Liquid nitrogen vapor is comparable to liquid nitrogen for storage of cryopreserved human sperm: evidence from the characteristics of post-thaw human sperm

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Objective: To compare the differences in the characteristics of post-thaw human sperm after storage in either liquid nitrogen (LN_2; −196°C) or LN_2 vapor (−167°C).
Design: Experimental study.
Setting: University hospital.
Patient(s): Thirty healthy volunteers who agreed to donate their normal semen samples for infertility or research were included in the study.
Intervention(s): Semen samples (n = 30) were divided into eight aliquots and frozen. Four aliquots of each human semen sample were stored in LN_2 (−196°C), and the other four aliquots were stored in LN_2 vapor (−167°C). After 1, 3, 6, or 12 months, samples were thawed and analyzed.
Main Outcome Measure(s): The motility was evaluated by the manual counting method. The viability was estimated by eosin staining. The morphology was analyzed by Diff-Quik staining. The sperm DNA integrity was determined with acridine orange fluorescent staining, and acrosin activity was assayed by the modified Kennedy method.
Result(s): The characteristics of post-thaw human sperm, including motility, viability, morphology, DNA integrity, and acrosin activity, showed no significant difference between LN_2 and LN_2 vapor storage for the different time periods.
Conclusion(s): LN_2 vapor was comparable to LN_2 in post-thaw sperm characteristics, suggesting that LN_2 vapor may be substituted for LN_2 for the long-term storage of human sperm. (Fertil Steril® 2015;104:1253–57. ©2015 by American Society for Reproductive Medicine.)
Key Words: Storage, human sperm, liquid nitrogen vapor, liquid nitrogen

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Human sperm cryopreservation has been extensively used in assisted reproductive technology centers and human sperm banks. The routine storage method uses liquid nitrogen (LN_2), in which all metabolic processes are arrested (1). However, it has been reported that infectious pathogens such as hepatitis B virus or human immunodeficiency virus can not only survive but also cross-contaminate other samples in the

same LN_2 container especially when the samples are not fully sealed (2–4). Therefore, it is necessary to seek safer measures to store cryopreserved sperm samples.

It has been reported that storage of cryopreserved specimens in LN_2 vapor is one way to prevent cross-contamination with occult infectious agents (5). However, the temperature of LN_2 vapor is higher than that of LN_2, which may affect the quality of cryopreserved human sperm (6). Although some studies have reported that there is no obvious difference in sperm quality between LN_2 vapor and LN_2 for human sperm storage, the duration of storage (from 3 days to 3 months) was short (7–9). These studies also did not evaluate the acrosin activity of preserved sperm, which is affected by cryopreservation (10) and can be considered as a predictor of sperm fertilizing capacity in standard IVF (11, 12). Moreover, owing to the reduced potential for cross-contamination, LN_2 vapor might be preferred for use in human sperm banks in China, where the quarantine of donor gametes is necessary until their infectious disease status can be determined after storage for at least 6 months (13). Therefore, it is necessary to determine the effect of LN_2 vapor for long-term storage on the quality of post-thaw human sperm.

In the present study, we compared the motility, viability, morphology, DNA integrity, and acrosin activity of post-thaw human sperm that had been stored in the LN_2 (−196°C) and LN_2 vapor (−167°C) for different durations after cryopreservation.

MATERIALS AND METHODS

Ethics Statement

The study protocol was approved by the Ethics Review Board of the Center for Reproductive Medicine of Shandong University. Informed consent for participation was obtained from all subjects.

Semen Collection and Assessment

Donors who visited the human sperm bank affiliated with Shandong University during the period June to September 2012 were recruited for the present study. Semen samples were obtained from a total of 30 male healthy donors who donated sperm for use in artificial insemination by donor or IVF to infertile couples and research after a recommended minimum of 3 days and a maximum of 7 days of sexual abstinence. All samples were collected by masturbation into a sterile container. The only ejaculates used in this study exhibited normal semen quality as follows: volume $\geq$3.0 mL, sperm concentration $\geq 60 \times 10^6$/mL, and progressive (PR) motility $\geq$50%. After seminal liquefaction, a routine semen analysis was performed to determine semen volume, concentration, motility, morphology, and round cells according to 5th edition of the World Health Organization (WHO) manual (2010) (14) for each sample. The semen samples that were not liquefied within 1 hour or in which the numbers of round cells exceeded 5×10^6/mL were excluded. The DNA integrity of sperm in fresh semen was evaluated by the acridine orange (AO) test.

Cryopreservation of Semen Samples

Each semen sample was mixed 1:1 ratio (v:v) with cryoprotectant medium, which mainly included 14% (v/v) glycerol, 20% (v/v) egg yolk, 0.072 M glucose, and 0.039 M saline sodium citrate (15) by pipetting. The mixture was equilibrated at room temperature for 10 minutes and then divided equally into eight cryovials (Greiner Bio-One). All cryovials containing the semen-cryopreservation medium mixtures were uniquely numbered and suspended in the liquid nitrogen vapor (10 cm above the level of LN_2, −80°C) for 25 minutes to allow initial slow-rate freezing. Then four aliquots of each sample were plunged into LN_2 (−196°C), and the remaining four aliquots were stored in the LN_2 vapor container (MVE 1500 Series-190 GB, −167°C) for 1, 3, 6, and 12 months until they were thawed and checked. The temperature −167°C is set up by this storage unit with the automatic induction and stably maintained by adding more LN_2 when the temperature is higher than −167°C through the LN_2 supply system Chart-MVE vapor freezer. It is the actual temperature of the highest boxes that is displayed on the control panel. A temperature probe is a part of this system and is located at the level of the highest boxes inside of the container. The locations of semen aliquots, the temperature probe, and the level of LN_2 in the LN_2 vapor container are shown in Supplemental Figure 1. The cryovials containing samples were located at a position in the tank that was at the same level as the monitoring probe (Supplemental Fig. 1). The temperature of the lower boxes is below −167°C.

Thawing of the Cryopreserved Sperm Samples

Samples were removed from storage in LN_2 or LN_2 vapor and immediately incubated in a 37°C water bath for 10 minutes. Then the water outside the cryovials was wiped. After the samples were thawed, the post-thaw parameters of motility, viability, morphology, DNA integrity, and acrosin activity were checked.

Determination of Sperm Motility

The motility parameters of the precryopreservation and thawed sperm aliquots were analyzed by the manual counting method in a Makler sperm counting chamber under phase-contrast microscopy (BX43; Olympus) at ×200 total magnification. In brief, a motile sperm was defined as a cell having a PR or nonprogressive (NPR) motion, with NPR sperm showing clear flagellar movement but without a change in position. Immotile sperms include all nonmoving cells without flagellar motion and sperm heads without a flagellum. To avoid the interobserver variation in the study, all evaluations were performed by a single observer.

Test of Sperm Viability

Sperm viability was assessed by the eosin technique as recommended by the WHO (14). For viability tests, approximately 5 μL of fresh or thawed semen was mixed with 5 μL of 0.5% eosin Y stain (Sigma) on a glass microscope slide and, after 30 seconds, examined under light microscopy (BX43;