CASE REPORT

Can prepubertal human testicular tissue be cryopreserved by vitrification?

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Objective: To assess vitrification of prepubertal human testicular tissue in vitro.
Design: Case report.
Setting: Academic research unit.
Patient(s): Two patients (6 and 12 years of age) who were to start gonadotoxic treatment for chronic granulomatous disease and acute lymphoblastic leukemia.
Intervention(s): Long-term (10-day) organotypic culture performed immediately after vitrification and warming. Fresh tissue and tissue cryopreserved by slow-freezing were used as control samples.
Main Outcomes Measure(s): Spermatogonial cell survival (MAGE-A4) and proliferation (Ki67) were evaluated by immunohistochemistry (IHC) and tubular integrity by light microscopy.
Result(s): Qualitative analysis revealed that histologic characteristics of spermatogonia and Sertoli cells were preserved, as were cell-cell cohesion and cell adhesion to the basement membrane, in vitrified tissue as well as in frozen and fresh control samples. Survival of spermatogonia and their ability to proliferate as evidenced by IHC was also confirmed in cultured fresh, slow-frozen, and vitrified tissue.
Conclusion(s): Vitrification, having the advantage of being a faster and more convenient method, shows promise as an alternative strategy to slow-freezing in the emerging field of immature testicular tissue cryopreservation. (Fertil Steril® 2011;95:2123.e9–e12. ©2011 by American Society for Reproductive Medicine.)

Key Words: Prepubertal human testicular tissue, cryopreservation, vitrification, slow-freezing, fertility preservation, long-term in vitro organotypic culture

The growing success of oncologic treatments and extension of such gonadotoxic therapies to benign pathologies make routine implementation of techniques aimed at preservation of reproductive function an increasingly important factor for long-term quality of life. Sperm banking is the only efficient and proven fertility preservation option currently available for male patients exposed to gonadotoxic treatment (1). However, this approach relies on the presence of spermatozoa and cannot be considered for prepubertal boys. Cryopreservation of prepubertal testicular tissue pieces has now emerged as an ethically acceptable strategy to preserve fertility in young boys (2–5). Controlled slow-freezing (SF) using dimethyl sulfoxide (DMSO) as a permeating cryoprotectant (CP) has proved to be a promising approach to preserve human immature testicular biopsies (6–8), although it is still considered to be experimental and there is currently no gold standard. Indeed, no effective cryopreservation protocol for immature testicular tissue has yet been established. In a previous study, a high proportion of spermatogonial (SPG) cells were lost and complete normal differentiation could not be achieved when a xenografting model was used to evaluate the efficiency of freezing and thawing processes (8). Vitrification (V) might be a better approach, because it avoids ice crystal formation and ensuing freeze injuries (9). We optimized a V protocol and observed survival, structural development, and integrity of prepubertal mouse testicular tissue in short-term (3-day) organotypic culture (10). Furthermore, we demonstrated SPG cell survival, proliferation, and differentiation, as well as Leydig cell expression of key steroidogenic enzymes, after V of prepubertal mouse testicular tissue maintained in long-term (10-day) organotypic culture (unpublished data). Using the long-term in vitro culture system we developed in the murine model, we investigated SPG cell survival and seminiferous tubule (ST) integrity after V of prepubertal human testicular tissue. These parameters are indeed of key importance for subsequent initiation of spermatogenic processes.

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CASE REPORT

Testicular tissue was obtained from two patients (6 and 12 years of age) who were to start gonadotoxic treatment for chronic

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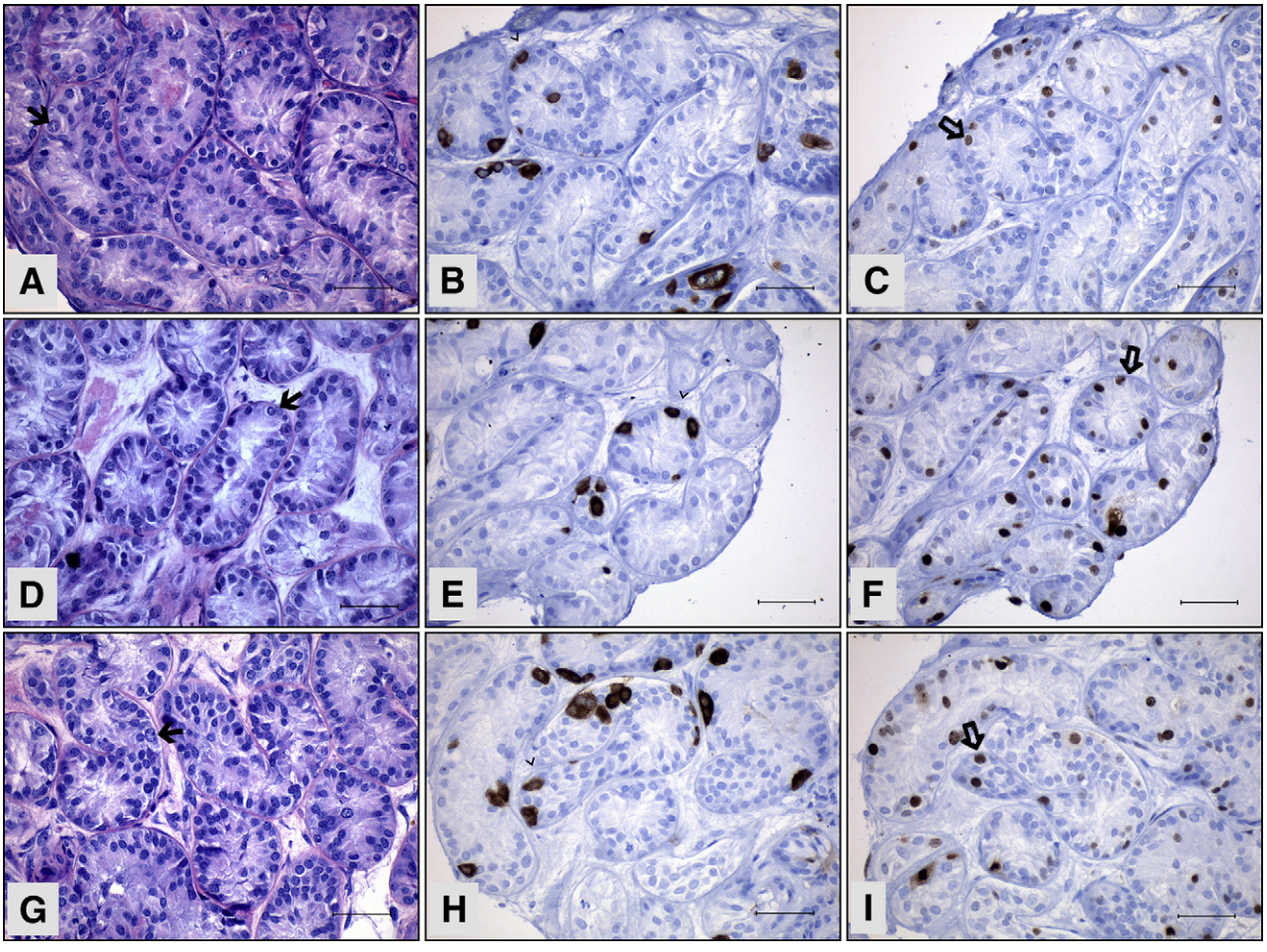
granulomatous disease and acute lymphoblastic leukemia. Testicular volumes were 4 and 6 mL, at Tanner stages I and II, respectively. After obtaining informed consent from the parents, <5% of testicular tissue was extracted from a single testis of each boy. Tissue samples were surgically retrieved under general anesthesia during the same anesthesia as used for central line placement required for administration of chemotherapeutic drugs. The tissue was then transfered in Hanks balanced saline solution (Invitrogen, Merelbeke, Belgium) at 4°C and transported on ice to the infertility unit for subsequent cryopreservation. No complications occurred during or after tissue retrieval.

The majority of the tissue was cryopreserved by SF for future clinical application and one-fourth was used for this pilot study. Tissue pieces of ~3 mm^3 were cryopreserved by both SF and V methods. Controlled SF was performed with a programmable freezer using a freezing solution made up of DMSO (0.7 mol/L) (Sigma, St. Louis, MO), sucrose (0.1 mol/L; Sigma) and human serum albumin (HSA; 10 mg/mL; Sanquin, Amsterdam, The Netherlands), as previously reported (7). Increased concentrations of CP and faster cooling and warming rates were used for V, according to the protocol we recently developed (10). Briefly, three dehydration steps were performed in increasing concentrations of DMSO (2.8 mol/L) and ethylene glycol (EG) (2.8 mol/L; Sigma) solution dissolved in a basic medium consisting of MEM/Glutamax I (Invitrogen) supplemented with 25 mg/mL HSA at 25% for 5 minutes, 50% for 10 minutes, and 100% for 10 minutes on ice. The samples were then placed on 0.3 mL insemination cryostraws (Cryo Bio System; L'Aigle, Normandie, France), which were cut with a scalpel, as described for vitrification of human ovarian tissue (11), and directly plunged into liquid nitrogen (LN_2). The cryostraws were then inserted into precooled cryovials, sealed, and stored for 24 hours in the LN_2.

After 24 hours of storage, the cryotubes were removed from the LN_2 and the straws were quickly immersed in a 37°C warming solution containing 1 mol/L sucrose dissolved in basic medium.

FIGURE 1

Histologic appearance of (**A, B, C**) fresh, (**D, E, F**) slow-frozen/thawed, and (**G, H, I**) vitrified/warmed testicular tissue from a 6-year-old boy after 10 days of in vitro organotypic culture and (**A, D, G**) hematoxylin-eosin (HE) staining or (**B, E, H**) immunohistochemical (IHC) MAGE-A4 and (**C, F, I**) Ki67 staining. Spermatogonial cells were identified on HE sections by their localization on the basement membrane, high nucleus-to-cytoplasm ratio, and white cytoplasmic halo around the nucleus (*black arrow*). Their survival was confirmed by IHC MAGE-A4 expression (*black arrowhead*) and their proliferative ability by Ki67 expression (*open arrow*). Original magnification ×200, scale bar 50 μm.



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