Development of a cryopreservation protocol for Leydig cells

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BACKGROUND: In the present study, we describe a procedure to cryopreserve the postnatal members of the Leydig cell lineage, including progenitor (PLC), immature (ILC) and adult (ALC) Leydig cells from, respectively 21-, 35- and 90-day-old rats. METHODS: The cells were resuspended in a culture medium supplemented with 1% bovine serum albumin (Dulbecco’s Modified Eagle’s Medium [DMEM]/F12) to a final concentration of 2 × 10⁶ cells/ml and the effects of varying concentrations of dimethylsulfoxide (DMSO) (5, 10, 15 or 20%) were assessed after freezing at −70°C and then storing in liquid nitrogen. After 12 months of frozen storage, these cells were thawed rapidly at 37°C and Trypan Blue exclusion staining and attachment to culture dishes were assessed as measures of viability. RESULTS: The trypan blue exclusion and attachment rates for Leydig cell stages were around 85% in the presence of 15% DMSO. After frozen storage, Leydig cell steroidogenic capacity in response to a range of LH doses, (0.01–100 ng/ml) was unchanged compared with freshly isolated control cells. Furthermore, the steady-state mRNA levels for Leydig cell specific transcripts were maintained. CONCLUSIONS: This study demonstrates that purified rat Leydig cells at a range of developmental stages can be frozen and that the cryopreserved cells retain normal function.

Keywords: Leydig cells; cryopreservation; hypogonadism; testosterone; LH response

Introduction

The common method used to treat male patients with hypogonadism is androgen replacement therapy. The goal of this method is to maintain physiological levels of serum testosterone. Androgen replacement therapy may increase muscle strength, improve osteoporosis, stabilize bone density and restore secondary sexual characteristics such as libido and erectile function (Bhasin and Bremner, 1997). There are several preparations, including unmodified testosterone itself (Aquilano and Dufau, 1985; Rommerts et al., 1982), oxytocin (Assinder et al., 2002) and insulin-like factor 3 (Burkhardt et al., 1994), that may not be produced during suppression by replacement therapy. These hormones may be beneficial for maintenance of spermatogenesis (Eddy et al., 1996; Ferlin et al., 2003; Frayne and Nicholson, 1995; Hess et al., 1997; Korach et al., 1996; Lubahn et al., 1993; Robertson et al., 1999; Toda et al., 2001). Therefore, the possibility that androgen replacement therapy does not substitute for non-functional Leydig cells deserves consideration. For this reason, alternate treatments, involving more physiological and longer-acting systems for androgen delivery, have been pursued. Transplantation of heterologous Leydig cells or gonadal tissue fragments has been proposed as a method for chronic testosterone replacement (Tai et al., 1989; van Dam et al., 1989). These approaches have been limited by the failure of the tissues and cells to produce testosterone. To overcome this problem, Leydig cell encapsulation in biocompatible and semipermeable
polymeric membranes has been shown to protect against immune rejection and to maintain viability while allowing for the secretion of desired therapeutic products, continuously and in response to specific physiologic stimulation (Machluf et al., 2003).

Although mature Leydig cells have been the primary sources for Leydig cell transplantation, techniques for transplantation of Leydig cell precursors are also advancing and the younger cells may afford advantages for this purpose because of their ability to survive and proliferate in vitro (Ge and Hardy, 1997). In parallel with the development of cell transplantation procedures, attempts have been made to cryopreserve Leydig cells. Towards this end Leydig cells from adult rats (Tai et al., 1994) and humans (Gao et al., 1994) have been successfully frozen and thawed. However, the viability of these cells as measured by testosterone production was not optimized. In addition, cryopreservation procedures for progenitor and immature Leydig cells have not been reported. In the present study, a method was developed to cryopreserve Leydig cells, at three developmental stages, by testing different freezing protocols. The survival and steroidogenic capacity under basal conditions and after maximal stimulation by Luteinizing Hormone, was measured. Leydig cells cryopreserved in Dulbecco’s Modified Eagle’s Medium (DMEM):ham’s F12 containing 15% dimethylsulfoxide (DMSO) maintained higher rates of viability and steroidogenesis compared to cells that were frozen in media without supplementary DMSO.

Materials and Methods

Animals

Sprague–Dawley rats (dams with litters of male pups, immature males and adult males) were purchased from Charles River Laboratories (Wilmington, MA). Male rats were 21, 35 and 90 days of age on the day of Leydig cell isolation. The animals were killed by asphyxiation with CO2. The animal protocol was approved by the Institutional Animal Care and Use Committee of the Rockefeller University (Protocol 91200).

Cell isolation

A complete description of the cell isolation procedure has been published (Ge and Hardy, 1997; Hardy et al., 1990; Salva et al., 2001). In brief, testes from 40 21-day-old rats were removed for isolation of progenitor Leydig cells (PLCs). Decapsulated testes were dispersed with 0.25 mg/ml collagenase (collagenase-D, Boehringer Mannheim Biochemicals, Indianapolis, IN) in medium 199 for 10 min at 34°C with shaking. The separated cells were filtered through two layers of nylon mesh, centrifuged at 250 x g and resuspended in 55% isotonic Percoll. Following density gradient centrifugation at 25 000 x g for 45 min at 4°C, the PLC fraction was collected between densities of 1.064 and 1.070 g/ml. The cells were washed with HBSS, centrifuged at 250 x g and resuspended in phenol red-free medium (DMEM-Ham’s F-12, D-2906, Sigma Chemical Co., St. Louis, MO) supplemented with 1 mg/ml bovine serum albumin (BSA). To isolate immature Leydig cells (ILCs), testes from 20 35-day-old rats were obtained and enzymatically dispersed with the following modifications to the above procedure. The testes were perfused with 1 mg/ml collagenase in medium 199 via the testicular artery before decapsulation. The ILC fraction was then collected from the Percoll gradient between densities of 1.07 and 1.088 g/ml. Fractions of adult Leydig cells (ALCs) were purified from the testes of six 90-day-old rats according to the method of Salva et al. (Salva et al., 2001). Before the Percoll density gradient centrifugation, collagenase-dispersed interstitial cells were elutriated in the Beckman JE-6B elutriation chamber (Palo Alto, CA) at a flow rate of 16 ml/min at 2000 rpm, after which ALCs were collected from the Percoll gradient between densities of 1.07 and 1.09 g/ml. Purities of Leydig cell fractions were evaluated by histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity. Enrichment of PLCs was typically to 90% purity (~90% of the cells were lightly stained). Of the remaining 10%, 6% were intensely stained. Based on previous cytological results (Shan et al., 1993), of the 4% that were unstained, fewer than 1% were macrophages. Both ILCs and ALCs were typically enriched to 92–95% and were stained intensely. Three to five separate batches of cells were used in the analyses of each Leydig cell stage. The typical yields for PLCs (40 rats), ILCs (20 rats) and ALCs (6 rats) were ~40, 20 and 20 x 10^6, respectively.

Cryopreservation

Immediately after cell isolation, viability (see below) was assessed and the cells were transferred on ice to a cold room (4°C). Cell suspensions in 0.5-ml aliquots (1–2 x 10^6 cells per ml) were prepared in 1.8-ml cryovials (Nunc, Life Technologies, Roskilde, Denmark), and an equal volume of 2× concentrated freezing medium was added to each. After gentle mixing by inverting the vials, samples were collected for viability assessment. The composition of the cryopreservation medium was based on the phenol red-free Leydig cell culture medium (LCM) that we have described previously: 1:1 DMEM:ham’s F12 supplemented with 1 mg/ml BSA, 1 mg/ml bovine lipoprotein and 25 mM HEPES (pH 7.2). The final concentrations of DMSO in the medium were 5%, 15% and 20% (Sigma, USA). After testing fetal bovine serum (FBS), we did not find that it affected cell viability and, considering that FBS contains hormones that could affect Leydig cell steroidogenic capacity, it was omitted from the cryopreservation medium.

Thawing and removal of DMSO

One week, three months and one year after cryopreservation, the vials were removed from liquid nitrogen and rapidly thawed by immersion in a 37°C water bath. Immediately after thawing, DMSO was removed by successive dilutions in 50 ml centrifuge tubes, with 20 ml of LCM. This was achieved by washing the cells three times through resuspension in the medium followed by centrifugation for 3 minutes at 30 x g, 4°C. The cell pellet was then resuspended in LCM medium.

Evaluation of cell viability

Cell viability was estimated by measuring the percentage of cells that excluded Trypan Blue staining (0.4%, GIBCO, Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, an aliquot of 1 x 10^5 cells/ml in a 0.5 ml test tube was mixed with 0.1 ml of 0.4% Trypan Blue stain and allowed to stand 5 min at room temperature. An aliquot of cells from the incubation was then loaded into a hemacytometer chamber for cell counting and numbers of non-viable (stained) and viable (excluded) cells were counted. Viability was calculated as the percentage of viable cells divided by the total cell count.

The attachment efficiency of the cells was also assessed as another index of viability. In brief, cell aliquots of 0.05 x 10^6 were cultured in 24-well plates in 1 ml of LCM in a humidified incubator at 34°C, 5% O2, 5% CO2, for 16 h. The numbers of attached (viable) and floating (presumed non-viable) cells in the culture wells were counted.
Attachment efficacy was calculated as the percentage of attached cells divided by the total cell count.

**Androgen production and radioimmunoassay**

The three different batches of isolated Leydig cells were incubated at a concentration of 0.5 × 10⁶ cells/ml (PLCs and ILCs) or 0.1 × 10⁶ cells/ml (ALCs) in a final volume of 1 ml for 3 h in a shaking water bath at 34°C. Incubations of triplicate samples were conducted in buffered LCM, with a maximally stimulating dose of LH (100 ng/ml) or with a substrate-saturating concentration of 2 ng/ml 22(R)-hydroxycholesterol (CHOL). The other two batches of PLCs and ALCs, which were cryopreserved for two years, were incubated with varying doses of LH (0 to 100 ng/ml) for 3 h. At the end of the incubations, media were stored at −20°C until measurement of androgen concentrations by radioimmunoassay. The androgen end products secreted by Leydig cells change during their postnatal development, with androsterone released by PLCs, 5α-androstan-3α,17β-diol by ILCs and testosterone by ALCs (Ge and Hardy, 1998). Accordingly, the spent media from PLC incubations were tested for androgen concentrations, while 5α-androstan-3α,17β-diol and testosterone were measured in, respectively, ILC and ALC samples. Radioimmunoassays for androsterone, 5α-androstan-3α,17β-diol and testosterone were performed as previously described (Cochran et al., 1981; Zamecnik et al., 1977). Interassay variation of the radioimmunoassay for all three androgens was between 7–10%. The intra-assay variation of the radioimmunoassay for all three androgens were within 10%. The results of three separate experiments were averaged for statistical analysis.

**Thymidine incorporation**

PLCs were incubated with or without 70 ng/ml IGF-I for 24 h whereupon the cells were labeled with [3H]thymidine (DuPont-New England Nuclear, Boston, MA) at 1 μCi/ml (specific activity 104.7 Ci/mmol) during the last 2 h of incubation. After labeling, the cells were washed twice with Dulbecco's phosphate buffered saline (PBS) and harvested. Cells were lysed in 0.5 ml hyamine hydroxide (ICN Radiochemicals, Irvine, CA) and radioactivity was measured in a liquid scintillation counter.

**Histochemical and immunohistochemical staining**

Histochemical staining for 3β-HSD activity was performed with 0.4 mM ethionolone as the steroid substrate (Payne et al., 1980) to assess cell purity. Immunofluorescent staining was performed using Leydig cells that were grown on microscope cover glasses to detect steroidogenic enzyme proteins. Cells were fixed with 4% formaldehyde, washed with PBS, and permeabilized with 0.1% w/v Saponin detergent in PBS + 10% normal serum. Nonspecific binding was blocked by incubation with 10% normal serum prior to addition of the primary antibody. Cells were incubated with different antibodies for 1 h at room temperature: 17α-hydroxylase (P450c17) polyclonal rabbit antibody provided by Dr. D. B. Hales, University of Illinois, Chicago, IL; P450 side chain cleavage enzyme (P450scs) polyclonal antibody from RDI Research Diagnostics, Inc., Flanders, NJ. Cells were then incubated with Alex488-conjugated second antibody for 1 h. Afterwards, the cells were counterstained with 4(6-diamidino-2-phenylindole) and mounted. The slides were examined under a Nikon fluorescence microscope with a filter suitable for selectively detecting the fluorescence of fluorescein isothiocyanate (green).

**RT-PCR**

Steady-state mRNA levels of the testosterone biosynthetic enzymes were evaluated, including: the mitochondrial P450scs gene Cyp11al, microsomal P450c17 (encoded by a single P450c17 gene Cyp17α), type I 3β-HSD (encoded by Hsd3b1) and type 3 17β-HSD (encoded by Hsd17b3). In addition, as the ability to synthesize testosterone first requires mobilization of cholesterol, performed by the steriodogenic acute regulatory protein (encoded by Star) upon stimulation by LH which binds to the LH receptor (encoded by Lhcr), these mRNAs were also measured. The mRNA levels for Cyp11a1, Cyp17, Hsd3b1, Hsd17b3, Star and Lhcr were analysed by RT-PCR and semi-quantitative measurement was performed relative to an internal control, ribosomal protein S16 (encoded by Rps16). The primers for Cyp11a1, Cyp17, Hsd3b1, Hsd17b3, Star, Lhcr and Rps16 were synthesized according to the published primer sequences (Ge and Hardy, 1998; Shao et al., 1995; Akingbemi et al., 2004).

First-strand cDNA synthesis from 400 ng total RNA was performed using avian myeloblastosis virus reverse transcriptase, random primers and deoxy-NTPs at 37°C for 75 min. The reaction was ended by heating at 95°C for 5 min. Then 1 μl of cDNA (at a 1:20 dilution) was added to the 50 μl reaction mixture for PCR analysis. PCR products were size-fractionated by gel electrophoresis (2% agarose) and stained with ethidium bromide, after which DNA bands from four or five reactions were quantified relative to ribosomal protein S16 (encoded by Rps16) that was used as the internal control. Quantitation was performed using an imaging system (Kodak Digital Sciences, Rochester, NY) after normalizing individual bands to the respective Rps16 density to correct for differences in gel loading. Preliminary studies showed that the targeted cDNAs were amplified linearly between 15–35 cycles of PCR and the accuracy of these measurements was spot-checked using real-time PCR.

**Statistics**

Statistical analysis of the changes in cell viability before or after thawing was performed by one-way ANOVA. Androgen levels in freshly isolated and cryopreserved cells were analysed using the Student’s t test to identify significant differences. All data are expressed as means ± SEM. Differences were regarded as significant at P < 0.05.

**Results**

**Effects of cryopreservation conditions on Leydig cell viability after thawing**

Initially, ALCs were tested for viability just after isolation, to evaluate the effects of different concentrations of DMSO in LCM. As judged by Trypan Blue staining, the viability of freshly isolated ALCs was 96 ± 0.9% (mean ± SEM, n = 4). Additions of 5 to 20% DMSO (to the medium) did not affect viability (Fig. 1A). However, cell viability was low when cells were stored in media without DMSO and thawed (Fig. 1B). The reduction in viability was prevented by addition of DMSO at 10 to 20% (Fig. 1B).

The rate of cell attachment onto culture well plate surfaces provided another parameter to judge viability. The attachment rate was highest when the medium contained 15% DMSO (Fig. 2). As judged by exclusion of Trypan Blue staining and attachment rate, addition of DMSO to the medium at concentrations as low as 5% achieves about 70% viability for PLCs and ILCs (data not shown), with 15% of DMSO in the medium achieving the highest viability. The effect of fetal calf serum (FCS) on ALC viability after thawing was also tested. Increasing the concentration of FCS in the DMSO-based
medium to 20% had no effect on survival after freezing and thawing. Given that FCS contains hormones and growth factors that may affect steroidogenic function, a serum-free cryopreservative medium was adopted. The present data suggest that 15% DMSO in FCS-free LCM is a suitable concentration for preserving Leydig cells at each of their three developmental stages.

Effect of cryopreservation conditions on androgen production

To determine whether cryopreserved Leydig cells were responsive to LH stimulation, androgen production under basal and LH-stimulating conditions was evaluated, comparing frozen-thawed cells with their freshly isolated counterparts. The frozen-thawed cells were cryopreserved for 12 months and then thawed to test testosterone biosynthetic capacity in vitro under basal and LH-stimulated (100 ng/ml LH) conditions. As shown in Figs 3 (PLC), 4 (ILC) and 5 (ALC), no statistical differences in androgen production (including androsterone, 5α-androstane-3α,17β-diol and testosterone) were observed after cryopreservation using 15% DMSO formulation, although there was an apparent trend towards increased basal androgen production in frozen-thawed cells. The primary androgens for each cell types are presented for PLCs (Fig. 3, androsterone), ILCs (Fig. 4, 5α-androstane-3α,17β-diol) and ALCs (Fig. 5, testosterone). The sensitivity of Leydig cells to LH stimulation after two years of frozen storage was also examined in the presence of varying LH concentrations. There were no significant changes in sensitivity to LH stimulation for ALCs (Fig. 6) and PLCs (data not presented). These results indicate that 15% DMSO can be used as a cryoprotectant to maintain steroidogenic pathways and LH signaling during frozen storage.

Proliferative capacity of PLCs

The proliferative capacities of thawed PLCs were evaluated in the presence of insulin-like growth factor-I (IGF-I). Proliferative activity occurred in the thawed cells, whether in the presence or absence of IGF-I. The stimulatory effect of IGF-I, ~2-fold in the presence of 70 ng/ml (Table 1) was similar in magnitude to our earlier observations. These results indicate that PLCs did not lose their proliferative capacity and their responses to IGF-I during frozen storage.

Effect of cryopreservation conditions on Leydig cell mRNA and protein levels

The mRNA levels for Lhcgr, Star, Cyp11a1, Cyp17, Hsd3b1 and Hsd17b1 were measured in cryopreserved Leydig cells.
cells. The results showed that frozen-thawed ALCs were unchanged compared to freshly isolated cells, with regard to the mRNA levels of these genes. The results for Star, Cyp11a1 and Hsd3b1 are shown in Fig. 7. The same was true for PLCs and ILCs (data not shown). As shown in Fig. 8, 3β-HSD activity signal intensities, as measured by histochemical staining, remained constant before and after cryopreservation, as did the P450c17 protein levels detectable by immunofluorescence.

Discussion
In the present study, we developed an optimized protocol to cryopreserve Leydig cells. After thawing, Leydig cells cryopreserved in the presence of 15% DMSO showed the highest viability compared to DMSO-free medium and medium with other concentrations of DMSO. Three different developmental stages of cells stored in 15% DMSO responded normally to LH stimulation and also expressed normal mRNA levels of Leydig cell specific genes including Lhcgr, Star, Cyp11a1, Cyp17,
The sensitivity of Leydig cells to LH stimulation was not different between freshly isolated and thawed cells. The proliferative capacity of PLCs with or without IGF-I stimulation was also intact after cryopreservation. This indicates that the composition of the cryopreservation medium was suitable for all three Leydig cell stages.

As a cryoprotectant, DMSO has been useful due to its low molecular weight and penetration capacity. This was demonstrated in a study that compared DMSO to other cryoprotecting agents such as glycerol and propanediol, for preservation of human fetal testis (Keros et al., 2005). However, the toxicity of DMSO is also a consideration for its application in clinical practice. Typically, the concentrations of DMSO used for frozen cell suspensions are 10–20% (Donahoe et al., 1977; Jezek et al., 2002; Shinohara et al., 2002). In the present study, the optimal concentration of DMSO was 15%, which was comparable to data obtained with testicular fragments (Donahoe et al., 1977; Jezek et al., 2002; Shinohara et al., 2002). When Leydig cells were thawed, the DMSO in the medium was removable by successive washes, leaving only trace amounts. Whatever DMSO remained did not interfere with Leydig cell function as shown in the present study. In fact, small amounts of DMSO may be beneficial for the maintenance of Leydig cell steroidogenesis, because it is

![Figure 5](image-url)  
**Figure 5**: Basal and LH-stimulated testosterone production by adult Leydig cells before or after one year of cryopreservation. ALCs were stored in medium containing 15% DMSO for one year. Cells were thawed and washed and cultured with 100 ng/ml of LH or without LH (basal). Bars show the mean (±SEM) (n = 4–6 batches of separate cell preparations).

<table>
<thead>
<tr>
<th>IGF-I concentration (ng/ml)</th>
<th>0</th>
<th>70a</th>
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<tbody>
<tr>
<td>Proliferative activity (cpm/10^4 cells)</td>
<td>5.28 ± 0.71</td>
<td>12.26 ± 2.10</td>
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Table 1: Proliferative capacities of thawed PLCs with and without IGF-I treatment

4 PLCs were incubated with or without IGF-I for 24 h and then incubated with [3H]thymidine for 2 h. Values represent the means ± SEMs for n = 6 replicate experiments.
thought to act as an antioxidant. Consistent with this hypoth-
esis, DMSO has been shown to stimulate Leydig cell steroido-
genesis and maintain the activity of cytochrome P450 enzymes
including P450scc and P450c17 (Abney and Meyers, 1987;
Myers and Abney, 1988; Quinn and Payne, 1984). In the
present study, exposure to DMSO in the cryoprotectant
medium was associated with improved basal testosterone
production.

The DMSO concentration could be lowered further to 5% in
the cryoprotectant medium. Although the 5% concentration
only provided about 50% viability for ALCs, the rate of viabi-
licity in PLCs and ILCs was maintained at 75% (data not shown).
The 5% DMSO concentration has been successfully used to
cryopreserve human fetal testes (Keros et al., 2005). Integrity
of the tissue and functional activity of the Leydig cells were
confirmed histologically and by assessing steroidogenic

![Figure 7](https://academic.oup.com/humrep/article-abstract/22/8/2160/646912)

**Figure 7:** Steady-state mRNA levels for Leydig cell specific genes in ALCs before or after one-year of cryopreservation
Adult Leydig cells were stored in medium containing 15% DMSO for one year then thawed and washed. Total RNA was prepared from freshly isolated or thawed cells. Levels of three representative mRNAs, Star, Cyp11a1 and Hsd3b1, are shown. The bars show the mean (± SEM, n = 4 batches of separate cell preparations)

![Figure 8](https://academic.oup.com/humrep/article-abstract/22/8/2160/646912)

**Figure 8:** Histochemical and immunofluorescent staining for Leydig cell specific proteins in ALCs before and after one year of frozen storage
ALCs were stored in medium containing 15% DMSO for one year then thawed and washed. In freshly isolated and thawed cells, 3β-HSD was assessed by enzyme histochemistry (Panel A) and P450c17 protein was visualized by immunofluorescence (Panel B). Magnification = ×200, Bar = 10 μm
competence in vitro (Keros et al., 2005). Although DMSO is known to be a toxic substance (at high concentrations), the viability of human testis tissue samples appear to be better after frozen storage with DMSO relative to glycerol or propanediol as cryopreservative agents (Keros et al., 2005). DMSO has also been used to cryopreserve human haematopoetic stem cells for clinical use (Larocca et al., 2005).

Mature Leydig cells obtained from humans and rats can survive after extended frozen storage, although the extent of cell viability was not assessed previously (Gao et al., 1994; Tai et al., 1994). Transplantation of isolated Leydig cells from rats and humans, either as suspensions of cells or cells encapsulated in micropheres, into the recipient male hosts has been shown to restore testosterone levels (Gao et al., 1994; Machluf et al., 2003; Tai et al., 1994). Furthermore, it has now been shown that thawed rat PLCs maintain their proliferative capacity with or without a stimulatory growth factor (IGF-1). We expect that cryopreservation will be useful to a mass quantities of Leydig cells that will be needed to achieve replacement levels of hormone production.

In summary, a cryopreservation protocol has been developed based on the addition of 15% DMSO to the culture medium for frozen storage. The medium maintains viability and steroidogenic function in three developmental stages of the Leydig cell. The potential utility of the cryopreserved medium is that it may enable investigators to amass large numbers of stored Leydig cells for use in transplantation, with the goal of achieving therapeutic androgen replacement.

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