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Development of a Cryopreservation Protocol for Type A Spermatogonia

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ABSTRACT: The aim of this study was to develop a cryopreservation protocol for type A spermatogonia. Testes from 5- to 7-month-old calves were collected, and type A spermatogonia were isolated using two-step enzymatic digestion and Percoll separation. Cells were resuspended in minimum essential medium (MEM) supplemented with 1% bovine serum albumin (BSA) in a final concentration of 6×10^6 per mL, and the effects of different cryoprotectants and freezing protocols were tested. Cells frozen/thawed in medium containing 10% fetal calf serum (FCS) and 1.4 M glycerol or dimethyl sulfoxide (DMSO) had a significantly ($P < .05$) higher percentage of living cells compared to medium with only FCS, whereas DMSO gave a significantly better cell survival rate than glycerol did. An increase in the concentration of FCS in the DMSO-based medium to 20% had no effect on survival after freezing and thawing. Furthermore, inclusion of 0.07, 0.14, or 0.21 M sucrose in DMSO-based medium resulted in a significant improvement of cell survival, cell proliferation in culture, and colonization efficiency in recipient testes. A controlled slow-freezing rate (1°C/min) resulted in significantly ($P < .05$) more viable cells than fast (5°C/min) freezing. However, noncontrolled-rate freezing, with a comparably low cooling rate, gave even better results than the controlled-rate slow freezing. Cryopreservation in MEM-based medium containing 10% FCS, 10% DMSO, and 0.07 M sucrose using a noncontrolled-rate freezing protocol appeared to be a simple and effective way to preserve type A spermatogonia, with a high yield of almost 70% living cells after thawing. Frozen/thawed spermatogonia survived in culture and retained the ability to proliferate as determined by colorimetric and bromodeoxyuridine incorporation assays. To test whether the stem cells among the A spermatogonia retained their ability to colonize the testis of a recipient mouse, bovine spermatogonia were transplanted. This resulted in colonization 2–3 months after transplantation. In conclusion, for the first time, a method specific for cryopreservation of type A spermatogonia, including spermatogonial stem cells was developed, which allows long-term preservation of these cells without apparent harmful effects to their function.

Key words: Freezing, spermatogonial stem cells, transplantation.
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Stem cells are generally defined as colonogenic cells capable of both self-renewal and differentiation (Fuchs and Segre, 2000; Weissman, 2000). In the testis of nonprimate mammals, the A single (A_s) spermatogonia serve as the source for continuous production of male gametes (de Rooij and Grootegoed, 1998). When A_s spermatogonia divide, the daughter cells either migrate away from each other and become 2 new stem cells, or they remain connected by an intercellular cytoplasmic bridge and become A paired (A_{pr}) spermatogonia. A_{pr} spermatogonia divide further to form chains of 4, 8, or 16 A aligned (A_{al}) spermatogonia. A_{al} spermatogonia differentiate into A_1 spermatogonia, which go through a series of 6 divisions in mouse and rat, resulting in A_2, A_3, A_4, intermediate, and finally, B spermatogonia. B spermatogonia through the last mitotic division give rise to primary spermatocytes. Finally, following the 2 subsequent meiotic divisions, spermatids are formed that differentiate into mature spermatozoa.

Isolation of pure type A spermatogonia (among them spermatogonial stem cells) from various species including rodents (Bellve et al, 1977; Morena et al, 1996; Van Pelt et al, 1996) and domestic animals (Dirami et al, 1999; Izadyar et al, 2000) has been described. In principle, these spermatogonia can be preserved in 2 ways, long-term culture or cryopreservation. So far it has not been possible to preserve pure populations of spermatogonia for longer than 1 week in culture (Dirami et al, 1999). However, when spermatogonia are cultured in the presence of serum and a feeder layer, some spermatogonial stem cells survive long-term culture and repopulate recipient testes after transplantation (Nagano et al, 1998).

An alternative and probably the best method for long-term preservation of spermatogonial stem cells is cryopreservation. To date, no attempt has been made to develop an optimal protocol to cryopreserve these cells. The method employed in spermatogonial stem cell transplantation experiments is one generally used for somatic cells and cell lines. This method results in the survival of ap-

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proximately 40% of cells in isolated germ cell mixtures after the freeze/thaw procedure (Avarbock et al, 1996; Lovell-Badge, 1996; Brinster, 1998). The cell suspensions used in these studies were not enriched for type A spermatogonia. Hence, the survival rate of type A spermatogonia in this procedure is not known. Nevertheless, at least some spermatogonial stem cells survive freezing/thawing with this method.

In the present study, a method was developed to cryopreserve pure populations of bovine type A spermatogonia by testing different freezing protocols and cryoprotectants. The survival and proliferation of type A spermatogonia after cryopreservation was studied in culture, and the functionality of spermatogonial stem cells among them was assessed by transplantation of these cells into recipient mouse testes.

Materials and Methods

Testis Collection and Cell Isolation

Testes from 3- to 7-month-old calves were collected from a slaughterhouse, placed on ice, and transferred to the laboratory within 2 hours. After decapsulation, testes were minced into small pieces and suspended in minimum essential medium (MEM; Gibco Life Technology, Paisley, Scotland) supplemented with 14 mM $NaHCO_3$, 4 mM L-glutamine (both from Sigma Chemical Company, St Louis, Mo), single-strength nonessential amino acids, 100 IU/mL penicillin, 100 μg/mL streptomycin, 40 μg/mL gentamycin, and 15 mM Hepes (all from Gibco). Seminiferous epithelial cells were enzyme-dispersed and separated by the method of Van Pelt et al (1996) with minor modifications. Briefly, minced testis pieces were suspended in MEM containing 1 mg/mL collagenase and trypsin (both from Worthington Biochemical Corporation, Freehold, NJ), hyaluronidase type II (Sigma), and 5 μg/mL DNAse I (Boehringer-Mannheim, Mannheim, Germany) and then incubated at 32°C for 60 minutes in a shaking water bath operated at 140 cycles/min. After the first enzymatic digestion, the suspension was centrifuged at 300 × *g* for 1 minute and the supernatant, which contains the interstitial cells, was removed. Then fresh MEM was added and mixed with the precipitate and centrifuged as described. This was repeated 3 times until the supernatant was completely clear. After 3 washes in MEM medium and after most interstitial cells were removed, seminiferous cord fragments were incubated in MEM containing collagenase, hyaluronidase, and DNAse for 45 minutes as described above. Cells were separated from remaining tubule fragments by centrifugation at 30 × *g* and after filtration through 77- and 55-μm nylon filters, pelleted, and subjected to differential plating to eliminate the somatic cells (myoid and Sertoli cells). The pooled cells were incubated overnight in MEM containing 10% fetal calf serum (FCS; Gibco) at 37°C. After removal of Sertoli and myoid cells, spermatogonia, which remained in suspension, were collected and loaded on a Percoll discontinuous density gradient for further purification (Van Pelt et al, 1996). Fractions containing more than 50% type A spermatogonia were washed, counted, and used. The purity of the cell suspension at the various steps was determined using Nomarski interference microscopy.

Cryopreservation

Immediately after cell isolation, viability was assessed (see below) and the cells were transferred on ice to a cold room (4°C) for further preparations. Cell suspensions in 0.5-ml aliquots (6×10^6 cells per mL) were prepared. Then, an equal volume of 2× concentrated freezing medium was added dropwise to the Eppendorf vial containing the cell suspension during a period of 10–15 minutes, and after gently mixing by inverting the vial, a sample was taken for viability assessment. The freezing media were based on MEM supplemented with 1 g of bovine serum albumin (BSA) per 100 mL (MEM/BSA) and contained (final concentrations): 1) 10 % (v/v) FCS; or 2) 10 % (v/v) FCS plus 1.4 M glycerol; or 3–6) 10% (v/v) FCS and 1.4M DMSO; and either 0, 0.07, 0.14 or 0.21 M sucrose; or 7) 20% (v/v) FCS and 1.4 M DMSO.

Different freezing protocols were compared using media 2 (glycerol) and 3 (DMSO). For the controlled-rate freezing protocols, the cell suspensions were packed in ''French'' straws (inside diameter, 1.6 mm, IMV, L'Aigle, France), which were heat-sealed. The volume of the straws was approximately 220 μL. All straws were completely filled, except for a small air bubble of approximately 10 μL. The straws were placed inside a programmable freezing cabinet (Planer 10, Cryotech Benelux, Schagen, The Netherlands), which had been set to a starting temperature of +5°C, and was then cooled with a linear rate of either −1°C/min or −5°C/min to −80°C, followed by cooling at a rate of −50°C/min to −120°C. Straws were then plunged into liquid nitrogen (−196°C).

For noncontrolled-rate freezing, 1.8-mL cryovials vials (Nunc, Life Technologies, Roskilde, Denmark) containing 1.0 mL of cell suspension in freezing medium were placed in an insulated (polystyrene) container at −80°C for at least 1 day and then plunged into liquid nitrogen. To monitor the temperature during the freezing protocol, one straw or vial containing the respective freezing medium was fitted with a copper/Constantan thermocouple (wires of 0.15 mm diameter). Typical runs are shown in Figure 1. The cells were thawed by swirling in 38°C water bath for 30 seconds (straws) or 2 minutes (vials). The contents of the straw or vial was transferred to a tube and diluted slowly by adding two volumes, dropwise, of MEM supplemented with 10% FCS. Then, the cells were pooled and centrifuged at 2000 × *g* for 5 minutes, the supernatant was removed, and the pellet was resuspended in MEM/BSA. A sample was taken for viability assessment, and the remainder of the cells were used for culture or transplantation experiments.

Evaluation of Spermatogonial Survival and Proliferation

Cultures were performed at 37°C in a humidified atmosphere with 5% CO_2, and medium was changed twice per week. To investigate the survival of spermatogonia after cryopreservation, cells (5×10^4) were cultured in 96-well plates containing 200 μL of MEM medium supplemented with 2.5% FCS for 1 week. Viability of cells during the isolation and cryopreservation steps and after 1 week of culture was determined using a mixture of Calcein-AM and ethidium homodimer (1 μM per each; the so-

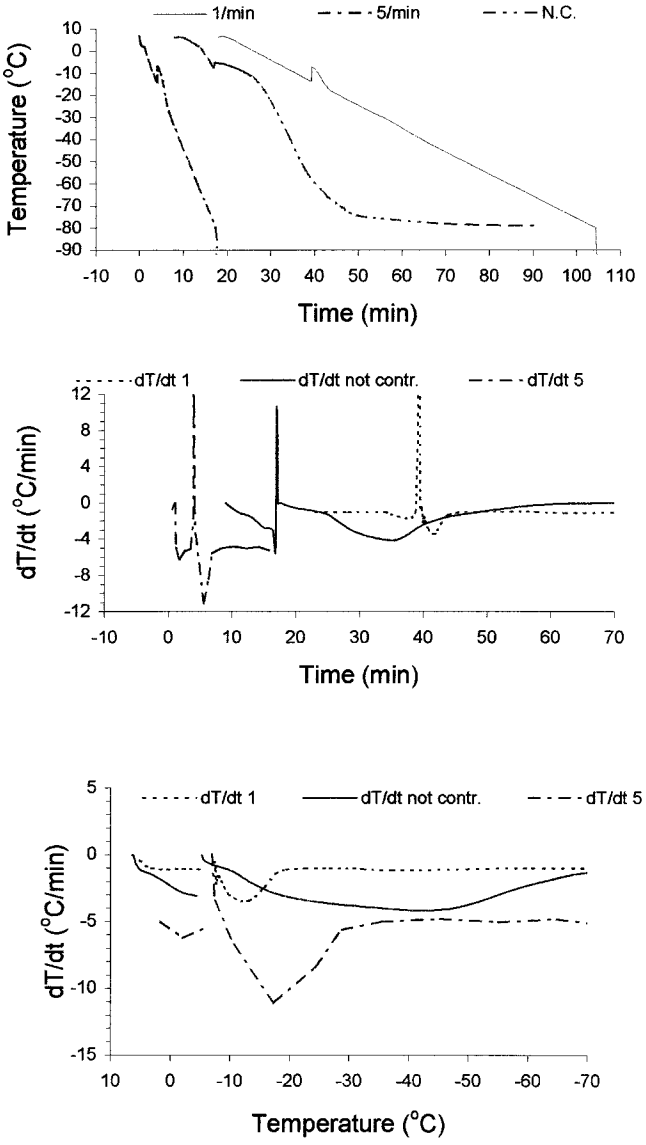


Figure 1. Temperature registration in samples during freezing of bovine type A spermatogonia in DMSO-based freezing medium using different freezing protocols. **(A)** cooling rate, **(B)** cooling rate as the function of time, **(C)** cooling rate as the function of temperature. Note that in all freezing programs, around −13°C ice nucleation occurs; however, the plateau is different with different freezing programs.

called live and dead kit, Molecular Probes, Eugene, Ore). During culture, a colorimetric assay was used to quantify proliferative activity, based on the cleavage of the tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate by mitochondrial dehydrogenases in viable cells (WST-1; Boehringer-Mannheim).

To study the proliferative activity of spermatogonia after cryopreservation, dolichos biflorus agglutinin (DBA), a marker for type A spermatogonia (Ertl and Wrobel, 1992) and bromodeoxyuridine (BrdU) incorporation was used. Therefore, cells (the same concentration) were cultured in 250 μL of medium in 8-well chamber slides (Nunc), and at days 4 and 7 of culture BrdU (0.3 mg/mL) was added to the medium, and after an incubation of 2 hours the slides were washed in PBS, fixed in Bouin solution, and the number of type A spermatogonia in the S phase of the cell cycle was determined using DBA-BrdU immunolabeling (see ''Immunohistochemistry'').

Preparation of Donor Cells, Recipient Testes, and Transplantation

Approximately 2–4 months after cryopreservation, the spermatogonia were thawed as described above and resuspended at a density of 20×10^6 cells/mL and kept on ice until transplantation. Adult NMRI mice HsdCpb (nu/nu, Harlan-Netherlands, Horst, Netherlands) were used as recipients. NMRI mice lack T cells and are immunodeficient; therefore, they were housed in specific pathogen-free conditions with all the food, water, and bedding autoclaved before use. The mice were housed in a 12-hour light:12-hour dark cycle at constant temperature, and were provided with food and water ad libitum. Recipient mice were given a fractionated dose of 1.5 and 12 Gy of x-rays in 24-hour intervals to destroy endogenous spermatogenesis. In a previous study we found that this irradiation protocol is enough to remove virtually all endogenous spermatogenesis and does not have any apparent harmful effect on supporting Sertoli cells (Creemers et al, 2002).

One month after irradiation, the recipient mice were anesthetized by intraperitoneal administration of a mixture of Fentanyl, Fluanison, and Midazolam (10 mg/kg). Testes were exteriorized through a midline abdominal incision, and donor cells (approximately 25 μL) were injected through a micropipette (Clark Electromedical Instruments, Reading, UK) via the efferent duct into the rete testis as described by Ogawa et al (1997). The contralateral testis was used as the negative control. Testes were harvested at 2–3 months after transplantation, were fixed in Bouin solution, and the colonization efficiency was assessed using DBA immunohistochemistry. The experimental protocol of this study followed the National Institutes of Health *Guidelines of the Care and Use of Laboratory Animals* and was approved by the animal care and use committee of Utrecht University.

Immunohistochmistry

Type A spermatogonia were distinguished using DBA immunohistochemistry as described by Ertl and Wrobel (1992). Bouin-fixed, paraffin-embedded testes (positive control) and cells cultured in glass chamber slides were used for immunohistochemistry. Briefly, after paraffin was removed (only for tissue sections) and sections were rehydrated, they were treated with 3% H_2O_2 (Merck, Darmstadt, Germany) for 10 minutes to inhibit endogenous peroxidase, and rinsed in phosphate-buffered saline (PBS). Incubation in 5% BSA in PBS for 15 minutes before lectin incubation was advantageous to block nonspecific adhesion sites. The sections were then incubated in DBA conjugated with horseradish peroxidase (DBA-HRP; EY Laboratories, San Mateo, Cal) 1:100 in PBS and 1% BSA for 1 hour at 37°C in a moist chamber. Following the lectin incubation the sections were rinsed in PBS 3 times. Staining of the DBA-HRP was obtained by treating the sections for 5–15 minutes with PBS containing 25 mg 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma), 1 mL of 1% nickel ammoniumsulfate solution, 1.25 mL of a 1% cobalt chloride solution, and 17 μL of 35% H_2O_2 per 50 mL.

Table 1. *Effects of different concentrations of FCS and sucrose on viability of bovine type A spermatogonia after cryopreservation with the conventional, nonprogrammed freezing protocol**

Additions to MEM-BSA	The Percentage of Viable Cells		
	Before Freezing	After Addition of Freezing Medium	After Thaw
10% FCS	86.0 ± 3.5	81.0 ± 3.6	35.5 ± 3.3[a]
DMSO + 10% FCS	86.0 ± 3.5	84.0 ± 2.8	49.3 ± 4.7[b]
DMSO + 20% FCS	86.0 ± 3.5	82.0 ± 4.1	48.5 ± 5.2[b]
DMSO + 10% FCS + 0.07 M sucrose	86.0 ± 3.5	83.0 ± 3.5	68.3 ± 2.8[c]
DMSO + 10% FCS + 0.14 M sucrose	86.0 ± 3.5	84.0 ± 2.4	66.5 ± 3.2[c]
DMSO + 10% FCS + 0.21 M sucrose	86.0 ± 3.5	85.5 ± 3.7	65.8 ± 4.2[c]

* Data represent means ± SEM of 5 independent experiments. Values with different superscripts in the same column are significantly ($P < .001$) different.

Then the slides were thoroughly rinsed in distilled water and, if necessary, counterstained with hematoxylin and Mayer for 1–2 minutes. The sections were dehydrated in a graded alcohol series, cleared in xylol, and mounted with Pertex (Cell Path; Compulink, United Kingdom). Negative control sections were incubated in 1% BSA in PBS without lectin.

To study the proliferative activity of the cultured spermatogonia more accurately, BrdU and DBA double fluorescence immunolabeling was employed. After rinsing in distilled water, the slides were transferred to 1% warm (60°C) periodic acid and incubated at 60°C for 30 minutes. After thoroughly rinsing in plain water and distilled water, the slides were washed in PBS for 15 minutes. To block nonspecific binding, the slides were incubated in 5% BSA in PBS for 10 minutes at room temperature, and subsequently overnight with anti-BrdU (Becton Dickinson, San Jose, Cal) 1:80 in PBS supplemented with 1% BSA at room temperature. After thoroughly rinsing in PBS, the slides were incubated in goat anti-mouse–Texas red (Jackson ImmunoResearch Laboratories, West Grove, Pa) 1:150 in PBS for 1 hour at room temperature in a dark chamber. After rinsing in PBS, the slides were incubated in DBA-fluorescein isothiocyanate (EY Laboratories) in PBS 1:50 for 1 hour at 37°C. After a long rinse in PBS, they were mounted in VECTAshield (Vector Laboratories, Burlingame, Cal), sealed with nail polish, and evaluated under a Nikon inverted light microscope equipped with an epifluorescence mercury lamp. Micrographs were made using Kodak Ektachrome EL 400 ASA film.

Statistical Analysis

The results are presented as means ± SEM. Statistical analysis was performed by two-sample *t*-test, and the difference was considered significant when P was $< .05$.

Results

Effect of Cryoprotectants on Viability After Thawing

More than 80% of the cells were viable after isolation (Table 1). Addition of any of the freezing media did not have a significant effect on viability. The viability after freezing and thawing was influenced by the freezing medium composition. Only 35% of the cells frozen in MEM containing 10% FCS survived after cryopreservation. Cells frozen in medium containing 10% FCS and 1.4 M glycerol or DMSO had a significantly ($P < .05$) higher percentage of living cells compared to medium with only FCS (Table 1), whereas DMSO gave significantly ($P < .05$) better cell survival than glycerol (Table 2). An increase in the concentration of FCS in the DMSO-based medium to 20% (Table 1) had no effect on survival after freezing and thawing. Inclusion of 0.07, 0.14, or 0.21 M sucrose in DMSO-based medium resulted in a significant ($P < .001$) improvement of cell survival (Table 1). In a preliminary experiment, similar cell survival was obtained with freezing medium containing trehalose instead of sucrose (not shown). Addition of 20% egg yolk to the medium containing 10% FCS, 1.4 M DMSO, and 0.07 M sucrose did not further improve cell survival (not shown).

Effect of Cooling Rate on Viability After Thaw

Controlled-rate slow freezing (1°C/min) resulted in significantly ($P < .05$) more viable cells than fast (5°C/min) freezing (Table 2). Noncontrolled-rate freezing with a

Table 2. *Effects of DMSO and glycerol under different freezing protocols on viability of bovine type A spermatogonia after cryopreservation**

Additions to MEM-BSA	Percentage of Viable Cells				
			Freezing Protocols		
	Before Freezing	After Addition of Freezing Medium	Controlled Rate, 1°C/min	Controlled Rate, 5°C/min	Noncontrolled Rate
DMSO + 10% FCS	83.0 ± 3.9	77.3 ± 6.2	54.9 ± 3.9[a]	38.9 ± 4.3[b]	59.7 ± 4.8[a]
Glycerol + 10% FCS	83.0 ± 3.9	77.5 ± 6.6	34.3 ± 4.6[b]	28.6 ± 3.9[b]	. . .

* Data represent means ± SEM of 3 independent experiments. Values with different superscripts are significantly ($P < 0.05$) different.

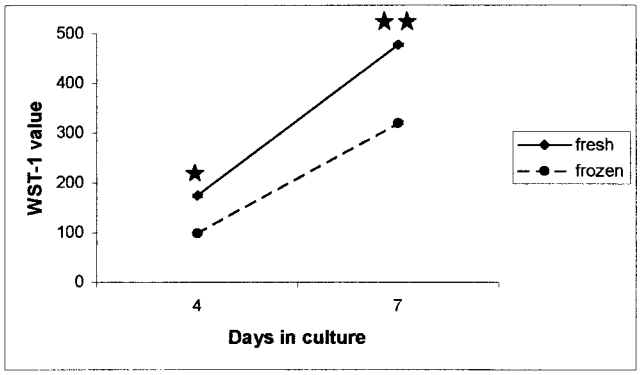


Figure 2. Viability and proliferative activity of fresh or frozen/thawed bovine type A spermatogonia in culture as determined by WST-1 colorimetric assay. Data represent means ± SEM of 3 independent experiments. *$P < .05$, **$P < .001$.

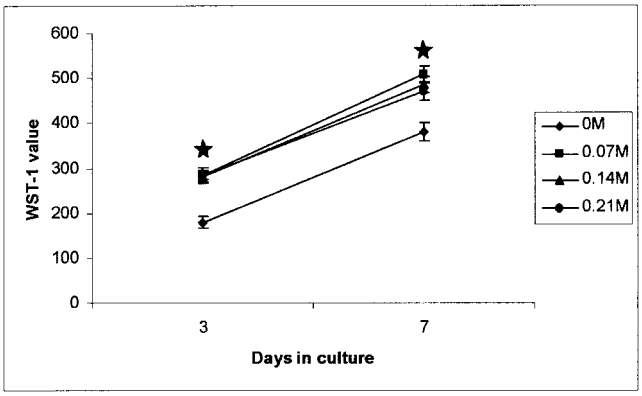


Figure 3. Effect of addition of sucrose during cryopreservation on proliferative activity of bovine type A spermatogonia during culture as evaluated by WST-1 colorimetric assay. Data represent means ± SEM of 3 independent experiments. *$P < .05$.

comparably low cooling rate resulted in an even higher percentage of living cells. Registration of temperature during freezing with different programs showed that ice nucleation occurs around −13°C in all programs; however, the noncontrolled method had a longer plateau after ice nucleation compared to the controlled methods, in which the plateau was much shorter, and the rate of cooling after ice nucleation was clearly higher (Figure 1a). Cooling rate as a function of time or as a function of temperature is separately demonstrated in Figure 1, b and c, respectively. The cooling rate remains higher in the 1°C/min method up to the point at which the temperature has decreased to −15°C. At lower temperatures, the cooling rate of the noncontrolled-rate method starts to increase and becomes higher than that of the 1°C/min method, but the cooling rate still remains lower than that of 5°C/min method.

Survival and Proliferation of Frozen-Thawed Spermatogonia in Culture

After 1 week of culture in MEM supplemented with 2.5% FCS, more than 50% of the frozen/thawed cells were viable as determined by live and dead staining (data not shown). In addition, the WST-1 value of the frozen/thawed cells was increased significantly ($P < .001$) during culture, indicating survival and proliferation of cells in culture (Figure 2). Survival and proliferation in culture, as determined by WST-1, was significantly ($P < .001$) higher in cultures of spermatogonia that had been frozen in the media containing 0.07, 0.14, or 0.21 M sucrose than those without sucrose (Figure 3). Immunolocalization of DBA and BrdU showed that after 1 week of culture, about 30% of the frozen/thawed cells were positive for the spermatogonial marker DBA and from those, about 5%–10% were positive for BrdU (data not shown). This is similar to that of the freshly cultured cells, indicating that the proliferative activity of spermatogonia in culture was not influenced by the freeze/thaw procedure.

Transplantation

To investigate the functionality of the spermatogonial stem cells among the purified A spermatogonia after cryopreservation, cells were transplanted into recipient immunodeficient nude mouse testes. Colonization efficiency was established by determining the proportion of tubule cross-sections containing DBA-positive cells and the number of DBA-positive cells per seminiferous tubule. Three months after transplantation of either fresh or frozen/thawed cells, groups of bovine type A spermatogonia, as detected by DBA staining, were found in the tubule cross-sections of the recipient mouse testes (Figure 4). Repopulation efficiency of the fresh cells was higher ($P < .01$) than that of cells frozen in the MEM/FCS/DMSO freezing medium. The repopulation efficiency of frozen/thawed spermatogonia was significantly ($P < .05$) higher for cells frozen in the MEM/FCS/DMSO freezing medium containing 0.07 M sucrose compared with cells frozen in the same medium but without sucrose. After transplantation of both freshly isolated and cryopreserved spermatogonial stem cells, no differentiated bovine germ cells were observed in the recipient mouse testes. No DBA-positive cells were found in the control testes, confirming the specificity of this identification method (Figure 5).

Discussion

In the present study, an optimal cryopreservation protocol for bovine type A spermatogonia was developed. First, the efficiency of different penetrating cryoprotectants (DMSO and glycerol) was studied. DMSO provided a better cryoprotection for type A spermatogonia than glycerol, resulting in the survival of approximately 50% of

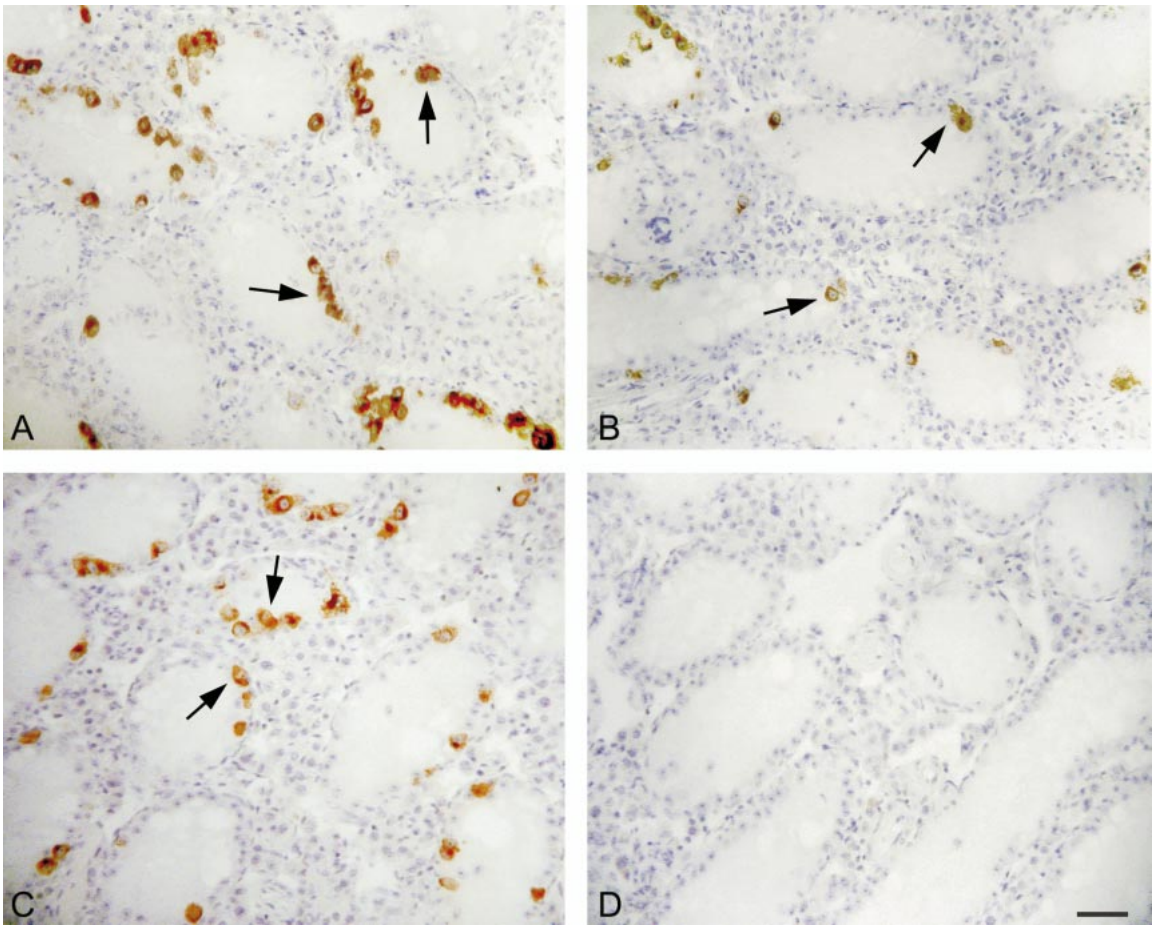
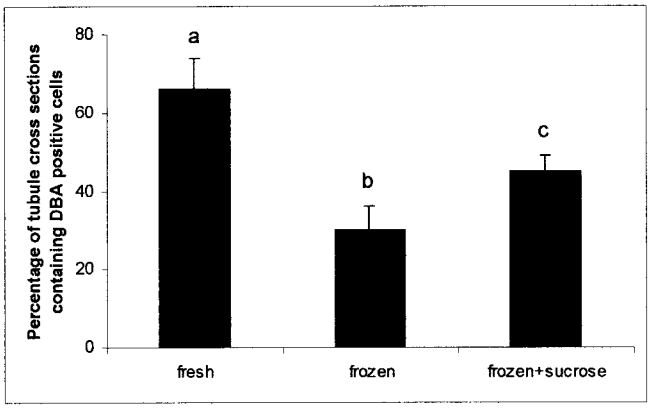


Figure 4. Tubular cross-sections of recipient mouse testes 3 months after transplantation with freshly isolated cells **(A)** or frozen/thawed cells in DMSO-based freezing medium in the absence **(B)** or presence **(C)** of 0.07 M sucrose. Note the presence of bovine type A spermatogonia (arrows) as detected by DBA immunostaining on the basal membrane of recipient testes. No DBA-positive cells were found in the contralateral control testes **(D)**. Scale bar = 50 μm.

spermatogonia after cryopreservation. A comparable survival rate was reported for the cryopreservation of hematopoietic stem cells using DMSO as cryoprotectant (Grilli et al, 1980; Donaldson et al, 1996). Second, the effect of the addition of other cryoprotectants to the freezing medium was tested. Addition of sucrose at all concentrations to the freezing medium significantly enhanced survival of spermatogonia, as well as postthaw proliferation in culture, and colonization efficiency in recipient testes. In the presence of sucrose, almost 70% of the original cell population survived the freeze/thaw procedure. Trehalose, a disaccharide of α-glucose, had the same effect on survival of spermatogonia (data not shown). Sugars are nonpenetrating cryoprotectants, which because of their positive influence on preserving cell membrane integrity, have been used successfully in cryopreservation protocols of many cell types, including embryos (Lazar et al, 2000; Oberstein et al, 2001) and gametes (Yildiz et al, 2000; Fabbri et al, 2001; Park et al, 2001; Sztein et al, 2001). In cryopreservation of hematopoietic stem cells, the polysaccharide hydroxy ethyl starch (HES) has been shown to improve cell survival (Donaldson et al, 1996; Walter et al, 1999).

The cooling rate during freezing is an important parameter for cell survival. Because much of the extracellular water is transformed into ice, the concentration of liquid water progressively decreases, leading to an extensive dehydration of the cells. Slow-cooling damage has been attributed to such phenomena as an increase of the external and internal solute (salt) concentration (Lovelock, 1953; Daw et al, 1973; Griffiths et al, 1979), the small size of the channels of unfrozen solution (Mazur and Rigopoulos, 1983), the mechanical stress of cell shrinkage (Mazur and Rigopoulos, 1983), and destabilization of membranes and proteins at low water potential (Crowe and Crowe, 1984; Rudolph and Crowe, 1985; Carpenter and Crowe, 1988). Increasing the cooling rate would reduce the time during which the cells remain vulnerable to the unfavorable conditions resulting from ice formation. Moreover, at higher cooling rates, intracellular dehydration, intracellular sol-



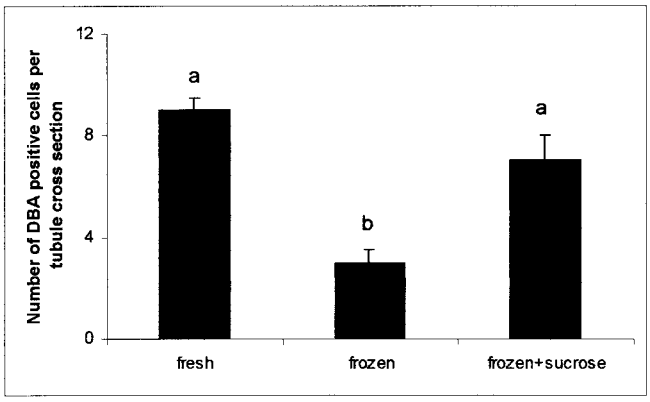


Figure 5. Colonization of bovine type A spermatogonia as detected by DBA staining in tubular cross-sections of recipient mice after transplantation of freshly isolated cells or frozen/thawed cells in DMSO-based freezing medium in the presence or absence of 0.07 M sucrose. **(A)** Percentage of tubules containing DBA-positive cells per tubule. **(B)** Number of DBA-positive cells per tubule cross-section. Data represent means ± SEM of 3 independent experiments. Bars with different superscripts are significantly different ($P < .05$).

ute concentration, and cell shrinkage may become less severe. However, when cooling rates are increased too much, dehydration may not be fast enough to prevent lethal intracellular ice formation (Mazur, 1963, 1977; Mazur et al, 1972). This mechanism is believed to be responsible for fast cooling damage, at least in a number of systems (Mazur et al, 1972; Bank, 1974; Mazur, 1977).

Different cell types may differ in optimal cooling rate, depending on the water content, size and shape of cells, and water permeability coefficient (Lp) of their cytoplasmic membrane. Small cells, such as red blood cells or spermatozoa, are known to have high permeabilities to water compared to other cells, and to tolerate ultrafast freezing in the range of 15–60°C/min, depending on species (McColm and Latter, 1986; Liu et al, 1998). Somatic cells, such as cumulus cells, blastocysts, and many cell lines (Dong and Xia, 1996; Nguyen et al, 2000; Saeed et al, 2000) can also resist rapid freezing but in relatively lower cooling rates. Hematopoietic stem cells have been successfully preserved at cooling rates between 1–5°C/min (Donaldson et al, 1996; Walter et al, 1999). There is scant information on the optimal cooling rate for spermatogenic stem cells. Two reports describe the use of a noncontrolled-rate freezing method for nonpurified spermatogonial populations, which were assumed to contain a small percentage of spermatogonial stem cells (Avarbock et al, 1996; Brinster, 1998). In our study, pure populations of bovine type A spermatogonia containing more spermatogonial stem cells were frozen successfully using a linear cooling rate of 1°C/min. The cell survival rate using a noncontrolled-rate freezing method was even better. In this method the cooling rate is variable, depending among other things, on the heat capacity of the sample and the liberation of heat of fusion. Up to the ice nucleation point, the cooling rate of the controlled slow-freezing rate method was higher than that of the noncontrolled-rate method. After ice nucleation, the cooling rate of the noncontrolled-rate method starts to increase and becomes higher than that of the slow freezing method, but it is still lower than with the fast-freezing method. The only range of temperatures in which the cooling rate of the noncontrolled-rate freezing protocol approaches that of the controlled fast-rate freezing protocol is between −30°C and −55°C (see Figure 1). The risk of ''fast cooling damage'' in this range is very small (Liu et al, 2000; Viveiros et al, 2002), which may explain why the linear cooling rate of 5°C/min appeared to be less suited for freezing bovine spermatogonia than the noncontrolled-rate freezing program. Because we have no data on the efficiency of controlled-rate freezing with rates lower than 1°C/min, the optimal cooling rate for bovine spermatogonia may be as low as or lower than 1°C/min, which is at the lower end of the range found to be optimal for hematopoietic stem cells (Donaldson et al, 1996; Walter et al, 1999).

We also demonstrated that frozen/thawed spermatogonia survive and proliferate in culture. Both the viability and proliferation of freshly isolated spermatogonia, as determined by the WST-1 colorimetric assay, were higher than that of frozen/thawed cells. This was mainly due to the diminished cell recovery following the freeze/thaw procedure and not to a lower proliferative activity after cryopreservation, because BrdU incorporation of frozen/thawed spermatogonia was similar to that of fresh cells. Reduced cell recovery following the freeze/thaw procedure was also reported by other investigators studying cryopreservation of nonpure spermatogonia from other species, including rodents (Avarbock et al, 1996; Brinster, 1998) and domestic animals (Dobrinski et al, 2000).

Purified spermatogonia consisted of a mixture of a few spermatogonial stem cells and A spermatogonia that were

already destined to develop into spermatozoa. To test the functionality of spermatogonial stem cells among these cells after cryopreservation, cells were transplanted into immunodeficient mouse testes. Three months after transplantation, frozen/thawed spermatogonia were found to have colonized recipient mouse testes, and groups of bovine spermatogonial stem cells, as detected by DBA staining, were found on the basal membrane of the tubule cross-sections of the recipient testes. Hence, spermatogonial stem cells maintain their functionality after cryopreservation. Both freshly isolated as well as cryopreserved bovine spermatogonial stem cells did not produce more advanced germ cells in the recipient mouse testes. This is probably due to the phylogenetic distance between the donor and the recipient species, as has been reported by other investigators working on xenogeneic spermatogonial transplantation (Dobrinski et al, 1999, 2000).

In summary, we developed an appropriate protocol for the cryopreservation of purified bovine type A spermatogonia, in which 70% survive the protocol and remain able to survive and proliferate in culture. The spermatogonial stem cells among these cells were found to remain functional and able to colonize recipient mouse testes after transplantation. These findings demonstrate that spermatogonial stem cells from a large domestic animal can be successfully cryopreserved, and are able to recover with full functional capability after freezing and thawing procedures. Cryopreservation of the male germ line effectively establishes the potential of generating, at any time, clones of the original male following spermatogonial stem cell transplantation to multiple recipients, and it has valuable implications in veterinary medicine as well as human medicine.

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