Transmission risk of hepatitis C virus via semen during assisted reproduction: how real is it?

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The risk of viral transmissibility in assisted reproduction is still a much-debated issue, especially for hepatitis C virus (HCV). HCV is a common causative agent for parenterally transmitted viral hepatitis. In addition, it has been incriminated in other routes of transmission, including sexual transmission and nosocomial infections. The management of infertility, in association with HCV, has sparked debates about the potential risk of spread of infection to virus-free individuals, embryos and/or semen. The lack of worldwide-accepted screening policies has helped to fuel this debate. Today, it is evident that there is a potential risk of spread of HCV through biological fluids, including semen, to other individuals. This risk can only be marginalized by the use of well-established criteria for safety in infertility centres, and by the use of proper initial detection and segregation of potentially hazardous materials. Techniques and protocols have been established to help the andrologist and embryologist to safeguard patients against such dangers, and should be imposed in all centres, allowing HCV-positive males to enter their assisted reproduction programmes.

Key words: hepatitis C virus/ICSI/IVF/sperm/viral transmission

Introduction

Many sero-discordant couples, in which the male partner is infected with hepatitis C virus (HCV) and the female is infection-free, wish to conceive children. Unfortunately, because of the risk of transmission of virus in semen, these couples are often recommended to use barrier methods of protection when engaging in sexual intercourse. Even though this may be a protection against possible viral transmission, it also eliminates any possibility of achieving pregnancy; in which case the couple is forced to seek alternative methods of procreation.

In general, viral infections have been shown to contribute to male infertility either by direct toxic effects on cells in the male reproductive tract, and/or indirectly by causing a local inflammatory, or immunological, reaction (Keck et al., 1998). The impact, if any, of HCV infection on male fertility is still being debated (Dore and Kaldor, 2000).

The HCV genome is a small, enveloped, positive sense, single-stranded RNA-encased linear virus, with ~10,000 nucleotides (Zein, 2000). Different HCV isolates from around the world show substantial nucleotide sequence variability throughout the viral genome (Cooreman and Schoondermark-Van de Ven, 1996). There are at least six major genotypes of HCV currently classified worldwide, each comprising multiple subtypes (Zein and Persing, 1996).

HCV is the major causative agent for parenterally transmitted non-A, non-B hepatitis. HCV infection is a major cause of chronic liver disease worldwide, with ≥85% of HCV-infected patients developing persistent infection, and almost 70% developing chronic hepatopathy with increased levels of liver enzymes (Choo et al., 1989). Chronic HCV infection may lead to cirrhosis and hepatocellular carcinoma (Centers for Disease Control and Prevention, 1997; Moyer et al., 1999).

Although HCV transmission is known to occur essentially by the parenteral route, it is estimated that in 40–50% of cases, the parenteral risk factor is not identified (Everhart et al., 1990). One potential mode of non-parenteral transmission is through body secretions. The potential infectivity of body fluids in HCV-positive-infected patients has been identified by detection of HCV RNA in saliva, ascites, breast milk, urine, faeces, semen and cervico-vaginal secretions (Liou et al., 1992; Numata et al., 1993; Young et al., 1993; Kumar and Shahul, 1998; Beld et al., 2000; Levy et al., 2000; Belec et al., 2003). Fortunately, since it is not a DNA virus, unlike the hepatitis B virus (HBV), and has no reverse transcriptase activity, unlike the human immunodeficiency virus (HIV), it therefore cannot succeed in DNA integration within infected cells, sperm or embryos (Steyaert et al., 2000).

Even though the importance of viral screening is well known, until today viral screening policies in assisted reproductive centres have been shown to differ in each country, and sometimes even between infertility centres in the same...
country (Abusheikha et al., 1999). At present, different protocols are observed in different IVF laboratories for detection and quantification of HCV RNA in both serum and semen, due to the lack of worldwide-accepted guidelines.

Performing assisted reproduction techniques in a patient with HCV infection may lead to a contamination risk for: (i) sero-negative partners of sero-positive patients (sero-discordant couples); (ii) sero-positive partners with different strains; (iii) the couple’s embryos; (iv) other patients attending at the centre during the same period; (v) other gametes, embryos or semen, originating from non-infected couples treated in the same period; (vi) technicians through infected cryopreserved semen storage and manipulation.

Transmission risk via sexual contact
Observations have shown that the route of HCV transmission may be sexual (Thomas et al., 1995; Scotto et al., 1996). To study the role of sexual transmission, some authors have collected epidemiological data (Bresters et al., 1993), while others have analysed the semen of HCV-infected patients for the presence of HCV RNA (Fried et al., 1992; Fiore et al., 1995). Such studies yielded contradictory results.

HCV is known to be mainly transmitted by the parenteral route, and the contribution of genital secretions to the spread of HCV is unclear. From an epidemiological point of view, discrepancies have been reported about the ability of HCV to be transmitted via sexual intercourse, with some reports supporting a possible role of this way of infection (Kao et al., 1996; Mesquita et al., 1997; Karmochkine et al., 1998; Halfon et al., 2001), whereas other studies found no evidence of increased prevalence of HCV infection in subjects with at-risk sexual behaviour (Wyld et al., 1997; Osella et al., 1998).

It has been generally estimated that 5% of all HCV cases are sexually transmitted; and in women, the risk for infection by HCV-positive partners is higher in relationships lasting >20 years (Dienstag, 1997). There is also an association between HCV positivity in males and risk for HCV positivity in their female partners. Female partners of anti-HCV-positive males are 3.7 times more likely to have anti-HCV antibodies than female partners of anti-HCV negative males (Thomas et al., 1995). In these cases, transmission is more feasible if exposure is repeated and long-lasting (Kao et al., 1996; Caporaso et al., 1998).

Furthermore, to determine whether HCV could be sexually transmitted, the frequency of HCV infection was studied in heterosexuals with multiple partners. The frequency of HCV infection in these individuals was found to be much higher than that in healthy women with a stable partner (Zhao et al., 1995).

In Egypt, Kumar suggested that HCV could be transmitted within couples (Kumar, 1998). This was founded on the observation that viral nucleotide sequence analysis for husband-and-wife pairs revealed significantly high levels of similarity (97–100%).

Although the role of sexual contact in such transmission is unclear, these and other studies (Alter, 1990, 1993, 1995; Everhart et al., 1990; Kao et al., 1992; Lissen et al., 1993; Akahane et al., 1994; Belec et al., 2003) indicate that the sexual transmission of HCV cannot be ruled out. Therefore even though the full extent of possible sexual transmission is not fully understood, it should not be neglected as a possible source of infection. Moreover, it is not clear whether assisted reproduction carries a greater risk for couples than would occur naturally. If proven that there is a definite risk involved, then HCV-discordant couples should be counselled to undergo assisted procreation when they feel the need for reproduction. In the meantime, they should be instructed to use barrier mechanisms, not for the purpose of safeguarding against an unexpected pregnancy, but to help curb any chance of spread during sexual contact.

Transmission risk via semen
For more than a decade, different studies have investigated HCV RNA in seminal samples, with detection levels varying according to the available methodology. Although the majority of earlier studies failed to document the presence of HCV RNA in seminal plasma, some recent publications have indicated its presence. Previous studies have reported the presence (Kotwal, 1993; Liou et al., 1992; Kotwal et al., 1992; Liu et al., 1994; Fiore et al., 1995; McKee et al., 1996; Tang et al., 1996; Leruez-Ville et al., 2000; Levy et al., 2000, 2002; Pasquier et al., 2000; Bourlet et al., 2002; Cassuto et al., 2002; Meseguer et al., 2002; Nyamathi et al., 2002; Pasquier et al., 2003; Pekler et al., 2003) or absence (Hsu et al., 1991; Fried et al., 1992; Terada et al., 1992; Caldwell et al., 1996; Semprini et al., 1998; Debono et al., 2000) of HCV RNA in seminal plasma of chronically HCV-infected males at varying prevalence.

The contradictory findings can be explained by differences in: (i) the processing techniques used for samples; (ii) the sensitivity of the assays designed to detect HCV RNA; and (iii) the presence of inhibitors in semen that may cause a false-negative evaluation to occur. Moreover, it is important also to note that the viral concentration in seminal plasma changes rapidly over time. Levy et al. (2002) observed that viral loads ranged from 40 to 1450 copies/ml in 12 sequential semen specimens over a period of <2 years in the same patient.

Molecular techniques used in seminal RNA extraction
The discrepant results available in the literature about the presence of HCV RNA in seminal plasma are related, at least in part, to the molecular techniques used and particularly to the wide range of protocols dedicated to RNA extraction. Those contradictory results could be explained, at least in part, by the heterogeneity of the populations studied and by the diversity and poor standardization of the techniques used for the extraction of RNA from semen and reverse transcription–real-time PCR (RT–PCR) protocols. Since there are a wide variety of techniques available in the literature, and no one technique has been fundamentally accepted, the type of detection technique used has been proven to have a bearing on the outcome.
Detection methods are commonly based on the amplification of well-defined sequences of viral nucleic acids. Overall, most studies that detected HCV RNA levels after one round of RT–PCR showed undetectable HCV RNA-positive sperm samples (Levy et al., 2000, 2002; Semprini et al., 2001). Nevertheless, Levy et al. (2000) noted that HCV RNA could be clearly identified by RT–PCR in semen, seminal plasma, after dilution (1:4), and even in the cell pellet obtained after centrifugation which contains leukocytes, sperm and round cells. Therefore, common PCR procedures, such as those employed to quantify the blood viral load, may not be sensitive enough to determine viral load in washed semen samples (Marina et al., 1998; Hanabusa et al., 2000; Pasquier et al., 2000).

Furthermore, in order to evaluate the efficacy of the different protocols for detection of HCV RNA in semen, a recent multi-centre study ran blindly tested and coded specimens in 12 French laboratories (Bourlet et al., 2003). All the centres used a qualitative RT–PCR technique to reach a conclusion on the presence or absence of HCV RNA in semen. The percentage of correct results ranged from 53.3 to 100%; the worst results being obtained when no centrifugation preceded the amplification extraction protocol. The beneficial effect of this centrifugation step can be related to its ability to remove most of the inhibitors of PCR, which may reach high concentrations in some seminal samples (Mayer and Anderson, 1995; Cohen et al., 1997). By contrast, the overall number of correct results was not correlated to the initial volume of sample used for the test.

Another way to determine the presence/absence of viral nucleic acids in post-wash sperm is the employment of nested PCR techniques, which increases the detection limit to one copy of viral RNA (Meseguer et al., 2002). This is confirmed by the recent report by Meseguer et al. (2002) in which they reported on finding HCV RNA with nested PCR in a previously RT–PCR-negative sample. Therefore, RT–PCR can not be considered a gold standard for detection of HCV RNA in semen, and a residual viral load cannot be excluded in previously RT–PCR-negative semen samples.

**False findings by PCR**

Since PCR can detect tiny amounts of viral RNA, stringent laboratory procedures must be employed to avoid false results. In order to avoid the occurrence of the false-positive results, strict laboratory procedures must be followed to avoid specimen contamination. Furthermore, a negative control should be included with each run to verify the results. In addition, to prevent false-positive results from cDNA contamination, dUTP-uracil-DNA-glycosylase should be added to all samples.

The occurrence of false-negative results due to the presence of PCR inhibitors in the ejaculates is also an important concern (Semprini et al., 1998; Debono et al., 2000; Levy et al., 2000). The discrepancy in detecting HCV RNA in semen could have been due to RNAse or lipoperoxidase inhibitors.

In particular, attention must be paid to false-negative PCR results due to the presence of Taq polymerase inhibitors in seminal fluid (Levy et al., 1997; Semprini et al., 1998). Lactoferrin, peroxides, and mostly zinc residues are thought to be the main substances that interfere with the action of Taq polymerases (Mayer and Anderson, 1995; Cohen et al., 1997).

It is also important to note that the detection of PCR inhibitors is essential, but must not represent the end-point of the analysis. Strategies must be adopted to nullify the effects of these inhibitors. One method would be to use a dilution method (e.g. 1:4) to dilute, or suppress, these PCR inhibitors; and thus allow a conclusion to be drawn for all patients. Without suppression, or dilution, of the Taq inhibitors in semen fractions, no result can be validated.

In conclusion, dilution of the samples can be criticized because of the risk of being under the detection limit, so testing RNA extraction protocols using silica to eliminate or decrease Taq inhibitors is preferred (Tachet et al., 1999). In addition, the use of nested PCR is recommended since it has a lower threshold for detection. Each seminal fraction should be tested individually with a positive control in each assay to further confirm that any negative findings are not false-negative.

**Safety of semen preparation techniques for assisted reproduction**

Observations have shown that HCV RNA detection, in previously positive semen samples, became negative after a three-layer Percoll gradient (McKee et al., 1996; Levy et al., 2000). The elimination of HCV RNA after a Percoll gradient centrifugation can be explained by the different steps of semen preparation (selection of motile sperm with a discontinuous gradient, elimination of leukocytes, immotile sperm and round cells, then subsequent washing of each fraction) (Levy et al., 2000). Furthermore, a discontinuous gradient is an easy procedure to select motile sperm, which also efficiently eliminates (or decreases) infectious agents such as cytomegalovirus (Levy et al., 1997), Chlamydia trachomatis (Levy et al., 1999) and HCV in infected ejaculates (Levy et al., 2000).

When the viral status of sperm fractions, obtained after density gradient centrifugation followed (Pasquier et al., 2000) or not (McKee et al., 1996; Levy et al., 2000, 2002; Cassuto et al., 2002) by a swim-up step, were studied, no HCV RNA was found in the purified 90% fraction of sperm, which is the one used for assisted reproduction. The absence of virus could be explained by the use of low-speed centrifugation, which is routinely used to select sperm for assisted reproduction which does not concentrate HCV in the 90% fraction (Cassuto et al., 2002).

**Safety of cryopreservation for assisted reproduction**

The potential risk of HCV transmission between semen samples and other samples stored in the same containers during cryopreservation has been raised. Within the framework of assisted reproduction technology, the gametes of HCV-positive patients, as well as the embryos obtained from them, are often routinely cryopreserved, as a safety net to ensure the absence of viral contamination.
protect against horizontal transmission (Letur-Könirsch et al., 2003). Although 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, proper measures should be taken to safeguard against the risk of such a transmission.

Viruses have been reported to have the ability to survive, and retain their virulence, in liquid nitrogen (Letur-Könirsch et al., 2003). Tedder et al. (1995) have previously reported on HBV contamination of negative bone-marrow samples cryopreserved in the same liquid-nitrogen tank. Therefore the threat of HCV transmission via cryopreserved bioproducts is both possible and real. For these reasons, it is necessary to assess the real safety of straws used for cryopreservation of human gametes and embryos, especially for routine use in assisted reproduction in conjunction with transmissible viral diseases.

Until recently, 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). Further, the storage of semen in cryovials placed in direct contact with liquid nitrogen was shown to present a risk because some of these vials may absorb liquid nitrogen through their caps (Clarke, 1999).

A report from the Royal Veterinary College has shown a relationship between leakage from plastic straws used for livestock semen cryopreservation and the method used to fill the straws (Russel et al., 1997). Furthermore, Letur-Könirsch et al., 2003 recently studied different types of cryopreservation materials [polyvinyl chloride, polyethylene terephthalate glycoc and high-security ionomeric resin (IR) straws] and the possibility of viral leakage in each. They concluded that the existence of a risk of straw contamination, in addition to the sealing instruments, is both valid and real, especially with ultrasound sealing. IR straws were the only type that they found to be safe under cryopreservation conditions. Furthermore, bleach decontamination was recommended as a way to diminish this possible risk.

This information is important because, at present, within the framework of assisted reproduction technology, the semen and/or gametes of virus-carrying patients as well as those derived from virus-free patients may be cryopreserved in the same liquid nitrogen tank. The use of highly secure straws for semen freezing for potentially infectious samples, and the storage of these straws in a separate cryotank dedicated to samples with infectious hazard, would therefore help to decrease the chance of nosocomial infections, or contamination to virus-free specimens (Benilha et al., 2002; Letur-Könirsch et al., 2003). Furthermore, the use of IR straws appears to be the safest under these conditions, but further studies are warranted to conclude the best protocol for proper cryostorage of biohazardous materials.

Strategies for assisted reproduction

One suggested strategy for sero-discordant couples, who need artificial insemination or IVF/ICSI, would be to use donor sperm. Unfortunately, not all IVF centres, countries, religions or individuals will accept this option. Many countries have laws regulating infertility treatment, including the source of the oocytes and sperm. For example, in Egypt, it is illegal to use donor sperm, even in the case of the possible transmission of infectious diseases. Furthermore, many religions and social orders do not allow for the use of donor sperm. Therefore, this option may be of limited value worldwide.

A second strategy would be to postpone IVF/ICSI trials in HCV-positive males until further laboratory testing has occurred. Semen would be submitted to selection by centrifugation in a discontinuous gradient, washing in BM1, followed by the swim-up technique (Levy et al., 2000). The motile sperm would be divided into two parts: one half frozen and the other half used to detect HCV RNA. If negative, the frozen, ‘safe’, ‘non-infected’ cryopreserved semen fraction could be used for assisted reproduction with minimal risk of viral contamination. This is the only method currently approved by the French biolaws (Cassuto et al., 2002).

Even though this method provides a safety net for detection of HCV and decreases the possible transmission risk in HCV-positive males, it also carries some drawbacks. First of all, not all centres have the capabilities to properly store and test semen for HCV. Furthermore, dividing the semen in half will decrease the amount of sperm remaining for use during the IVF procedure, therefore decreasing the chances for success of finding enough motile sperm for IVF, or for ICSI, on the day of the ovum retrieval, therefore jeopardizing the cycle. Moreover, this technique would only be valid in cases of ejaculated sperm, with adequate number and motility. Therefore, it would not be appropriate for cases where surgical retrieval of sperm is needed, or with poor motility, since it is known that freezing sperm results in decreased motility, vitality and number of sperm cells (Donnelly et al., 2001) with, as an eventual consequence, poor oocyte fertilization and pregnancy rates during assisted reproduction treatment.

Besides, the need for routine cryopreservation in HCV-positive male partners is questionable since it has been demonstrated by previous studies that the routine technical protocols for sperm preparation for assisted reproduction can eliminate the presence of the virus.

Further, in cases where testicular sperm extraction (TESE) is needed, this option is very debatable. Cryopreservation of sperm in cases with severe testicular pathology, where very small numbers of sperm are retrieved from a few minute foci, is both difficult and may result in total loss of viability. In such cases, sperm may not be retrieved from a repeated TESE (Mansour et al., 2003).

Safety of HCV-positive semen in assisted reproduction

Even though HCV has been detected in the semen of some HCV-positive patients, no purified sperm fraction (i.e. 90% fraction) has been shown to be HCV positive, thereby suggesting a very low risk of virus transmission. Moreover, embryo culture media sampled on the day of transfer has been reported to contain no detectable levels of HCV RNA (Cassuto et al., 2002; Sifer et al., 2002; Devaux et al., 2003).
Preliminary evidence suggests that, in HCV sero-positive males, assisted reproduction with ejaculated sperm may be a safe procedure. Semprini et al. (2001) carried out 2300 assisted reproductive procedures (IVF and intrauterine insemination), using sperm samples selected by gradient centrifugation followed by swim-up, in HIV-positive men, 62% of whom were co-infected with HCV, and not a single woman sero-converted either for HIV or HCV. In addition, Levy et al. (2002) reported on a pregnancy in an HCV RNA sero-discordant couple (male positive) without female partner sero-conversion after a classic IVF with cryopreserved HCV RNA-negative sperm obtained with gradient selection and subsequent swim-up.

Regrettably, there are limited data available in the literature on the safety of assisted reproductive treatment performed with testicular sperm. In such cases, blood contamination increases the viral load of the sample and the risk of HCV transmission in comparison with procedures done with ejaculated sperm purified by gradient selection and swim-up (Manno et al., 2003). Even though, in a recent report, Manno et al. (2003) reported on a case of TESE/ICSI in an HCV chronically infected sero-discordant couple with HCV RNA-positive seminal plasma. In this case, the female partner also did not sero-convert following the assisted reproductive treatment trial.

Nevertheless, because the presence of HCV in semen implies a possible risk of nosocomial contamination, safety regulations must be strictly applied in assisted reproduction laboratories. Thus, to curb this risk, safety rules must be strictly respected in all assisted reproduction laboratories that include infertile couples with HCV positive men (Steyaert et al., 2000).

Conclusions

Since the whole philosophy of assisted reproduction is built on the hopes of increasing the chances of success in couples with previous lack of natural procreation, we believe that a rational, informed consent by the involved parties is essential for the inclusion of individuals with infectious diseases into assisted reproductive treatment cycles. Furthermore, we believe that even though it is the right of any couple seeking medical help to be able to have the appropriate care, it is also the right of an infertility clinic to deny access to their facilities if this care is not present, or if the management is not willing to carry the risk of transmission. Furthermore, it is our belief that these centres should refer these couples under question to specialized care facilities that can better care for their individual needs. The goal of any assisted reproductive treatment plan is to provide the couple with a live, healthy offspring, and without undergoing complications for the couple, other patients or the staff members. So it is important to conclude that worldwide recommendations and guidelines are needed to help contain the spread of disease, while not violating the basic human right of access to treatment.

Since HCV sero-positive does not seem to decrease the success rates of IVF, and the rate of vertical and horizontal transmission is limited, there seems to be no evident reason to discourage hepatitis C sero-positive patients from attempting IVF. However, care must be taken on the part of the IVF centre to limit any transmission risk. This reluctance to undertake assisted reproductive treatment 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 assisted reproductive treatment.

The management of HCV-infected men enrolled in programmes of medically assisted reproduction is highly dependent upon the definition of standardized protocols of detection of HCV RNA in semen. There is an urgent need for the preparation of worldwide-accepted guidelines for semen preparation, detection of HCV RNA in semen and the provision of safety for all parties involved. These data may be useful for counselling and management of couples who seek assisted reproduction, and also for further studies analysing the risk for HCV transmission in infertility clinics.

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