Optimizing human semen cryopreservation by reducing test vial volume and repetitive test vial sampling

Christian F. S. Jensen, B.Sc.,[a,d,e] Dana A. Ohl, M.D.,[a] Walter R. Parker, M.D.,[a] Andre M. da Rocha, Ph.D.,[b] Laura M. Keller, A.A.S.,[b] Timothy G. Schuster, M.D.,[a] Jens Sonksen, M.D., Ph.D.,[d,e] and Gary D. Smith, Ph.D.[a,b,c]

[a] Departments of Urology, [b] Obstetrics and Gynecology, and [c] Physiology, University of Michigan, Ann Arbor, Michigan; [d] Department of Urology, Herlev University Hospital, Herlev, Denmark; and [e] Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Objective: To investigate optimal test vial (TV) volume, utility and reliability of TVs, intermediate temperature exposure (−88°C to −93°C) before cryostorage, cryostorage in nitrogen vapor (VN_2) and liquid nitrogen (LN_2), and long-term stability of VN_2 cryostorage of human semen.
Design: Prospective clinical laboratory study.
Setting: University assisted reproductive technology (ART) laboratory.
Patient(s): A total of 594 patients undergoing semen analysis and cryopreservation.
Intervention(s): Semen analysis, cryopreservation with different intermediate steps and in different volumes (50–1,000 μL), and long-term storage in LN_2 or VN_2.
Main Outcome Measure(s): Optimal TV volume, prediction of cryosurvival (CS) in ART procedure vials (ARTVs) with pre-freeze semen parameters and TV CS, post-thaw motility after two- or three-step semen cryopreservation and cryostorage in VN_2 and LN_2.
Result(s): Test vial volume of 50 μL yielded lower CS than other volumes tested. Cryosurvival of 100 μL was similar to that of larger volumes tested. An intermediate temperature exposure (−88°C to −93°C for 20 minutes) during cryopreservation did not affect post-thaw motility. Cryosurvival of TVs and ARTVs from the same ejaculate were similar. Cryosurvival of the first TV in a series of cryopreserved ejaculates was similar to and correlated with that of TVs from different ejaculates within the same patient. Cryosurvival of the first TV was correlated with subsequent ARTVs. Long-term cryostorage in VN_2 did not affect CS.
Conclusion(s): This study provides experimental evidence for use of a single 100 μL TV per patient to predict CS when freezing multiple ejaculates over a short period of time (<10 days). Additionally, semen cryostorage in VN_2 provides a stable and safe environment over time. (Fertil Steril® 2015;103:640–6. ©2015 by American Society for Reproductive Medicine.)
Key Words: Semen cryopreservation, test vial, liquid nitrogen vapor, fertility preservation

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/jensenc-semen-cryopreservation-test-vial-volume-sampling/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

Sperm cryopreservation is an essential tool in preservation of fertility in oncology and vasectomy patients, and when male partners are unavailable during assisted reproductive technology (ART) procedures. Although years of research have resulted in improvements of sperm cryopreservation, patients are still facing loss of a significant amount of spermatozoa with decrease in motility and viability after thawing of samples (1, 2).

Cryosurvival rates [(post-thaw motility/pre-freeze motility) × 100] are usually reported to be ~50%, with higher rates in healthy donors and lower rates in patients cryopreserving for different pathologies (3–6). Some laboratories use pre-freeze semen parameters to predict the varying cryosurvival between individuals (3), however this prediction is not accurate in all patient subgroups (3, 6). In addition, intersample variability exists with

Received August 21, 2014; revised and accepted December 10, 2014; published online January 10, 2015.
C.F.S.J. has nothing to disclose. D.A.O. has nothing to disclose. W.R.P. has nothing to disclose. A.M.d.R. has nothing to disclose. L.M.K. has nothing to disclose. T.G.S. has nothing to disclose. J.S. has nothing to disclose. G.D.S. has nothing to disclose.
Reprint requests: Gary D. Smith, Ph.D., Department of Obstetrics and Gynecology, University of Michigan, 6428 Medical Sciences Building I, 1301 E. Catherine St., Ann Arbor, Michigan 48109-0617 (E-mail: smithgd@umich.edu).

Fertility and Sterility® Vol. 103, No. 3, March 2015 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2014.12.107

varying cryosurvival rates from sample to sample in the same patient (7). Interindividual and intersample variability is not of great concern in healthy donors, because their good pre-freeze semen parameters leave room for a decrease in semen quality after thaw. This is not the case in patients with low semen parameters who are banking sperm for testicular cancer and other malignancies (3, 4, 6, 7). In these cases it is important to estimate the cryosurvival accurately. This is done by aliquotting a test vial (TV) that may be used specifically to assess post-thaw motility shortly after cryopreservation. In this way the cryosurvival rate can be calculated and the patient can be counseled regarding the expected procedure and the estimated number of semen samples that need to be frozen to meet the anticipated sperm needs for the selected procedure. However, the benefit of doing a test thaw remains unproven, because the utility and reliability of TVs have not been reported.

Cryostorage in liquid nitrogen (LN_2) has been the method of choice for years owing to superior post-thaw motilities compared with other procedures (8). The better results in LN_2 can be explained by LN_2 providing temperatures below the glass transition temperature of water, which is frequently quoted to be around −130°C (9). Once below the glass transition temperature, cellular activity and ice formation which induce spermatozoa cryodamage cease (10). However, of concern with LN_2 immersion as the cryostorage environment, intratank hepatitis B was transmitted from a bone marrow sample cryostored in LN_2, resulting in acute hepatitis B infection (11). Other viruses, including hepatitis C virus and human immunodeficiency virus, can survive in LN_2 (12).

Although the risk of cross-contamination in cryopreservation of human semen remains theoretic, effort to reduce this risk should be adopted by all ART laboratories (12). Assessment of patient viral and infectious disease status before semen cryopreservation may not be possible, depending on the time course of initiating treatment. Additionally, testing for viral and infectious agents at a time of unrelated life-threatening disease diagnosis may have unwanted and possibly deleterious emotional and psychologic effects. Therefore, storing all potentially infectious cryopreserved human tissues, including semen samples, in gaseous environments is an option and has not been reported to facilitate viral cross-contamination. Nitrogen vapor (VN_2) routinely provides temperatures below −150°C and post-thaw motility similar to LN_2 (5, 13, 14).

After evaluating the methodologies used in studies comparing LN_2 and VN_2 cryostorage, and before initiating VN_2 storage in our laboratory, specific questions arose regarding procedural cryopreservation when storing in VN_2, including: 1) optimal TV volume; 2) utility of TVs to predict cryosurvival of future ART procedure vials (ARTVs) within the same sample and between samples in the same patient; 3) requisite of an intermediate temperature exposure (−88°C to −93°C for 20 minutes) after initial cooling to 4°C and before cryostorage; 4) equivalence of VN_2 and LN_2 storage; and 5) long-term stability of VN_2 cryostorage of human semen. Therefore, our objectives were to investigate the minimum optimal volume of TVs, the efficacy of pre-freeze semen parameters and TVs in predicting cryosurvival of ARTVs and the necessity of obtaining a TV for every ejaculate a patient supplies. In addition, experiments were designed to confirm that storage of semen in VN_2 was similar to LN_2 and if an intermediate temperature step was necessary for cryopreservation when storage was in VN_2. Finally, evaluations were performed to determine if VN_2 storage provided a stable environment for cryostorage of semen over time.

MATERIALS AND METHODS

Semen Samples and Analysis

All semen samples were obtained from patients who presented to the University of Michigan ART laboratory either for semen cryopreservation or as part of an andrology evaluation. Samples were collected both off site and on site. When collected off site, samples were maintained at body temperature and delivered within 30 minutes of collection. Samples were allowed to liquefy completely at 37°C for ≥20 minutes. Manual semen analysis was performed within 1 hour of collection. Sperm concentration was obtained by counting immobilized sperm with a hemocytometer. Motility was estimated on a 37°C microscope stage with the use of a semen wet mount on a covered slide, and resulted in determination of percentage motility, percentage of sperm with forward progression, and qualitative assessment of progressive motility score (15) as: 1, sluggish; 2, slow; 3, good; or 4, vigorous/rapid. Morphology was not assessed. Institutional Review Board approval was obtained for this study. Additionally, the authors have no conflict of interest to disclose related to this study.

Cryopreservation, Storage, and Thaw

All samples were cryopreserved after analysis with serially mixing of raw semen and TEST-yolk buffer with glycerol (Irvine Scientific) to reach a 1:1 volume solution over 10 minutes and aliquotted at volumes ranging from 50 μL to 1.5 mL (dependent on the experiment) into cryovials (Cryogenic Vials, 1.8 mL; Nunc-368632). The established protocol involved cryovials on cryocanes being first placed into a glass beaker containing a thermometer and 22°C water, then placing that water beaker into an ice bath until the thermometer read 4°C. In some experiments, canes were then placed in a Handi-Freeze freezing tray (Taylor-Wharton), placed into the neck of a liquid nitrogen storage tank at a level between 38 and 29 cm above LN_2 providing a VN_2 environment between −88°C and −93°C for 20 minutes (intermediate temperature step) before plunging into LN_2 where they remained immersed until thawed (three-step protocol). Further description of individual experiments will describe if the intermediate temperature step was used before storage in the vapor freezer where temperatures are below −150°C. Samples were thawed at room temperature for 30 minutes.

Test Vial Volume and Intermediate Temperature Step Evaluation

Ten normozoospermic samples were cryopreserved in 1.8-mL cryovials with and without the intermediate step of VN_2 exposure (−88°C to −93°C; two-step versus three-step