Validation of safety procedures for the cryopreservation of semen contaminated with hepatitis C virus in assisted reproductive technology

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BACKGROUND: In France, assisted reproductive technologies involving a hepatitis C virus (HCV)-infected man require the cryopreservation of potentially infected semen (in order to establish the presence of HCV), hence the need for a safe and secure storage system. We evaluated the safety of high-security straws for the conservation of semen containing HCV RNA under routine conditions. METHODS: Ionomeric resin (IR) straws were filled with seminal plasma spiked with different concentrations of HCV RNA and sealed using a thermo-solder. After a 4% sodium hypochlorite treatment and/or cryopreservation for 7 days in liquid nitrogen, the outside ends of each straw were rinsed with RNAse-free water. RESULTS: No HCV RNA could be detected in any of the water samples. Additional samples included the rinsing water from straws sealed by thermo-solder and from the heating wire used to cut the end of straws containing HCV-positive semen. The latter samples were found positive for both HCV RNA and the protamine-2 gene expressed by spermatozoa. CONCLUSIONS: These results demonstrate the safety of IR straws, the filling system and the thermo-solder for cryopreservation of semen containing HCV in liquid nitrogen. Decontamination of the straw after sealing and the use of disposable scissors to open the straws are strongly recommended.

Key words: assisted reproductive technology/cryopreservation/hepatitis C virus/straws/viral safety

Introduction

French guidelines subject assisted reproductive technology (ART) using semen from hepatitis C virus (HCV) chronically infected patients to strict control, including detection of HCV RNA in seminal plasma and/or in spermatozoa (Anonymous, 2001). Standardized protocols are now available for the detection of HCV RNA in these fractions (Bourlet et al., 2002; 2003). Our technique, combining a pre-dilution of the sample in the same volume of RNAse-free water, a centrifugation step (at least 20 000 g for 1 h), a silica-based extraction method and an RT–PCR amplification performed with the Cobas Amplicor Assay (Roche Diagnostics, Meylan, France), allows a sensitivity of ~50 IU of HCV RNA per ml of seminal plasma. A recent study suggests that the transcription-mediated amplification (TMA) assay is a highly sensitive test for the detection and the quantitation of HCV RNA in semen (Pekler et al., 2003).

When appropriate techniques are used, HCV RNA is detected in 12–38% of the seminal plasma from HCV chronically infected subjects, depending whether they are co-infected or not by HIV (Leruez-Ville et al., 2000; Pasquier et al., 2000; 2003; Bourlet et al., 2002; Pekler et al., 2003). Before its use in ART, the potentially HCV-infected semen has to be cryopreserved in straws stored in liquid nitrogen containers. The safety of the straws is of high importance, in order to avoid cross-contamination that could occur during filling and sealing steps, storage in liquid nitrogen and opening of the straws, as reported previously (Tedder et al., 1995; Fountain et al., 1997).

High-security ionomeric resin (IR) straws (Cryo Bio System, L’Aigle, France) have recently been validated for the storage of semen containing HIV (Benïfa et al., 2000; Letür-Könirsch et al., 2003); however, no data are available regarding their safety for semen from HCV-infected subjects. The aim of this study was to evaluate the safety of IR straws when they are filled with seminal plasma containing HCV RNA, and of the procedures surrounding their use.

Materials and methods

Seminal samples

Semen samples were collected by masturbation from 11 subjects who tested negative for HCV antibodies, after they gave an informed written consent. Seminal plasma was separated from the pellet by
centrifugation at 800 g for 10 min, within 2 h after ejaculation, and kept frozen at ±80°C until use. The seminal samples were not pooled, and were rendered anonymous and used in the study without consideration of their origin.

**Design of the study**

After thawing, seminal fractions were spiked with a blood plasma containing 5 × 10^6 IU/ml of HCV RNA taken from a chronically HCV-infected patient. High-security IR straws were filled with 100 µl of seminal plasma using a pump, as recommended by the manufacturer, and sealed at both ends using a thermo-solder (SYMS® System; Cryo Bio System).

Before testing, the straws were submitted to different treatments: (i) disinfection of the extremity of the straw with no subsequent cryopreservation; (ii) no disinfection and no cryopreservation; (iii) disinfection before cryopreservation and before thawing; (iv) disinfection only before cryopreservation; (v) disinfection only before thawing; and (vi) cryopreservation without disinfection. The decontamination step was performed by soaking the straws in a 4% sodium hypochlorite solution for 10 min and bleaching with a sterile compress. Cryoconservation was performed for 8 days in liquid nitrogen after progressive freezing at ±20°C and ±80°C.

For each experiment, three HCV RNA concentrations were tested: 500 000, 50 000 and 5000 IU/ml. Control straws filled with semen spiked with blood plasma from HCV-negative subjects were tested in parallel.

After optimal treatment, the end of each straw was soaked in 1 ml of RNase-free water for 10 min at room temperature. The soaking water was then frozen at ±80°C in aliquots of 250 µl until use. Some of the straws containing semen spiked with HCV RNA-positive or -negative blood were opened using a heating wire system in order to analyse their contents.

To test the safety of the filling and sealing steps, an empty straw was sealed at the end of the study. A wipe-test was also performed on the sealing system and on the heating wire used to open the straws and on the forceps used for this. The straws and swabs were rinsed in RNase-free water and each water sample was frozen as described above.

**Detection of HCV RNA by RT–PCR assay**

The qualitative detection of HCV RNA in rinse water was performed using the Cobas Amplicor HCV 2.0 assay (Roche Diagnostics). For semen samples, we used a modified sensitive protocol combining a high-centrifugation step and a silica-based RNA extraction followed by the Cobas assay amplification (Bourlet et al., 2002). The sensitivity of the test was of ~50 IU HCV RNA per ml of seminal plasma.

**Human protamine-2 gene amplification**

To test for potential contamination of the sealing and opening systems by semen, the DNA and mRNA from the protamin-2 gene, a human seminal housekeeping gene (Miller et al., 1994), was investigated as described previously (Bourlet et al., 2002).

**Influence of the cryoprotectant on the RT–PCR sensitivity**

To evaluate the impact of the cryoprotectant on the sensitivity of the RT–PCR, eight straws (two for each of the three concentrations of HCV RNA mentioned above and two negative controls) were filled with 100 µl of seminal plasma diluted 1:1 in glycerol containing cryoprotectant (Medicult™; Lyon, France).
Results

Influence of the cryoprotectant on the RT–PCR assay

The content of the six straws filled with seminal plasma containing HCV RNA and diluted 1:1 in glycerol containing cryoprotectant tested positive for HCV RNA; the two negative controls were HCV RNA-negative and -positive for the internal control (Table I). These results indicate that the cryoprotectant did not inhibit the RT–PCR assay.

Analysis of the rinsing water

The rinsing water samples resulting from the straw ends all tested negative for HCV RNA. One sample was non-interpretable, as the internal control had been inhibited. These data show the absence of contamination of the exterior part of the straws via the sealing system and during cryopreservation in liquid nitrogen, even in the absence of disinfection steps. All the seminal plasma recovered from the straws spiked with HCV RNA-negative blood were negative for HCV RNA, demonstrating that no cross-contamination occurred during the sealing or storing steps.

Material controls

The wipe-test performed on the sealing system tested negative for HCV RNA and for both DNA and mRNA of the protamine-2 gene, as did the rinse water from the end of the empty straw sealed at the end of the study (Figure 1, lanes 2 and 3). In contrast, the wipe-test performed on the heating wire used to open the straws was found positive for the protamine-2 gene and weakly positive (optical density in the grey zone in two successive experiments) for HCV RNA (Figure 1, lane 4). The wipe-test performed on the clip used during this opening step was found positive for both HCV RNA and protamine-2 gene (Figure 1, lane 5).

Discussion

The present study evaluated the safety of IR straws filled with seminal plasma spiked with HCV RNA throughout the overall procedure, including filling, sealing, liquid nitrogen storage and opening. To measure the risk of contamination, seminal plasma samples were artificially contaminated with much higher HCV RNA loads (5000–500 000 IU/ml) than those found in semen from HCV naturally infected men (<3000 IU/ml) (Leruez-Ville et al., 2000; Bourlet et al., 2002). Regarding the evaluation of the filling step, all the corresponding rinse-water samples of the straw ends were found negative for HCV RNA in all experiments except one, the internal control of which was inhibited, possibly due to the presence of RNase on the external surface of the straw. The safety of the sealing procedure was corroborated by the absence of HCV RNA in the rinse water of the empty straw sealed at the end of the experiment and the absence of HCV RNA and protamin-2 gene amplification in the wipe-test performed on the sealing system. Our results are in agreement with those of Lettier-Könirsch et al. (2003), who tested straws spiked with HIV culture supernatants. In their study, using an experimental design similar to ours, a single sample out of 32 rinse water samples (from the end of a non-decontaminated straw) was found positive for HIV RNA; the authors concluded that this was a false-positive RT–PCR result, because of the absence of amplification of HIV RNA on the sealing material and in the rinse water of the same straw after cryopreservation.

Another important point stressed herein is the lack of inhibitory effect of the cryoprotectant on the RT–PCR assay, allowing the detection of HCV RNA in all cryopreserved semen samples.

In addition, the present study validated the safety of the cryopreservation step of IR straws, as illustrated by the absence of HCV RNA in the rinse water and in the control seminal plasma from straws kept in liquid nitrogen. This is of importance, since cryovials were shown to be permeable to liquid nitrogen during storage (Clark, 1999). In fact, liquid nitrogen is an excellent survival medium for most infectious agents, as illustrated by straws contaminated by hepatitis B virus (Tedder et al., 1995) and tanks by Aspergillus sp. (Fountain et al., 1997). This contamination of the straw content can originate from outside the straw (during the filling or

Figure 1. Detection of (A) HCV RNA and (B) protamin-2 gene products from rinse-water samples corresponding to different steps of cryopreservation conditions of high security straws. The results in (A) are expressed as optical density (OD) of the RT–PCR assay (HCV Amplicor; Roche Diagnostics); the grey zone (undetermined results) ranged between 0.15 and 1. Lane M, size marker; lane 1, positive control (human semen spiked with 5000 IU/ml of HCV RNA); lane 2, wipe-test performed on the sealing system; lane 3, rinse water from the end of the empty straw sealed at the end of the study; lane 4, wipe-test performed on the heating wire opening system; lane 5, wipe-test performed on the forceps used during the opening step; lane 6, water control. The electrophoresis image in (B) corresponds to the detection of the protamine-2 RNA (154 bp) and DNA (310 bp) by RT–PCR.
sealing step), or during storage, due to a permeability defect. Another potential source of contamination is the step of opening the straw, after thawing, by splashing of the straw content on and around the material. In contrast with the previous steps, the opening system using a heating wire was at high risk of contamination in this study, as demonstrated by the detection of both HCV RNA and protamine-2 gene products in the rinse water of the wipe-tests carried out on the system itself and on the forceps used to handle the straw. These results, not yet reported by others, indicate that this opening system, and others similar to it, should be eliminated and replaced by disposable scissors.

In conclusion, this study validates the safety of the filling procedure of the IR straws and of their cryopreservation in liquid nitrogen tanks when used with samples containing high loads of HCV RNA. The availability of DNase- and RNase-free straws is an additional benefit, since their content has to be tested by molecular technology. These straws are particularly recommended for use by laboratories performing ART with semen from subjects with chronic viral diseases. In addition, in order to satisfy the rules of standard precautions, these recommendations should be extended to all re-usable biological material stored in liquid nitrogen.

References


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