Medically assisted procreation and transmission of hepatitis C virus: absence of HCV RNA in purified sperm fraction in HIV co-infected patients

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Objective: The risk of hepatitis C virus (HCV) transmission in medically assisted procreation (MAP) is debated and some researchers have proposed to exclude MAP for HCV-positive infertile patients. The objectives of this study were to assess the presence of viral RNA in the final preparation of density gradient semen fractions collected from men with chronic HCV and HIV co-infection participating in a MAP program, and to assess whether HIV co-infection influences the rate of the presence of HCV RNA in the semen.

Design and methods: The study was based on a cohort of 170 HCV-infected male patients (93 HIV co-infected) participating in a MAP program in a French center. Semen samples were subjected to standard MAP sperm preparation, using density-gradient centrifugation with 40 and 90% layers. All aliquots were tested with a commercially available HCV RNA assay (Roche Monitor), adapted for use with semen after a nucleic HCV RNA extraction modification (Organon Technika).

Results: Seminal plasma samples from 19 (11%) patients were HCV RNA positive. The positive HCV viral load in semen was less than 600 IU/ml. None of the 90% fractions from HCV-infected patients were HCV RNA positive. Among the 93 co-infected patients, 10 were positive for HCV RNA in semen and three were HIV/HCV RNA positive in semen.

Conclusions: Although HCV RNA was found in the semen of 11% of patients, no purified sperm fraction, or spermatozoa used in MAP were HCV RNA positive. The 90% purified sperm fraction discards the virus and must be used with care in MAP.

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Introduction

The risk of viral transmissibility in assisted reproduction is still a much-debated issue, especially for hepatitis C virus (HCV) [1]. The recent finding of HCV RNA in seminal plasma led to guidelines for managing HCV chronically infected patients who participate in medically assisted procreation (MAP) [2]. Most authors have used sensitive

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polymerase chain reaction (PCR) techniques to detect HCV RNA in seminal plasma [3–8]. However, even if sexual transmission, at a low rate, is clearly demonstrated, the role of semen has never been established, and the use of spermatozoa from men chronically infected with HCV in MAP may lead to contamination of the female partner [9,10]. The management of HCV-infected men enrolled in MAP programs depends on the definition of standardized protocols of detection of HCV RNA in semen [11]. The discrepancy in results on the presence of hepatitis C virus (HCV) RNA in seminal plasma of men chronically infected by this agent is due, at least in part, to the molecular techniques used and particularly to the wide range of protocols for RNA extraction [12,13]. A multicenter quality control study has provided recommendations on HCV RNA determination in seminal plasma [14]. Only one study detected HCV RNA in seminal plasma and in the motile spermatozoon fractions of semen from men chronically infected with HCV attempting medically assisted reproduction [12]. There is thus a need for international guidelines.

The management of infertility, in association with HCV, has sparked debate about the risk of spreading the infection to virus-free individuals and embryos, and semen. The lack of worldwide-accepted screening policies has fueled this debate. Today, it is evident that there is a risk of HCV spread through biological fluids, including semen. This risk can only be controlled by the use of well-established criteria for safety in infertility centers, and by proper initial detection and segregation of potentially hazardous materials [15]. The main objective of this study was to assess the presence of viral RNA in the final preparation of density gradient semen fractions and spermatozoa collected from men with chronic viral hepatitis C participating in an assisted-reproduction program. The secondary objective was to assess whether HIV co-infection influences the rate of the presence of the HCV RNA in the semen.

Methods

Patients

One hundred and seventy-one male patients (mean age 40 years; age range 32 to 52 years) entering a MAP program were included in the study after they gave their fully informed written consent. These men were all tested positive for HCV RNA in blood by reverse transcriptase (RT)-PCR. Among these patients, 93 were HIV positive and all were hepatitis B surface antigen negative. Sequential samples of semen were obtained from 18 men: 16 gave two samples (mean delay between collections, 11.3 ± 8 months), one man gave three samples within 8 months, and one man gave four samples within 25 months.

Samples

Plasma samples were separated from blood by centrifugation and frozen at -80°C until use. After 3 days of sexual abstinence, semen samples were obtained by masturbation into a sterile container and were processed within 2 h of ejaculation. One milliliter of the semen sample was centrifuged at $800 \times g$ for 10 min, and the seminal plasma was separated from the cell pellet and stored at -80°C until further use. Another milliliter was submitted to centrifugation through a two-layer discontinuous gradient (2 ml of 40, and 90% of SupraSperm, Medicult, France). The motile spermatozoa fraction were recovered from the 90% fraction, observed by microscope to count them and to check for the absence of white blood cell contamination, and kept frozen at -80°C as aliquots of 500 000 cells until use (motile spermatozoa fraction).

Detection of HCV RNA in blood plasma

The qualitative and quantitative detection of HCV RNA in blood plasma was performed by the Cobas AMPLICOR and Cobas monitor HCV assay (version 2.0; Roche Diagnostics, Meylan, France), according to the instructions of the manufacturer.

Detection of HCV RNA in seminal plasma

RNA extraction was performed using silica beads (NucliSens; Organon Teknika S.A., Fresnes, France): 200 μl of thawed fraction 1 and 5.8 μl of the internal control from the Roche Cobas AMPLICOR assay were mixed in a lysis tube (9 ml). After centrifugation $1500 \times g$ for 2 min, 50 μl of silica was added. After incubation for 10 min at room temperature, a new centrifugation step of $1500 \times g$ for 2 min was performed. The supernatant was eliminated, and the residue was washed five times: two with 1 ml of washing buffer, two with 1 ml of 70% ethanol, and the last with 1 ml of acetone. The centrifugation during washing step was $10\,000 \times g$ for 30 s. After acetone elimination, the pellet was dried during 10 min at 56°C. For each pellet we added 50 μl of elution buffer and incubated the tubes for 10 min at 56°C. After centrifugation at $10\,000 \times g$ for 2 min, 30 to 35 μl of elute was diluted with specimen Roche Amplicor kit diluent to obtain 200 μl of sample in a new tube. This centrifugation step removes most of the inhibitors of PCR, which may reach high concentrations in some seminal samples: lactoferrin, peroxides, and particularly zinc residues. RT and qualitative PCR were performed using the Cobas AMPLICOR HCV assay (version 2.0) according to the instructions of the manufacturer.

For the quantification of HCV RNA in seminal plasma, the RNA extraction step was similar to that for the qualitative protocol (we used in this case HCV RNA Monitor QS), except that the eluent was diluted with specimen Roche Amplicor Monitor kit diluent to obtain 1 ml of sample in a new tube.

The sensitivities of the qualitative and quantitative assays were determined by using serial two-fold dilutions (from 160 to 10 copies/ml) or 10-fold dilutions (from 10 000 to 10 copies/ml), respectively, in HCV-negative seminal plasma of blood plasma from an HCV-infected patient, which had been quantified previously by the Cobas AMPLICOR HCV Monitor assay. Then, each dilution was extracted and tested in six independent experiments. Sensitivity of the qualitative assay was 100 copies/ml (2 log) and 600 IU/ml (2.78 log) for the quantitative assay.

Detection of HIV RNA in blood plasma

HIV RNA in blood plasma was detected by Cobas Monitor HCV assay (Roche Diagnostics), according to the instructions of the manufacturer. Sensitivity of the quantitative assay was 40 copies/ml (1.6 log).

Detection of HIV RNA in seminal plasma

The same method was used to detect HIV RNA in seminal plasma as for HCV RNA. For HIV RNA we used 500 μl of thawed fraction 1 and 6.7 μl of the internal control from the Roche Cobas AMPLICOR Monitor HIV assay. The eluted sample was diluted with specimen diluent of Roche Amplicor Monitor HIV kit to obtain 100 μl of sample in a new tube.

The sensitivities of the quantitative assays were determined by using serial two-fold dilutions (from 320 to 20 copies/ml) or 10-fold dilutions (from 4000 to 4 copies/ml), respectively, in HIV-negative seminal plasma of blood plasma from an HIV-infected patient, which had been quantified previously by the Cobas AMPLICOR HIV Monitor assay. Then, each dilution was extracted and tested in six independent experiments. Sensitivity of the quantitative assay was 40 copies/ml (1.6 log).

Detection of HCV RNA in spermatozoa motile fractions (90% fraction)

We tested 24 spermatozoa motile fraction 2 from 21 men whose seminal plasma was positive for HCV RNA. RNA was extracted by the same method (Organon Technika) and in the same conditions as for seminal plasma. RT and qualitative PCR were performed according to the instructions of the manufacturer.

Statistical analysis

Values were compared by the chi-squared test, Fisher test, or Wilcoxon test. A P value of less than 0.05 was considered significant.

Results

Detection of HCV RNA in blood and seminal plasma

All 170 patients included in the study were positive for HCV RNA in blood plasma. Seminal plasma samples from 19 of 170 patients (11.1%) were found to be positive for HCV RNA by the qualitative test. No PCR inhibitors were detected in seminal plasma specimens.

Among the 93 (54.7%) co-infected patients, 10 were found positive for HCV in semen. Among these 11 patients, three had HIV positive in semen. The HIV positive status does not influence the positivity of the HCV RNA in the semen (chi-squared $P = 0.96$).

Detection of HCV RNA in spermatozoon fractions

The corresponding motile spermatozoa (fraction 2) collected from patients whose seminal plasma was HCV RNA positive were tested for HCV RNA. All fractions tested remained negative for HCV RNA and for HIV DNA when HIV RNA was found in semen.

Figure 1 shows the distribution of the presence of HIV RNA and HCV RNA in blood plasma and seminal plasma.

Sequential determination of HCV RNA was carried out on semen from 18 patients. Among them, seven were

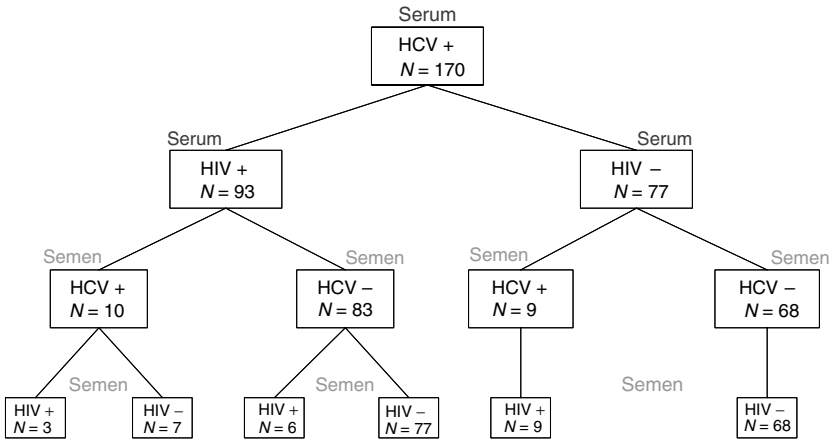

Fig. 1. Distribution of the presence of HIV/hepatitis C virus (HCV) RNA in both blood plasma and seminal plasma.

Table 1. Sequential analysis of 18 patients.

Patient	First result	Second result	Third result
1	POS	POS	NEG
2	POS	POS	ND[a]
3	POS	POS	ND
4	POS	NEG	ND
5	POS	NEG	ND
6	POS	NEG	ND
7	POS	NEG	NEG
8	NEG	NEG	ND
9	NEG	NEG	ND
10	NEG	NEG	ND
11	NEG	NEG	ND
12	NEG	NEG	ND
13	NEG	NEG	ND
14	NEG	NEG	ND
15	NEG	NEG	ND
16	NEG	NEG	ND
17	NEG	NEG	ND
18	NEG	NEG	ND

[a]Not defined.

found HCV RNA positive after the first test; the other 11 patients were HCV RNA negative. All 11 patients found HCV RNA negative at the first test were also negative at the second test. Among the seven patients HCV RNA positive after the first test, three were HCV RNA positive at the second test. Neither of the two patients with three tests were HCV RNA positive after the third test (Table 1).

Detection of HCV RNA and HIV RNA in serum

Median HCV RNA titer for the 170 patients was 684 140 IU/ml (range: 29, 23 690 000) and median HIV viral load for the 93 co-infected patients was 45 copies/ml (range: 20, 142 000) (Table 2).

Table 2. Demographic characteristics of the patients studied.

Hepatitis C virus infected (*N*)	170
Hepatitis C virus genotype (*N*)	
1	35
2	1
3	24
4	5
NA	105
Viral load hepatitis C virus (median)	684140 IU/ml
Fibrosis (*N*)	
0	23
1	28
2	37
3	19
4	8
NA	55
HIV/hepatitis C virus co-infected [*N* (%)]	93 (55%)
Known duration of HIV infection (years)	14 ± 4.22
Viral load HIV (median)	45 copies/ml
Antiretroviral therapy (*N*)	93
CDC stage (*N*)	
A	90
B	2
C	1
CD4 cell count (mean ± SD)	573 ± 294 cells/μl

The mean blood viral load was not significantly higher in patients positive for HCV RNA in semen than in those negative for HCV RNA in semen: 565 624 versus 540 448 IU/ml, Wilcoxon test $P > 0.05$ (Table 2).

Among the 93 patients with HIV co-infection, three of the 10 patients with HCV RNA positive in the seminal plasma were HIV RNA positive in the seminal plasma versus six of 83 with HCV RNA negative in the seminal plasma ($P > 0.05$).

Among the 93 patients HIV/HCV co-infected, 10 were found positive for HCV RNA in the seminal plasma versus nine out of 77 HCV mono-infected patients with HCV RNA positive in the seminal plasma ($P > 0.05$).

Discussion

This study on a large number of HCV chronically infected patients showed that the seminal plasma of 21 of 171 (12.3%) subjects were positive for HCV RNA by the RT-PCR technique. Previous studies on HCV RNA in the semen of infected subjects yielded conflicting results [3,4,8,16] but all failed to detect any HCV-positive seminal sample in semen, mainly due to the sensitivity of the assays available at these dates and the presence of PCR inhibitors. In addition, three reported that relatively high proportions of patients were positive for HCV RNA in this compartment: eight of 21 (38%) patients, four of 20 (20%) patients, four of 32 (12.5%) in the studies of Leruez-Ville *et al.*, Pasquier *et al.* and Bourlet *et al.*, respectively [3,12,13].

The present authors and others have found PCR inhibitors in seminal plasma, as demonstrated by the absence of detection of internal control in a standardized commercial technique [8,12,16]. To address this point, the main cause of discordant results in the studies on this topic, a multicenter quality control investigated the detection of HCV RNA in seminal plasma in several laboratories in France [14]. To eliminate PCR inhibitors in seminal plasma, many modified extraction procedures were described, including high-speed centrifugation and silica-based extraction [3]. On the basis of the results of this multicenter study, simple consensus techniques for the detection of HCV RNA in seminal plasma can be proposed: (1) the volume of specimen suggested for the test is 200 μl of seminal plasma, [17] – a predilution of the sample in the same volume of RNA-free water (1 : 1) is recommended [17]; (2) the extraction step is based either on the NucliSens test as recommended by the manufacturer or on the Amplicor HCV extraction assay necessarily preceded by a centrifugation step of the specimen (at least 20 000 × *g* for 1 h); (3) the amplification step is performed by RT-PCR using the Cobas Amplicor assay according to the manufacturer's instructions. The

methodology proposed in the present follows that recommended by this multicenter study.

The results we observed are consistent with the positivity of seminal plasma results of Bourlet *et al.* (12.5 versus 14.5% in patients without HIV co-infection) and seem representative of the actual rate of positivity in this compartment. In the present study, however, none of the corresponding 90% fractions or spermatozoa of the positive seminal plasma were positive. These results validated by the detection of the internal control in the RT-PCR method in these compartments, without detection of HCV viral genome, differ markedly from those of Bourlet *et al.* [12].

The finding of HCV RNA in fractions of semen and in spermatozoa calls for reinforced precaution for men whose blood is chronically infected with HCV and who are candidates for MAP. We suggest that seminal plasma be systematically evaluated for the absence of viral RNA in order to ensure that MAP is performed with samples free of HCV RNA. For those subjects found to be positive, motile spermatozoa should be investigated for the presence of HCV RNA and only spermatozoon fractions that test negative should be used for MAP. French authorities have made the similar recommendations for patients exhibiting HCV RNA in seminal plasma, that is, the use of cryopreserved cells that tested negative at the time of sampling [2]. However, no methods to search for the HCV in spermatozoas were validated or standardized and this recommendation must be re-evaluated after definition of standardized protocols of detection of HCV RNA in semen to avoid the assessment difficulty due to PCR inhibitors in the seminal plasma [15].

In addition to previous studies on the same topic, this study reports the compartmentalization of HCV RNA between seminal plasma and blood. Even though HCV RNA is intermittently detected in seminal plasma in a few patients, we excluded the possibility that spermatozoa support HCV replication because no spermatozoa fractions exhibited the presence of HCV RNA [18]. One of the weaknesses of the studies based on the single detection of HCV RNA in semen is that HCV is intermittently detected in seminal plasma [19]. Indeed, the limitation of the present study was that the analysis was conducted on several ejaculates only in some men but not in the overall population of patients. The notion of analyzing several ejaculates when the first detection is negative must be evaluated in clinical practice as suggested by Pasquier *et al.* [19]. The HIV-positive status does not influence the presence of the HCV RNA in the semen, and this finding must be taken in consideration because HCV sexual transmission is more frequent in patients co-infected by HIV [20]. These last points indicate that sexual transmission of HCV may be due to infected blood passed during intercourse abrasions of mucosa rather than through HCV-infected semen [9]. Regarding the mutual influence of HCV on HIV and inversely on the presence of the virus in seminal plasma, it seems that there is no influence of the HCV status on the presence of the HIV RNA in the seminal plasma, and there was no influence of HIV co-infection on the presence of HCV RNA in seminal plasma.

Surprisingly we found no correlation between the HCV RNA titer in serum and its detection in semen and processed fractions, as found in the study of Bourlet *et al.* [12].

In conclusion, neither final fractions nor spermatozoa were found HCV RNA positive. Thus medically assisted procreation may be indicated for patients with HCV. The uneventful deliveries of infants in the cohort evaluated in the present study are encouraging. Nevertheless, in France, recent legislation has dictated that seminal plasma should be systematically tested for the presence of HCV RNA in this population [14]. When HCV is present in semen, safety regulations must be strictly applied in assisted reproduction laboratories. French regulations recommend that HCV must be searched for in MAP routine practice.

International guidelines should be developed through studies on larger populations of HCV chronically infected individuals [21].

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