasonically only at the free non-cotton-plugged end. High-security IR straws were filled with a pump, as recommended by the manufacturer (Cryobiosystem), and sealed by thermosoldering of both ends.

To test cryopreservation conditions, each straw was placed in an empty conical 15 ml tube (TPP, Falcon). Each tube was capped and then placed in a container of liquid nitrogen for 7 days.

Experimental protocols

Two experiments were performed with eight straws of each type (four testing the cotton-plugged end and four testing the free end). The only difference between the two was that, for the second, the outside of the straw was decontaminated after filling and sealing by swabbing with a bleach-impregnated compress (Chlorine concentration: 36%).

Before each of these two experiments and for each type of straw, the sealing instruments were decontaminated with bleach (pre-experiment sealing-system controls). After filling and sealing the straw, the end being tested was rinsed with 1 ml of RPMI medium (rinse 1: R1) that was collected and frozen at –80°C until all samples were subjected to HIV-1-RNA detection at the same time. The straw was then dried with a sterile gauze and placed in the TPP tube. All the tubes prepared in this way were progressively exposed to the cold as follows: –4°C for 30 min, –20°C for 30 min, then immersed in liquid nitrogen for 7 days.

On day 7, the tubes were removed from the liquid-nitrogen tank and the test end was again rinsed, before thawing, with 2 ml of RPMI; 1 ml was recovered and immediately frozen at –80°C (R2) until HIV-1-RNA testing. The test end of the straw was then left soaking in the remaining 1 ml of RPMI for 20 min, at which time this soaking medium (S) was collected and immediately frozen at –80°C until HIV-1-RNA testing. Controls consisted of one Falcon tube filled with 100 µl of HIV-1-containing supernatant and 1 ml of RPMI medium, and another was filled with RPMI medium.

At the same time, two empty, unsealed straws were tested under the same conditions as straw material controls. Two other empty sealed straws were also tested as controls for the closing systems after decontamination of the sealing instruments with bleach. Finally, after each experiment, two other empty sealed straws were tested to control for the possible contamination of the sealing instruments (post-experiment sealing-system control).

For HIV-1-RNA detection, each sample was thawed and then 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 RT-polymerase chain reaction (RT-PCR) followed by nested PCR (Titan RT-PCR kit, Boehringer, Mannheim, Germany), using primers specfic to the HIV-1 protease gene and loaded into 1% Agarose gels. Sterile water was used as the negative PCR control.

Results

Results of experiments 1 and 2, as a function of HIV presence or absence in the R1, R2, S media collected from each end and each type of straw are given in Table I.

PVC straws

Experiment 1: HIV was detected in R1 media collected from the sealed non-cotton-plugged ends of all four straws tested, whereas R2 and S for this end were positive only for one straw. For the non-sealed cotton-plugged end, no HIV was detected in any of the four R1 samples, whereas one straw yielded positive R2 and S media.

Experiment 2: HIV was detected in the R1, R2 and S samples from the sealed non-cotton-plugged ends of two of the four straws tested. For the non-sealed cotton-plugged ends, none of the tested samples contained HIV.

Control straws: Pre-experiment sealing-system control straws were all negative. After experiment 1, they all remained negative for the non-sealed cotton-plugged end, but all R1, R2 and S samples from the sealed, non-cotton-plugged ends were positive. After experiment 2, the R1, R2 and S media from controls for the two ends tested were negative.

Table I. Presence of HIV-1 at each end of the three types of cryopreservation straws used in experiments 1 and 2

Sample	PVC			PETG			IR		
	R1	R2	S	R1	R2	S	R1	R2	S
Straw material control									
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Non-cotton-plugged end	–	–	–	–	–	–	–	–	–
Pre-experiment-1 sealing-system control									
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Non-cotton-plugged end	–	–	–	–	–	–	–	–	–
Experiment 1									
Cotton-plugged end	–	+	+	–	–	–	+	–	–
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Non-cotton-plugged end	+	–	–	+	–	–	–	–	–
Non-cotton-plugged end	+	+	+	+	+	+	–	–	–
Non-cotton-plugged end	+	–	–	+	+	+	–	–	–
Non-cotton-plugged end	+	–	–	+	+	+	–	–	–
Post-experiment-1 sealing-system control									
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Non-cotton-plugged end	+	+	+	–	–	–	–	–	–
Experiment 2									
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Non-cotton-plugged end	–	–	–	–	–	–	–	–	–
Non-cotton-plugged end	+	+	+	–	–	–	–	–	–
Non-cotton-plugged end	+	+	+	+	+	+	–	–	–
Non-cotton-plugged end	–	–	–	–	–	–	–	–	–
Post-experiment-2 sealing-system control									
Cotton-plugged end	–	–	–	–	–	–	–	–	–
Non-cotton-plugged end	–	–	–	–	–	–	–	–	–
Controls									
HIV-1 control	+	+	+	+	+	+	+	+	+
RPMI control	–	–	–	–	–	–	–	–	–

R1 = first RPMI rinse; R2 = second RPMI rinse; S = soaking medium; + = HIV-1 positive; – = HIV-1 negative.

PETG straws

Experiment 1: HIV was detected in the R1 media collected from the sealed, non-cotton-plugged end of the four straws tested. Among these four straws, only one yielded negative R2 and S samples. In contrast, R1, R2 and S samples from the non-sealed, cotton-plugged ends remained negative for the four straws tested.

Experiment 2: Only one of the four straws yielded HIV-positive R1, R2 and S specimens from its sealed, non-cotton-plugged end. R1, R2 and S samples from the non-sealed, cotton-plugged ends were negative. For this type of straw, pre- and post-experiment 1 and 2 controls were negative.

IR straws

Experiment 1: Concerning R1 samples, tested for each of the two sealed ends, one of the four straws was positive at the cotton-end. R2 and S specimens from both ends were negative.

Experiment 2: The R1, R2 and S samples from the two ends of the four straws were negative, as were those from the pre- and post experiment 1 and 2 controls.

Discussion

Viruses can survive in liquid nitrogen and retain their virulence. Indeed, hepatitis B virus contamination of negative bone-marrow samples cryopreserved in the same liquid-nitrogen tank has been reported (Tedder *et al*., 1995).

It is known that HIV is present in seminal plasma cells (Zagury *et al*., 1984; Stewart *et al*., 1985; Centers for Disease Control, 1990; Dulioust *et al*., 1998; Tachet *et al*., 1999) and, despite antiretroviral therapy, remains in the seminal fluid of 2–50% of the patients (Eron *et al*., 1998; Luizzi *et al*., 1998; Vernazza *et al*., 1998; Zhang *et al*., 1998). Furthermore, although the separation and removal of the infective fraction of the ejaculates of HIV-positive men by density-gradient centrifugation and the absence of seroconversion of the mother and child after more than 2000 intrauterine inseminations using these purified sperm fractions is reassuring (Semprini *et al*., 1992; 1997, 1998; Brechard *et al*., 1997b; Marina *et al*., 1998a, 1999; Tur *et al*., 1999), doubt persists regarding HIV-1 infection of the spermatozoa themselves (Baccetti *et al*., 1994; Bagasra *et al*., 1994; Nuovo *et al*., 1994; Muciaccia *et al*., 1998).

For all these reasons, it is necessary to assess the real safety of straws used for cryopreservation of human gametes and embryos. Only a report from the Royal Veterinary College 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). In a previous study (Benifla *et al*., 2000), we investigated the safety of PVC, PETG and IR straws at 37°C. But, because the straw material was not designed for and might not be safe at 37°C, we conducted the present study under cryopreservation conditions.

Our results obtained with PVC straws suggest three possible types of contamination. First, the straw material or the cotton plug might be permeable, because only one straw among the four tested yielded HIV-positive R2 and S samples at the non-sealed cotton-plugged end, whereas the R1 specimen had been negative. Second, the sealing system might be defective, either splashing the straw contents at the moment of sealing or the seal not being impervious, leading to contaminaton of the outside of the straw which was not eradicated by R1 rinsing. This contamination persisted in liquid nitrogen, as it was found in R2 and S samples. Decontamination of the outside of the straw with bleach after sealing did not totally eliminate this risk, but it must be recalled that we used a high HIV load in these experiments. Third, the sealing instrument might become contaminated, as shown by the presence of positive post-experiment-1 controls. This point must be emphasized for the safety of performing the procedures entailed. Indeed, splattering of a straw's contents and contaminating the ultrasound-sealing block can, in turn, contaminate the outside of subsequent straws. However, the negative post-experiment using two control straws would seem, *a priori*, to eliminate this latter hypothesis in this case.

Our results obtained with PETG straws suggest only one type of contamination: defective sealing procedure with splashing of straw contents during ultrasound sealing with contamination of the outside of the straw or the permeability of the seal. The persistence of HIV, despite decontamination of the outside of the straw with bleach might be explained by the high virus load used in these experiments. However, this explanation does not allow us to completely eliminate the possibility that ultrasound sealing somehow damaged the straw and led to defective imperviousness.

The positivity of one IR straw in experiment 1 is difficult to explain satisfactorily in terms of our three hypotheses. Contamination of the sealing apparatus cannot be advanced because the controls remained negative. The same holds true for the imperviousness of the seal or straw material because the R2 and S samples from all the straws tested were negative. The only explanation could be an artefact of PCR. Thus, it would seem that, under the cryoconservation conditions of our experiment, the straw material and the sealing system are 'safe' for HIV-1 and the external decontamination of the straw with bleach has no additional impact. This safety criterion of IR straws was previously suggested based on experiments conducted with different strains of bovine viruses (Decuadro-Hansen *et al*., 2000).

Taken together, our findings suggest the existence of a risk of straw contamination during sealing, especially with ultrasound, and a risk of contamination of the sealing instruments. Bleach decontamination seems to be a way to diminish this risk. IR straws appeared to be safe for HIV under cryopreservation conditions used in this experiment. This information is important because, at present, within the framework of ART, the gametes of virus-carrying patients as well as the embryos derived from these gametes are susceptible to being cryopreserved and other than a strategy of solitary quarantine, the risk of contamination of the other samples stored in the same tank must be considered. This possibility must also be considered for the emergency freezing of specimens before the serological results of the donors are known and for all viral infections during the incubation period before serological detection is possible.

Until now, the permeability status of different types of straws was unknown, particularly those used routinely to cryopreserve human gametes or embryos (Bahadur and Tedder, 1997a,b; Janssens, 1997), and the storage of semen in cryovials placed in direct contact with liquid nitrogen was shown to present a risk because some of these vials absorb liquid nitrogen through their caps (Clarke, 1999). Even if cross contamination of gametes and embryos stored in the same tank has not yet been reported, to ensure the safety of these cells during storage, two types of quarantine with complementary approaches have been proposed. Either all the patients' samples quarantined in a liquid-nitrogen tanks for 6 months are destroyed should one of the patients seroconvert during this period (Janssens, 1997); or two successive 3-month quarantines, enabling the identification, based on serological tests conducted before freezing and 6 months later, of the 'safe' tanks and those at risk, i.e., patients who became positive or were not tested, and their separation accordingly (unpublished data). In the latter approach, it remains to be determined whether these straws should be used. Thus, quarantine in liquid-nitrogen tanks does not definitively resolve all the safely problems. For this reason, Tomlinson and Sakkas (2000) proposed conservation in liquid-nitrogen vapour. Despite the real advantages and the demonstration that sperm and embryo survival did not differ from that of liquid-phase storage, the authors concluded that no concrete clinical evidence supports the better safety of vapour storage.

Conclusion

Finally, impervious straws would assure better safety of cryopreserved specimens in nitrogen tanks provided that certain conditions of use are respected. According to our observations, IR straws clearly meet all the requirements for the storage of HIV. It must be kept in mind that we tested only HIV-1 because of its small size, in comparison with hepatitis B and C viruses. However, we do not know if the molecular weight is an adequate criterion for the evaluation of straw impermeability. One can easily imagine molecular affinities with transport across the straw wall, varying as a function of the virus and straw materials. Also, complementary studies will be conducted with hepatitis B and C viruses under the same experimental conditions.

In conclusion, under cryopreservation conditions, IR straws would appear to be safe for HIV in ART. Among the three types of straws tested, the ultrasound-sealing system may be source of 'risk' in terms of straw imperviousness.

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