Separation of human immunodeficiency virus type 1 (HIV-1) from motile sperm using a continuous density gradient and subsequent swim-up

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HIV infection is becoming a controllable chronic infection with the introduction of the highly active antiretroviral therapy (HAART), and patients with this infection are now able to make plans for the future. Numerous serodiscordant (HIV-positive male and HIV-negative female) couples seek medical assistance to bear offspring with minimum risk to the negative female partner and child, which can be accomplished using processed (washed) sperm. HIV-1 elimination from the semen by density gradient centrifugation and the swim-up procedure, which is the standard processing procedure, depends both on the sperm motility and the physical properties of the virus-containing particles. To establish a simple method for recovering sperm from poor quality semen, we developed the tilted-tube rotation method to generate a continuous density gradient, and succeeded in recovering motile sperm even from the semen of seropositive males with severe “male factors” using this continuous density gradient. This review summarizes the recent progress in the underlying principles and technical aspects of semen processing for elimination of HIV-1.

Keywords HIV-1; Spermatozoa; Semen processing; Infertility; Assisted Reproductive Technology

1. Introduction

The mortality rate of AIDS has decreased by more than 80% after the introduction of the highly active antiretroviral therapy (HAART) in 1996. With AIDS thus becoming a controllable chronic infection, patients with this infection are now able to make plans for the future, and numerous serodiscordant (HIV-positive male and HIV-negative female) couples seek medical assistance to have a child with minimum risk to the negative female partner and child, which can be accomplished using processed (washed) sperm.

In this review, we summarize the recent progress in the processing of semen for elimination of HIV-1, and the underlying principles and technical aspects; the clinical results of assisted reproductive technology using processed semen are also reviewed.

2. HIV-1 in semen and its clinical biology

2.1 HIV-1 in semen and its origin

Semen is now well recognized as a carrier body fluid of the HIV-1 virus, after it was demonstrated that HIV-1 could be transmitted horizontally by donor insemination.

The HIV-1 virus is reported to exist mainly as free viral particles in the seminal plasma and in the CD4(+) lymphocytes in the semen. The possibility that the sperm itself may contain the viral gene is discussed in the next section.

The main reservoir of HIV-1 virus in the male genital tract (MGT) has not yet been precisely identified, and may be influenced by the disease status of the patient: is the MGT infected with HIV, or is the patient receiving antiretroviral treatment, e.g., HAART. In fact, Krieger et al showed that the majority of HIV particles found in the semen arise distal to the vas deferens, and Paranjpe demonstrated phylogenetic differences between HIV particles derived from the seminal plasma and seminal cells.

Recently, Anderson et al proposed three different sources of the HIV-1 virus particles found in semen, based on the results of phylogenetic analysis of HIV-1 in the blood and semen of chronically infected, previously untreated patients; 1) direct import of virus from the blood, 2) oligoclonal amplification within the seminal tract, and 3) autonomous and sustained replication in the seminal tract (so-called compartmentalization).

2.2 HIV-1 in sperm

It still remains to be established whether HIV particles are attached to the spermatozoa, whether spermatozoa can be infected with HIV, or whether spermatozoa themselves can transmit HIV-1 infection.
The presence of HIV-1RNA or proviral DNA in the spermatozoa has been demonstrated by 1) electron microscopy and PCR, and 2) in-situ PCR of spermatozoa and its progenitor cells. Recently, Muciaccia et al reported identifying HIV-1 DNA in the ejaculated abnormal spermatozoa from a seropositive male. These findings are endorsed by the fact that a specific virus receptor has also been identified on the surface of the spermatozoa.

While other researchers have reported the absence of HIV-1 particles or nucleic acid in sperm, it should be emphasized that there is a distinct possibility that the sperm itself may be a carrier of transmissible HIV-1.

2.3 HAART and HIV-1 in semen

Some researchers have suggested that effective HIV-1 treatment essentially renders a patient non-infectious. If this were true, the semen of a prospective father would be HIV-1-free during effective HAART therapy, and there would be no need for semen processing before ART.

Recently, it was reported that the risk of HIV-1 transmission through semen would become negligible (but not zero) if the following three conditions were met; (1) the HIV-infected patient is receiving antiretroviral therapy with excellent treatment adherence; (2) the blood viral load has consistently been undetectable (<40 copies per mL) for more than 6 months; (3) no other STDs are present in either of the partners (so-called Swiss statement).

However, previous studies have shown that intermittent HIV RNA shedding in the semen can occur even in treated patients with undetectable HIV-1 viral load. It is widely accepted that certain anatomical sites in the body represent “sanctuary” sites, where antiretroviral drugs permeate poorly, allowing the selection of drug-resistant strains, and that these are important sources of virological failure of therapy; the male genital tract is considered as one such sanctuary site. Zhang et al. reported the existence of latently infected cells harboring replication-competent virus particles in the male genital tract.

Although HAART would greatly reduce, and in some cases even eradicate, the infectious HIV-1 from the semen, there still remains the risk of sexual HIV-1 transmission, and it is necessary to process the semen before assisted procreation.

3. Principle and technologies of HIV-1 virus elimination

3.1 Principle of the density gradient centrifugation / swim-up procedure for eliminating HIV-1 from semen

HIV-1 elimination from the semen by density gradient centrifugation and the swim-up procedure, which is the standard processing procedure, depends both on the sperm motility and the physical properties of the virus-containing particles.

Figure 1. Principle of HIV-1 elimination from the semen. Density gradient centrifugation removes most of the free virus particles, and also non-sperm cells like epithelial cells and lymphocytes, from the seminal fluid, while some cells (e.g. non-viable cells which are shrunk because of cell membrane damage) remain in the heavy-density fraction (middle, after density gradient). In view of the possibility of the heavy-density cells being infected with HIV-1 virus, the pellet obtained after density gradient centrifugation cannot be considered as being suitable for insemination. However, when the swim-up procedure is employed subsequently, only the motile sperm swim up to the upper fraction, leaving the heavy-density cells at the bottom of the tube (right, after swim up).
The sedimentation kinetics of motile sperm in density centrifugation is thought to be highly dependent on the sperm motility. During density gradient centrifugation, the spermatozoa are thought to align in parallel to the centrifugal force vector with their heads, being the heaviest part of the cells, directed centrifugally. Since density gradient media, like Percoll or Pureception, exert no effects on the motility or ultrastructure of the spermatozoa, it is conceivable that the motile sperm will move downward across the gradient according to their motility. Indeed, Gorus et al.\textsuperscript{xxvi} reported that whereas the immotile spermatozoa showed banding in the density region of 1.06 to 1.09, the motile spermatozoa penetrated into denser regions as a function of their velocity and centrifugation time. Thus, if we choose the appropriate centrifugation time which would allow only motile sperm to come down to the bottom, but not the immotile virus particles, we may be able to establish a rapid and powerful method for separating motile sperm from free viral particles in seminal plasma and CD4(+) lymphocytes.

Although density gradient is a powerful tool to decrease the number of HIV-1 particles in the sperm fraction, it is still not perfect, and an additional swim-up procedure is considered to be essential to improve the HIV-1 elimination efficiency. It is conceivable that aggregated viruses or degenerated lymphocytes might also come down very steeply to the bottom during centrifugation, because of the high density gradient and small particle size. Indeed, it has been reported that around 5% of the sperm fractions obtained from HIV-1 patients after density gradient centrifugation alone remain positive for HIV-1, making potential HIV-1 transmission even through the processed semen possible\textsuperscript{xxvi}.

Furthermore, contamination often occurs during the harvesting step after gradient separation. While the upper layers are aspirated, small amounts of virus from the original semen fraction or upper gradient fraction might descend into the pellet of motile sperm. To avoid this risk, Politch et al. introduced a new sperm-processing device, the double sperm tube, with a discontinuous gradient of sperm separation medium formed inside\textsuperscript{xxix}.

### 3.2 Sedimentation kinetics of HIV-1

To develop an effective centrifugation method, it is necessary to calculate the buoyant density and sedimentation kinetics of HIV-1 particles. We determined, in a previous study, the buoyant density and sedimentation kinetics of HIV-1 particles (MOLT-4/LAI strain) in Percoll and Pureception using isopyknic ultracentrifugation and continuous density gradient centrifugation\textsuperscript{xxiv}.

#### 3.2.1 Buoyant density

To calculate the buoyant density, concentrated HIV-1 LAI strain (0.2 ml) was mixed with 2.5 ml of 65% Percoll or 2.5 ml of 50% Pureception (final viral concentration, $1.15 \times 10^6$/ml). After centrifugation at 16 400 \(\times g\) for 20 min (Percoll) or 11 400 \(\times g\) for 20 min (Pureception), aliquots of the postcentrifugation suspension (approximately 0.25 ml each) were fractionated beginning at the bottom of the tube (Experiment 1).

After ultracentrifugation, the distribution peaks of HIV-1 RNA and p24 antigen were calculated as existing at approximately 1.042 g/ml in Percoll (Figure 2). Accordingly, the buoyant density of HIV-1 in Percoll was estimated to be 1.042 g/cm\(^3\); the buoyant density of HIV-1 in Pureception was also estimated to be 1.042 g/cm\(^3\) (Figure 3). Interestingly, however, the viral distribution had a single peak in Percoll, but showed two peaks in Pureception, with a small amount of the virus in the higher-density fraction (bottom of the tube) in addition to the main peak.

![Figure 2](Image)

**Figure 2.** Viral RNA load in each density fraction of Percoll. Fractions were collected from the bottom and analyzed for density, HIV-1 RNA (■), and p24 (●). The amounts of HIV-1 RNA and p24 were plotted against the density of each fraction using a semilogarithmic scale. A single peak was observed for both HIV-1 RNA and p24 at the position representing a density of 1.042.
3.2.2 Sedimentation velocity

To estimate the sedimentation kinetics of HIV-1 particles, concentrated HIV-1 virus of the same strain as in Experiment 1 was overlaid on top of the continuous density gradient and centrifuged. The 3-ml continuous gradient was prepared by mixing two pairs of solutions in a pump for column preparation (Bio-Rad, Philadelphia, PA): either a mixture of 80% Percoll and Hanks’ solution, or a mixture of 90% Pureception and sperm-washing medium. After centrifugation at 1600 × g for 5, 10, 20 or 40 min, aliquots of the postcentrifugation suspension (approximately 0.25 ml) were fractionated beginning at the bottom of the tube.

In both the continuous density gradient media, most HIV-1 particles were found in fractions with a specific gravity of less than 1.04, even after 40 min of centrifugation (Fig. 4). In Pureception, however, small viral accumulations were observed at the bottom of the tube (data not shown), a finding that was not observed in Percoll.

3.3 Other technical implications; continuous density gradient and avoidance of contamination

Since sperm recovery by density gradient centrifugation depends mainly on the motility of the sperm through the density gradient, it is somewhat difficult to recover motile sperm from the semen of seropositive males with “male factors.” For such cases, therefore, the development of a more effective procedure to collect the motility-impaired sperm without decreasing the HIV-1 elimination efficiency was considered to be necessary.
Berger et al. compared the recovery rates of spermatozoa between continuous and discontinuous Percoll density gradients. They reported that although the continuous Percoll gradient was more effective as compared to the discontinuous gradient for separating motile sperm, the requirement for a high-speed centrifuge and rotor for the former would make the procedure impractical in many cases.

To establish a simple method for recovering sperm from poor-quality semen, we developed the tilted-tube rotation method for generating a continuous density gradient, and have succeeded in recovering motile sperm even from the semen of seropositive males with severe “male factors.” In brief, 2 ml of Percoll (80%) is overlaid with 2 ml of Hanks balanced salt solution in a sterilized disposable centrifuge tube and rotated with the tube kept tilted (Figure 5).

![Diagram of the tilted-tube rotation method](image)

**Figure 5.** Preparation of a continuous density gradient by the tilting-tube method. Two milliliters of Percoll (80%) was overlaid on 2 ml of Hanks balanced salt solution in a sterilized disposable centrifuge tube and rotated with the tube kept tilted. The prepared continuous gradient shows good linearity (lower figure).

The semen from the infected male is overlaid on the prepared continuous gradient before centrifugation at 1600 g for 20 min. The average sperm count recovered after the centrifugation and swim-up procedure were 45% and 5%, respectively. The elimination efficiency of HIV-1 using this method was 99.999%.

We also applied some technical improvements for the recovery of motile sperm from the tube after the swim-up procedure. We utilized a sterilized thumbtack with a silicone protector (Figure 6) to punch a small hole at the bottom of the tube, and an aliquot of medium containing motile sperm was recovered through this hole. The protector is necessary to protect the staff from any accident. Then, in order to introduce the sperm suspension to the bottom of the swim-up tube without allowing it to come in contact with the HIV-1 free medium of the upper layer, an outer and inner embryo transfer tube was utilized (Figure 7).

![Diagram of the thumbtack with silicone protector](image)

**Figure 6.** Thumbtack with a silicone protector to punch a hole at the bottom of the centrifuge tube.
Figure 7. Preparation of the swim-up apparatus using an embryo transfer tube. In order to introduce the sperm suspension to the bottom of the swim-up tube without allowing it to come in contact with the HIV-1-free medium of the upper layer, an outer and inner embryo transfer (ET) tubes were utilized. First, the outer sheath of the ET tube was set in the centrifuge tube containing the swim-up medium (1). After introducing the inner (embryo transfer) tube inside the outer sheath (2a), the sperm suspension prepared by density gradient centrifugation was underlaid beneath the medium (3b,c), so that the potentially contaminated inner tube would not directly come into contact with the culture medium. After 45 minutes, swim-up sperm was recovered from the surface of the medium (4d).

Using this method, we have processed 159 specimens from 81 seropositive men, and have found only two (1.3%) specimens to be HIV-1-positive, as examined by nested RT-PCR. When these processed specimens were applied for IVF-ICSI, 16 pregnancies were achieved among 69 IVF-ICSI trials in 30 couples, and no evidence of horizontal or vertical transmission was observed in any of the 18 babies from 14 deliveries xxxi.

3.4 Recent advances in semen-processing techniques

The efficiency of sperm washing in removing HIV-1 might also vary according to the amount of virus present in the sample. Fiole et al. xxxii evaluated the relationship between the seminal HIV-1 viral load and the efficiency of a standardized sperm washing procedure in removing HIV-1 RNA from the semen samples. In their experiment, semen samples obtained from healthy HIV-1 seronegative men were mixed with various concentrations of a concentrated HIV-1 virus fraction and incubated for 30 min before being processed by the Percoll discontinuous density gradient and the swim-up procedure. They reported persistence of viral RNA even after two standard washing procedures in semen samples containing high concentrations of HIV-1.

Loskutoff et al. xxxiii reported an interesting washing method utilizing trypsin to remove the virus from the sperm surface. Since the standard sperm-processing procedure, namely, density gradient centrifugation and the swim-up procedure sometimes fails to remove the virus, this novel method should be considered as a useful alternative option, while considering the risk of some contamination with trypsin.

4. Clinical trials utilizing processed sperm for Assisted Reproductive Technology

4.1 IUI

Semplini et al. were the first to report a sperm-processing protocol to isolate spermatozoa in 1992, in order to allow pregnancies in HIV-serodiscordant couples with a male infected partner xxxiv. The method of conception that they utilized was IUI. Although detection of the HIV genome in semen was not technically possible at that time, no case of seroconversion of the female partner was reported. Thereafter, the same author reported conducting artificial inseminations in more than 2,000 HIV-discordant couples using their swim-up method, with not a single case noted of HIV transmission xxxv.

Although IUI is a more patient-friendly method, since it is inexpensive and less invasive, the major drawbacks of IUI for infertility treatment of serodiscordant couples are 1) the necessity of immediate PCR check for the absence of virus,
and 2) the relatively high risk of transfer of HIV-1-harboring CD4(+) lymphocytes (as compared with IVF/ICSI, see below). Savasi et al reported high pregnancy rates with IUI, and they emphasized the 1) necessity of checking for female infertility factors before IUI, 2) use of fresh sperm, and 3) necessity of controlled ovarian stimulationxxxi.

Because HIV-1 testing of processed semen on the same day is difficult in most laboratories, cryopreservation of spermatozoa is necessary to allow viral validation. Since freeze-thawing deteriorates both the sperm motility and viability, intrauterine insemination (IUI) with thawed spermatozoa might not result in successful pregnancyxxxii and IVF-ICSI may be necessary. Thus, only a limited number of clinics perform IUI.

### 4.2 IVF/ICSI

IVF/ICSI is necessary for the serodiscordant couples who 1) experience repetitive failed IUI, 2) have female infertility problems, and 3) fail to yield a sufficient number of sperm after semen processing. Marina et al were the first to report the results of artificial insemination programsxxxiv and intracytoplasmic sperm injection (ICSI) in serodiscordant patientsxxxv.

Since this procedure predominantly utilizes freeze-thawed sperm, there is some concern about deterioration in the sperm DNA integrityxxxvi, especially in chronically infected individualsxxxvii. However, Frainais et al demonstrated no impairment of sperm DNA after the freeze-thawing procedure in HIV-1 infected men, using terminal uridine nick-end labeling. Also, HIV-1 infection does not seem to deteriorate either the embryo development or the ICSI outcomexxxviii. Furthermore, there is the remote risk of the fertilized sperm harboring infectious HIV-1 particle(s), and consequently, of the infected embryo becoming the source of infection of the pregnant mother. There is also an experimental report of vertical transmission of the HIV-1 gag gene by human spermxxxix. However, this type of infection seems to be extremely rare, since there is no report of the birth of an infant with congenital HIV-1 infection to those serodiscordant couples.

### 4.3 Clinical efficacy of assisted reproduction using processed and PCR-checked semen

The efficacy of HIV-1 transmission through sexual intercourse is relatively low; Gray et al reported that the estimated risk of male-to-female transmission is about 1 event per 1000 coital acts (0.0011 per act)xl. Accordingly, large numbers of trials were needed to confirm the clinical efficacy of assisted reproductive techniques using processed semen.

Bujan et al recently reported the cumulative results of assisted conception at eight centers in Europexli. Out of the 3390 assisted reproduction cycles (2840 intrauterine inseminations, 107 in-vitro fertilizations, 394 intra-cytoplasmic sperm injections and 49 frozen embryo transfers) using processed semen, there were no cases of female seroconversion occurring following treatment in the 3272 cycles for which the results were known; thus, the calculated contamination risk was zero (95% CI, 0–0.09%), endorsing the efficacy of this clinical procedure.

### 5. Conclusion

Several possibilities to bear their own children are now available for serodiscordant couples with HIV infection; data on the safety of medically assisted procreation (IUI, IVF and ICSI) using processed semen are accumulating. Medical professionals treating these couples should have a precise knowledge of the basic science, and patients must be informed about the various solutions available for having children safely, including the advantages and disadvantages of each, with only a very remote risk of HIV-1 transmission through these treatments.

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