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Follicle-Stimulating Hormone and Testosterone Stimulation of Immature and Mature Sertoli Cells *in vitro*: Inhibin and N-cadherin Levels and Round Spermatid Binding

JOHN LAMPA, JOS W. HOOGERBRUGGE,† WILLY M. BAARENDS,† PETER G. STANTON, KRISTEN J. PERRYMAN, J. ANTON GROOTEGOED,† AND DAVID M. ROBERTSON

From Prince Henry's Institute of Medical Research, Victoria, Australia and the †Department of Endocrinology and Reproduction, Erasmus University Rotterdam, Rotterdam, The Netherlands.

ABSTRACT: The *in vitro* response of Sertoli cells isolated from adult rat testes to testosterone (T) and follicle-stimulating hormone (FSH) treatment was investigated. Sertoli cells from >70-day-old Sprague-Dawley rats were isolated by a combined enzymatic treatment followed by the removal of the majority of contaminating germ cells with immobilized peanut agglutinin lectin. Sertoli cells were then cultured for 6–10 days, forming a confluent layer with a cell viability of >83% and 74–77% purity. The contaminating cells were peritubular cells (4–6%), pachytene spermatocytes (4–5%), round spermatids (<2%), elongated spermatids (<1%), and degenerating germ cells (14.8%). The proportion of degenerating germ cells decreased from 14.8% to 8.6% between days 6 and 10 in culture. After a prestimulation culture period of 4 days, FSH treatment over a 2-day period resulted in a dose-related increase of inhibin with a median effective dose (ED_{50}) value of 36.7 ± 20.4 ng/ml in comparison with an ED_{50} value of 4.4 ± 0.9 ng/ml obtained with immature Sertoli cell cultures from 20-day-old rats. Mature Sertoli cells, in contrast to immature Sertoli cells, were unresponsive to combined FSH + T treatment for the production of the cell adhesion protein N-cadherin. FSH treatment promoted the *in vitro* binding of round spermatids isolated from adult testis to adult Sertoli cells in coculture. It is concluded that mature Sertoli cells in culture are responsive to FSH in terms of inhibin production and round-spermatid binding. The lack of an FSH + T–induced increase in N-cadherin or round spermatid binding is attributed to either a reduced sensitivity, or an alteration in the regulation of mature Sertoli cells by FSH + T.

Key words: Testis, tissue culture, cell adhesion molecules, spermatogenesis.

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Previous *in vivo* studies have shown that the maturation of round spermatids through stages VII to VIII of the rat spermatogenic cycle is highly dependent on testosterone (T) and on follicle-stimulating hormone (FSH; O'Donnell et al, 1994, 1996). Reduction of intratesticular T to low (<5%) levels *in vivo* despite normal or near-normal serum FSH levels resulted in the detachment of step 8 round spermatids from the seminiferous tubule epithelium. Spermatid reattachment can be initiated by increasing the intratesticular T levels (O'Donnell et al, 1996). It was thus hypothesized that cell adhesion molecules dependent on T and probably on FSH were involved in the interaction between Sertoli cells and round spermatids. The cell adhesion molecule N-cadherin has been localized to the Sertoli cell–round spermatid junction in the rat *in vivo* (Newton et al, 1993; Byers et al, 1994), and *in vitro* studies using immature (20 days old) rat Sertoli cells in culture have shown increased levels of N-cadherin in response to stimulation by a combination of T and FSH (Perryman et al, 1996). In addition, immunoneutralization studies using N-cadherin antiserum showed an inhibition of the binding of purified round spermatids to immature Sertoli cells *in vitro* (Perryman et al, 1996). However, to date, *in vitro* studies of this kind have not been undertaken with Sertoli cells isolated from adult rats, as appropriate isolation methods were not available. It was anticipated that adult Sertoli cell cultures would respond differently to immature Sertoli cell cultures because of their increased cellular complexity (Kelly et al, 1991), previous association with germ cells (Russell, 1980; Parvinen, 1982), and apparent decrease in FSH responsiveness (Steinberger et al, 1978; O'Shaughnessy, 1979; Toebosch et al, 1989) due to changes in enzyme (e.g., phosphodiesterase) activities (O'Shaughnessy, 1979; Means et al, 1980). This study assessed the FSH and T responsiveness of cultured Sertoli cells isolated from adult (>70 days old) rats by a new procedure with regards to inhibin and N-cadherin levels and round spermatid binding to these cells *in vitro*. A comparison with Sertoli cells isolated from immature rat testis was also undertaken.

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Correspondence to: Peter Stanton, PhD, Prince Henry's Institute of Medical Research, P.O. Box 5152, Clayton, Victoria 3168, Australia. E-mail: Peter.Stanton@med.monash.edu.au

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Materials and Methods

Hormones, Enzymes, Chemicals

Testosterone (Sigma Chemical Co, St. Louis, Missouri) was prepared as a 1 mg/ml stock in 100% ethanol and was diluted in media DMEM (Dulbecco's modified Eagle medium)/0.2% BSA (bovine serum albumin) for mature Sertoli cells and in DMEM/0.1% BSA for immature Sertoli cells. The media was supplemented with 1 mM L-glutamine, nonessential amino acids, and sodium bicarbonate (Trace Biosciences, Sydney, Australia). Human FSH (HPAC hFSH, 2350 IU/mg, LH content 35 IU/mg) was provided by the Endocrine Society of Australia (Sydney). Trypsin (bovine pancreas, 110 U/mg), collagenase A (*Clostridium hystolyticum*, >0.15 U/mg), and hyaluronidase (ovine testes, 1000 U/mg) were obtained from Boehringer Mannheim (Mannheim, Germany). Peanut agglutinin lectin was obtained from Vector Laboratories (Burlingame, California). Matrigel was purchased from Collaborative Research (Bedford, Massachusetts). Sodium deoxycholate was obtained from BDH (Poole, England). DNase (type III) was from Sigma. Rats were obtained from the Monash University central animal facility. This project was undertaken with the approval of the institutional animal ethics committee under the guidelines of the National Health and Medical Research Council of Australia.

Sertoli Cell Isolation and Culture

Immature Sertoli Cells—Immature Sertoli cells were isolated from 19–20-day-old Sprague-Dawley rats as previously described (Dorrington et al, 1975; Perryman et al, 1996). Sertoli cells were plated on Matrigel (unless otherwise stated; see below) at a density of 2.5 million cells/ml per well in 24 well culture plates (Costar, Oneonta, New York) for 4 days. Cells were routinely cultured in DMEM/0.1% BSA for a total of 6 days at 37°C in 5% CO_2, 95% air, with medium renewal every second day. Hormonal treatments (FSH and/or T) in 1 ml DMEM/0.1% BSA were added, after a 4-day preincubation period, for a period of 2 days.

Mature Sertoli Cells—Mature Sertoli cells were prepared from Sprague-Dawley rats (>70 days of age). Additional and more detailed aspects of this isolation procedure will be presented elsewhere (J.W. Hoogerbrugge, unpublished data). One testis was decapsulated, teased apart, and placed in a 150-ml conical flask containing 1 mg/ml trypsin, 1 mg/ml collagenase A, and 0.5 mg/ml hyaluronidase in 20 ml of 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 1.1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.2–7.4 (PBS$^+$) and shaken in a DM10 orbital mixer incubator (Ratek Instruments, Melbourne, Australia) at 90 cycles/minute (amplitude, 1 cm) at 32–34°C for a period of 45 minutes. The resulting tubule fragments were washed in 30–40 ml 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, and 8.09 mM Na_2HPO_4, pH 7.2–7.4 (PBS) and allowed to sediment for 2 minutes at unit gravity. The volume was readjusted to 20 ml with PBS and shaken (120 cycles/minute) at 32–34°C for 30 minutes. Cell aggregates were removed every 5 minutes during this period. The cell suspension was divided into two, and PBS$^+$ was added to bring the volume of each aliquot to 50 ml in conical, 50-ml polypropylene tubes. DNase (40–160 ng in PBS) was added at this stage if required. The suspension was then processed by centrifugation at 175 × *g* for 3 minutes. Each cell pellet was resuspended in 50-ml PBS$^+$ and filtered through a 200-μm nylon mesh. The filtrates were processed by centrifugation at 110 × *g* for 3 minutes and resuspended again in a total volume of 50 ml PBS$^+$. This wash step was repeated four to five times, until a clear supernatant was obtained. The cell pellets were combined and resuspended in 10 ml PBS$^+$. To remove contaminating germ cells, 10 × 6 well plates (Costar) were precoated with peanut agglutinin lectin (PNA) by incubating 150 μg PNA/1.5 ml PBS$^+$ per well for 1 hour at 37°C. After removal of nonbound PNA, 350 μl cell suspension per well was added to the first five PNA-coated plates and shaken on the orbital shaker for 30 minutes at 50 cycles/minute at 32–34°C. Nonbound cells were then transferred to the remaining PNA-coated wells and were incubated for a further 30 minutes. The enriched Sertoli cells were pooled, processed by centrifugation (110 × *g* for 3 minutes), resuspended in DMEM/0.2% BSA (1 million cells/ml per well), and plated onto glass coverslips (14 mm diameter, precoated with Matrigel [1:8 dilution in medium, 10 μl per well] 4 hours before use) in 24-well plates (Costar) at 37°C. The cells were then cultured for a total of 6 or 8–10 days (depending on the experiment) in DMEM/0.2% BSA, with medium replacement on days 3 and 4 for the 6-day cultures and daily from day 3 to day 8 for the 10-day cultures. Hormones (FSH and/or T) were added in 1 ml DMEM/0.2%BSA for the final 2-day period for the inhibin and cadherin studies. For studies on round-spermatid binding, see below.

Studies were undertaken to evaluate the effectiveness of the lectin absorption procedure for the removal of the contaminating germ cells. Following the first lectin purification step, the proportion of contaminating germ cells was 24%, and after a second PNA step, this proportion was further reduced to 20%, indicating that there would be little advantage with additional repeats. However, this final lectin step was considered important in order to reduce the background levels of bound germ cells to the lowest level possible prior to the round spermatid–Sertoli cell adhesion assay (see below).

Cell Quantitation

Quantitation of cell numbers was performed on an Olympus BX-50 microscope (Tokyo, Japan), as recently described (Meachem et al, 1998). Nuclear profiles of Sertoli cells, peritubular cells, pachytene spermatocytes, round spermatids, and degenerating and unknown cells that did not intersect the forbidden boundaries were counted on a set of two unbiased counting frames placed directly on the video screen. A total of 100 fields were selected by a systematic, uniform random-sampling scheme generated by the Castgrid software with the use of a motorized stage (Multicontrol 2000, ITK, Lahnau, Germany). The percentage purity of each cell type was calculated as a proportion of the total cells counted in 100 fields and was used to estimate the purity of Sertoli cells in both the mature and immature Sertoli cell cultures.

For all cultures, inhibin, cadherin, and round spermatid binding were expressed per Sertoli cell; these numbers were determined by morphometric counting of Sertoli cell nuclei of n = 3–6 coverslips per hormone treatment. The within-assay variation, expressed as a coefficient of variation between coverslips,

for this method with immature and mature Sertoli cell cultures was 11 and 9.6%, respectively. For both immature and mature Sertoli cells, the proportion of Sertoli cells bound to the Matrigel-coated well surface at the completion of the culture period was approximately 10% of the number of cells plated.

Round Spermatid–Sertoli Cell Adhesion Assay

Round spermatids (steps 1–9) were isolated from adult Sprague-Dawley rats (>60 days old) as previously described, by a combined elutriation centrifugation–Percoll density gradient isolation procedure (Meistrich et al, 1981; Perryman et al, 1996). Mature Sertoli cells (1.0×10^6 cells in 1 ml) were plated onto Matrigel-coated glass coverslips (details as above) and were cultured for an initial 8-day period as described above. Immature (19–20 days old) Sertoli cells were cultured for 4 days (0.75×10^6 cells/0.3 ml) in 16-well, Matrigel-coated chamber slides (Nunc, Naperville, Illinois), as previously described (Perryman et al, 1996). Hormone treatments were added with purified round spermatids (0.6×10^6 cells/0.3 ml per well for immature Sertoli cells; 1×10^6 cells/1 ml per well for mature Sertoli cells) in McCoy's 5A culture medium, and the co-cultures were incubated at 32°C for 48 hours. The mature Sertoli cell wells were then washed five times with McCoy's 5A culture medium, and the coverslips were fixed in Bouin's fixative for 5 minutes. Binding of round spermatids to Sertoli cells was determined as described above, by a single counting frame.

Immunoassays

N-Cadherin RIA—At the completion of the hormone treatment period, Sertoli cells were solubilized for 2 hours with 250 μl/well of 0.5% (w/v) sodium deoxycholate (DOC) in Dulbecco's phosphate buffer (10 mM $NaH_2PO_4.H_20$-Na_2HPO_4, 154 mM NaCl, and 0.68 mM $CaCl_2$, pH 7.4) at room temperature. Solubilized cells from 4 wells with the same hormone treatments were pooled and processed by centrifugation ($2500 \times g$ for 30 minutes at 4°C). The resulting supernatant was removed and assayed for N-cadherin by a double antibody radioimmunoassay (RIA; Perryman et al, 1996). The N-cadherin RIA consisted of an antiserum raised in rabbits to amino acid sequence YFHLRAHAVDINGNQV (N-cadherin$^{238-252}$), as previously described (Perryman et al, 1996) with the iodinated peptide as tracer and standard.

Inhibin Immunofluorometric Assay—A two-site inhibin immunofluorometric assay (IFMA; Robertson et al, 1997) was employed. This assay has been shown to detect all known αC subunit-containing inhibin forms in human serum and follicular fluid (Robertson et al, 1997). The specificity of this assay is similar to that seen previously with the Monash inhibin RIA, which has been applied to rat Sertoli cell culture medium (Risbridger et al, 1989). The captured and labeled antibody used was a caprylic acid IgG cut of a sheep polyclonal antibody (#41) raised against human inhibin αC subunit fusion protein. The IgG fraction used as a label was immunopurified by fractionation on a column of immobilized bovine inhibin αC subunit fusion protein and then biotinylated. The assay consisted of incubation of human recombinant 32K inhibin standard (100 μl, WHO 91/624, 39–10,000 pg/well in culture medium), or medium sample (100 μl) and Tris buffer (100 μl, 50 mM Tris /HCl [pH 7.4], 154 mM NaCl, and 0.5% BSA) for 2 hours at room temperature in a 96-well microtiter plate precoated with capture antibody. The wells were washed, biotinylated antibody (100 ng/200 μl buffer) was added, and the plate was incubated for 2 hours at room temperature. The wells were washed, Eu-streptavidin (50 ng/200 μl) was added, and the plate was incubated for 30 minutes prior to extensive washing, addition of enhancement solution, and counting in a Wallac 1234 fluorometer (Wallac, Turku, Finland). The sensitivity of the IFMA was 390 pg/ml. The between-assay variation, based on the repeated measurement of a Sertoli cell culture medium sample used as a quality control, was 12% ($n = 6$).

Statistical Analyses

Significant differences at the $P < 0.05$ level between groups were determined by Peritz's F test for multiple comparisons between means (Harper, 1984). Data are expressed as mean ± standard deviation. Median effective dose (ED_{50}) values for dose-response curves were estimated by line-fitting procedures by the Allfit computer program (version 2.7, NIH, Bethesda, Maryland).

Results

The characteristics of the mature rat Sertoli cell cultures and those of parallel immature rat Sertoli cell cultures are presented in Table 1 and Figure 1. The mature Sertoli cells contained an ovoid nucleus of average diameter 15 μm (Fig. 1A) with a single prominent nucleolus and positive cytoplasmic immunocytochemical stain for inhibin (data not shown). Mature Sertoli cells exhibited larger nuclear and whole-cell size when compared with immature Sertoli cells in culture (Table 1). After 6 days in culture, mature Sertoli cells exhibited a purity of 74.4%, compared with 91.6% for immature Sertoli cells, with the cellular contaminants being peritubular cells (4.3%), as shown by immunocytochemical staining of smooth muscle α-actin (data not shown) and round spermatids (<1%) and pachytene spermatocytes (5.4%), as identified morphologically following hematoxylin and eosin staining. Degenerating cells (classification based on a shrunken appearance with pyknotic nuclei) comprised 14.8% of the total cell culture. By extending the culture duration from 6 days to 8–10 days, the proportion of degenerating cells in the mature Sertoli cell cultures decreased from 14.8 to 8.6%, while the proportion of other cells present remained relatively unchanged (Table 1; Fig. 1).

Secretion of Inhibin by Cultured Sertoli Cells

For all cultures, inhibin, N-cadherin, and round-spermatid binding were expressed per Sertoli cell, as determined by morphometric counting of Sertoli cell nuclei. Mature Sertoli cells cultured initially for 4 days, followed by a 2-day treatment period with FSH (total, 6 days), produced a dose-related increase in inhibin (Fig. 2) with an ED_{50}

Table 1. *Comparison of characteristics between immature and mature Sertoli cells* in vitro. *Data are presented as the mean ± SD. The immature Sertoli cells were assessed after a total of 6 days in culture, whereas the mature Sertoli cells were assessed after either 6 or 8–10 days in culture*

Characteristic	Immature—6 days (*n*)	Mature—6 days (*n*)	Mature—8–10 days (*n*)
Viability	90–95% (8)	86–90% (6)	83–89% (4)
Purity (%)			
Sertoli cells	91.6 ± 1.6 (3)	74.4 ± 3.3 (3)	76.8 ± 4.6 (4)
Peritubular cells	6.9 ± 2.4 (3)	4.3 ± 2.1 (3)	6.0 ± 1.6 (4)
Degenerating cells	—*	14.8 ± 2.7 (3)	8.6 ± 5.1 (4)
Pachytene spermatocytes	—*	5.4 ± 0.4 (3)	4.3 ± 1.1 (4)
Round spermatids	—*	0.3 ± 0.2 (3)	1.7 ± 1.3 (4)
Elongated spermatids	—*	<0.1 (3)	0.7 ± 0.3 (4)
Unidentified cells	1.6 ± 0.8 (3)	0.9 ± 0.4 (3)	0.9 ± 0.8 (4)
Sertoli cells			
Nuclear diameter (μm)†	12.1 ± 0.8 (3)	15.4 ± 1.4 (3)	14.8 ± 2.0 (4)
Plated cell area (μm²)‡	572 ± 12 (1)	nd	1960 ± 257 (4)
FSH ED_{50}			
Inhibin (ng/ml) (− Matrigel)	3.9 ± 0.3 (3)	nd	nd
Inhibin (ng/ml) (+ Matrigel)	4.3 ± 1.5 (3)	36.7 ± 20.4 (3)	nd
N-cadherin (ng/ml)§	9.4 ± 2.1 (3)	no dose response (3)	nd
Testosterone ED_{50}			
N-cadherin (ng/ml)§	65.6 ± 29.2 (3)	no dose response (3)	nd

n indicates the number of separate cultures; nd, not determined.
* Included with unidentified cells.
† Determined as the average of 50-cell nuclei/culture from 3 separate cultures.
‡ Calculated as the ratio of the total well area/number of Sertoli cell nuclei. The Sertoli cells under these conditions formed a confluent cell layer.
§ Assayed in the presence of testosterone (28 ng/ml) or FSH (1 μg/ml), respectively.

value of 36.7 ± 20.4 (n = 3) ng/ml (Table 1). The addition of T to these cultures had no effect on basal or FSH-stimulated inhibin levels (Fig. 3). The corresponding ED_{50} value obtained with immature Sertoli cell cultures was 4.3 ± 1.5 ng/ml (n = 3, Table 1). A comparison with ED_{50} values from cultures on plastic alone gave similar values (3.9 ± 0.3 ng/ml n = 3, Table 1). Inhibin levels in mature Sertoli cells initially cultured for 8 days with a 2-day FSH or FSH + T treatment period (total, 10 days) were reduced to 49.5% compared to the 6 days (total) culture period (data not shown).

Hormonal Control of N-cadherin Levels by Cultured Sertoli Cells

Mature Sertoli cells cultured for 6 days (as above) were unresponsive to FSH (1,000 ng/ml), T (28 ng/ml), or a combination of FSH and T, in terms of N-cadherin levels (Figs. 2, 3). In contrast, immature Sertoli cells showed a threefold increase in N-cadherin levels following stimulation with FSH and T, with an ED_{50} for T of 9.4 ± 2.1 ng/ml and an ED_{50} for FSH of 65.6 ± 29.2 ng/ml (Table 1; Figs. 2, 3) and were unresponsive to FSH or T alone. No changes in N-cadherin levels were observed between treatment groups for mature Sertoli cells cultured for a total of 10 days, as above (data not shown).

Hormonal Control of Round Spermatid Binding by Cultured Sertoli Cells

For the round spermatid binding experiments, the culture period for the mature Sertoli cells was extended to 10 days total (8 days pretreatment and 2 days treatment), in order to reduce the proportion of degenerating cells, as described above. Binding of round spermatids to mature Sertoli cells (when expressed as number of round spermatids bound per Sertoli cell) was not stimulated by FSH and T in combination (Fig. 4), although FSH treatment alone significantly ($P < 0.05$) increased the round spermatid binding by 2.5-fold (Fig. 4), compared with the binding in controls. By paired t-test, there was a significant difference ($P < 0.05$) between the FSH-treated group and either the T-treated or FSH + T–treated groups. Immature Sertoli cell cultures (6-day total), in contrast,

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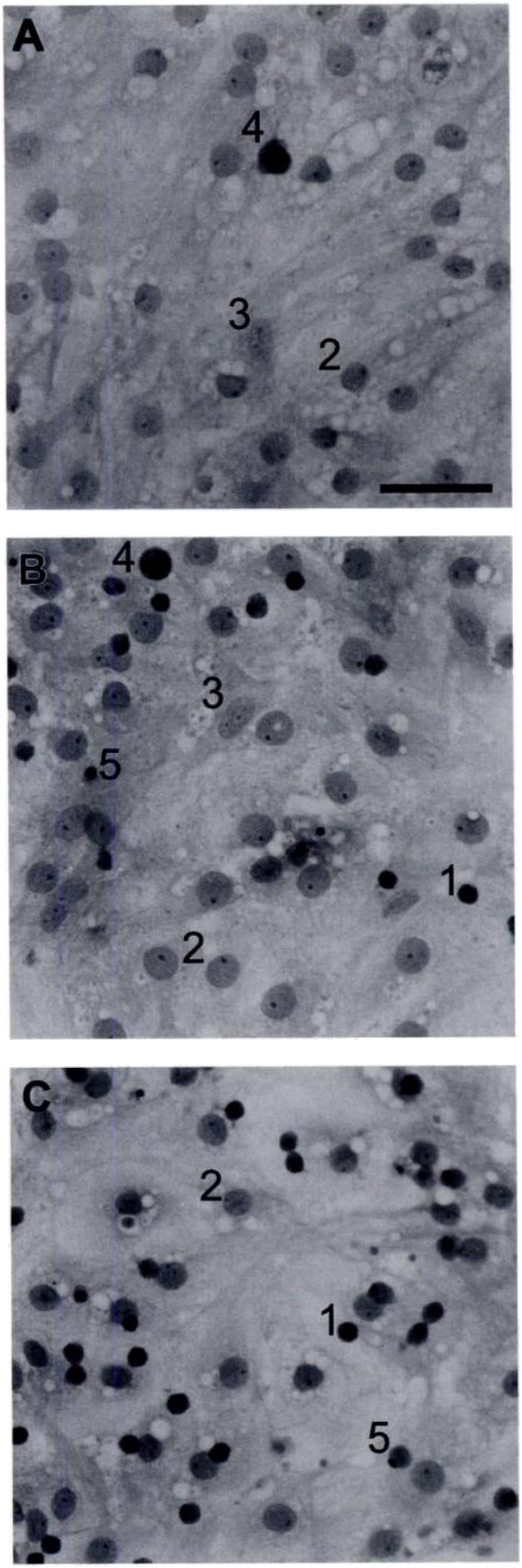
FIG. 1. Micrographs of cultured mature rat Sertoli cells and co-cultures of rat Sertoli cells with added round spermatids. Rat Sertoli cells were cultured for 8 days prior to the addition of purified round spermatids, with a further co-culture period of 2 days at 32°C. **Panel A**, Sertoli cells alone. **Panel B**, Sertoli cells with round spermatids added in medium alone. **Panel C**, Sertoli cells with round spermatids added in the presence of FSH (1,000 ng/ml). Numbers on the figures identify the following cell types: 1, round spermatid; 2, Sertoli cell nuclei; 3, peritubular cell; 4, pachytene spermatocyte; 5, degenerating germ cell. Scale bar, 50 μm.



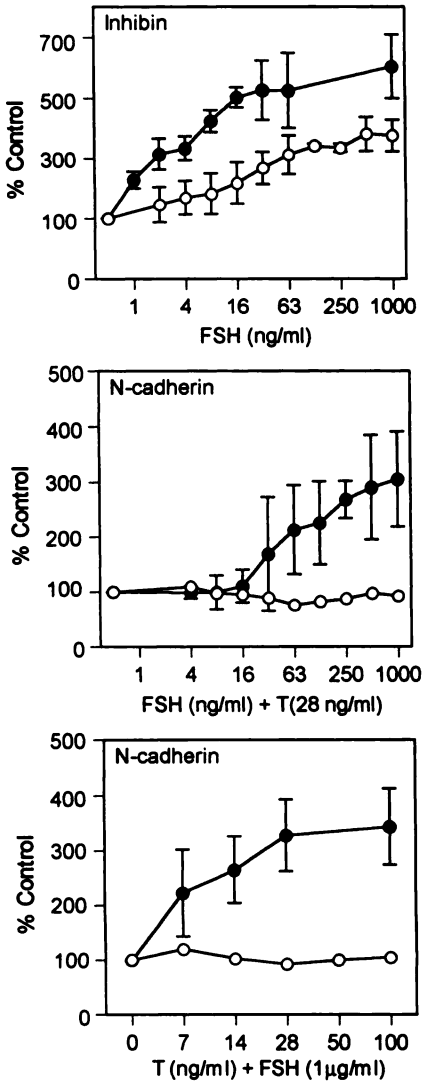


FIG. 2. Inhibin and N-cadherin responses of immature and mature Sertoli cells *in vitro* to graded doses of FSH. Immature (20 days old, [●]) and mature (>70 days old, [○]) rat Sertoli cells were cultured on Matrigel for a period of 4 days, then stimulated with increasing doses of FSH (0–1000 ng/ml) for 48 hours in the presence of testosterone (T, 28 ng/ml). Inhibin results for both mature and immature Sertoli cell cultures are the mean ± SD from three separate cultures. N-cadherin results for immature Sertoli cell cultures are the mean ± SD from three separate cultures, while results from mature Sertoli cell cultures are the average of two experiments. Results are determined as protein levels (ng) per million Sertoli cells, counted morphometrically, and are presented as percent of basal values (immature and mature Sertoli cells; inhibin, 44.3 and 171 ng/million Sertoli cells, respectively; N-cadherin, 1.2 and 3.9 ng/million Sertoli cells, respectively).

showed a significant response with FSH + T but not with FSH or T alone (Fig. 4).

When the data for the number of round spermatids per Sertoli cell nuclei are expressed as the number of round spermatids per 10,000 μm^2 of Sertoli cell culture area, for immature Sertoli cell cultures, a ratio of 0.058 was observed, similar to that previously observed by Perryman

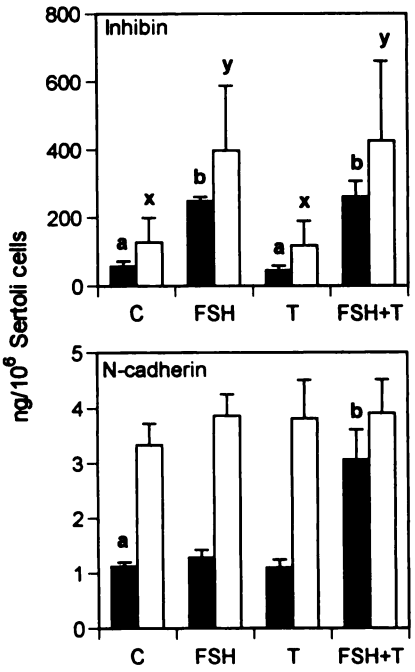


FIG. 3. Hormonal control of inhibin and N-cadherin in immature and mature Sertoli cells *in vitro*. Immature (20 days old; filled columns) and mature (>70 days old; open columns) rat Sertoli cells were cultured for 4 days, then stimulated with FSH (1,000 ng/ml), testosterone (T, 28 ng/ml), FSH (1,000 ng/ml) + T (28 ng/ml), or medium alone (C) for 2 days. Values are expressed as nanograms of inhibin or N-cadherin per million Sertoli cells. The Sertoli cell number was determined morphometrically on day 6 of culture. Data is mean ± SD from three experiments. For a vs. b and x vs. y, $P < 0.05$.

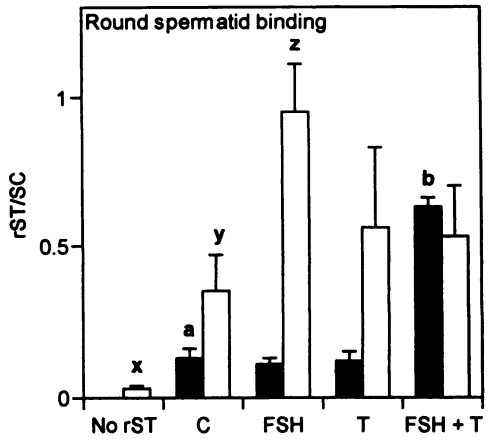


FIG. 4. Hormonal control of round spermatid binding to Sertoli cells from immature and mature rats *in vitro*. Immature (20 days old; filled columns) and mature (>70 days old; open columns) rat Sertoli cells were cultured for 4 or 8 days then stimulated with FSH (1,000 ng/ml), testosterone (T, 28 ng/ml), FSH (1,000 ng/ml) + T (28 ng/ml), and medium alone (C) for 2 days. Immature Sertoli cells were pretreated for 4 days prior to hormonal stimulation for 2 days, whereas mature Sertoli cells were pretreated for 8 days prior to hormonal stimulation for 2 days. Values are expressed as number of round spermatids (rST) bound per Sertoli cell (SC). "No rST" = no added round spermatids or hormones. The round spermatid and Sertoli cell numbers were determined morphometrically on day 6 or 10 of culture. Results for the immature Sertoli cell culture are presented as the mean ± SD of triplicate wells of a representative culture, while results for the mature Sertoli cell culture are the mean ± SD from three separate cultures. For a vs. b and for x vs. y vs. z, $P < 0.05$.

et al (1996). The corresponding ratio for adult Sertoli cells was 0.25.

Discussion

This study has shown that Sertoli cells isolated from adult rats are receptive to FSH stimulation with regard to inhibin production and round spermatid binding but are unresponsive to FSH and/or T treatment in relation to Sertoli cell N-cadherin levels. These findings point to differences in hormone responsivity between immature Sertoli cells and mature Sertoli cells in culture.

One likely explanation for the decreased FSH + T sensitivity of the mature Sertoli cell cultures in terms of N-cadherin levels is that the mature Sertoli cells are already fully differentiated and thus no longer responsive to FSH + T. Evidence of differentiation can be seen with regard to the size of the mature Sertoli cell, which, under culture conditions similar to those used for immature Sertoli cells, occupied a 3.4-fold greater surface area with a 1.7-fold greater nuclear volume. In addition, the levels of N-cadherin observed in mature Sertoli cell cultures were comparable with those seen in fully stimulated (FSH + T) immature cultures, suggesting a fully activated system. Furthermore, maximal binding of round spermatids to mature Sertoli cells was observed to be approximately 1 round spermatid per Sertoli cell, whereas in comparison, only 0.65 round spermatid per Sertoli cell was bound in the fully stimulated immature cultures. These data collectively suggest that the mature Sertoli cell is better equipped to facilitate round spermatid binding; however, the extent of binding is still far less than that observed *in vivo*, where 6–8 round spermatids per Sertoli cell have been noted (Orth et al, 1988; Meachem et al, 1996). The factors responsible for this disparity are unclear at this point; however, one variable may relate to the proportion of round spermatids in the round spermatid preparation that are available or able to bind. Previous data (Russell, 1977; Cameron and Muffly, 1991) have suggested that the strong adherence of round spermatids to Sertoli cells requires the formation of an ectoplasmic specialization junction apparatus that can only occur at a substage of step 7/8 spermatids. Spermatids at this stage represent a small proportion (<15%) of the total round spermatid preparation isolated, and thus an improved assessment can only occur with their isolation and with further characterization of the *in vitro* junctions formed between these cells and mature Sertoli cells.

Several reports have described the isolation of Sertoli

cells from sexually mature rats (Steinberger et al, 1975, 1978; Wright et al, 1989, Karzai and Wright, 1992, Simpson et al, 1992). The major difficulty experienced by researchers has been the removal of the very large number of contaminating germ cells not found in immature Sertoli cell cultures. The germ cell contamination can be reduced by rendering the rat cryptorchid (Simpson et al, 1992) or by the use of an extensive washing protocol (Wright et al, 1989; Karzai and Wright, 1992) that, however, results in a low cell yield. In the present procedure, PNA has been effective in removing a very high proportion of germ cells; however, even after two separate cycles of the PNA absorption procedure, contaminating cells were still present. This contamination was reduced by extending the pretreatment period from 4 to 8 days.

In order to match the culturing conditions with those used previously for the immature cell cultures, the mature Sertoli cells were cultured for an initial pretreatment period of 4 days plus a hormone treatment period of 2 days. However, under these conditions, the proportion of degenerating germ cells was sufficiently high to prevent reliable quantitation of the binding of added round spermatids. By extending the pretreatment period to 8 days in culture under conditions not conducive for germ cell survival (i.e., 37°C; Cameron and Muffly, 1991), the proportion of these contaminating cells was reduced from 14.8 to 8.6%. Under these conditions, the Sertoli cells were still sensitive to FSH, as seen by their inhibin responsiveness, although the levels of inhibin produced were lower than those seen after 6 days in culture. The proportion of contaminating degenerating cells was sufficiently reduced after 8 days to undertake the round spermatid binding studies.

The present procedure has the advantage of speed of cell isolation and purification with high yields and acceptable viability. Based on the number of Sertoli cells/testis determined by stereological methods, it is estimated that an adult Sprague-Dawley rat has 39 million Sertoli cells per testis (Meachem et al, 1996). The yield of Sertoli cells per testis obtained by the present procedure was determined to be 30%, based on an analysis of Sertoli cell number plated under subconfluent conditions. Our data also demonstrate that the final Sertoli cell purity depends on the culture duration.

Following a prestimulation culture period of 4 days, FSH stimulation of inhibin secretion by mature Sertoli cells over a 2-day period resulted in a 3.4-fold change with an ED_{50} value of 37 ng/ml, compared with a 5.5-fold increase with an ED_{50} value of 4.3 ng/ml for immature Sertoli cells cultured under the same conditions. These data suggest that mature Sertoli cells are approximately eightfold less sensitive than immature Sertoli cells to FSH stimulation in terms of inhibin production, and these results are consistent with previous reports documenting the relative refractoriness of the mature Sertoli cells *in vivo* (O'Shaughnessy, 1979; Parvinen, 1982) and *in vitro* (Steinberger et al, 1978) to FSH treatment. The basis for this difference in ED_{50} values may reflect the longer exposure of mature Sertoli cells to circulating FSH, thus rendering them less sensitive to exogenous FSH or to the effects of the contaminating germ cells. It is also possible that the high basal inhibin level may not allow further stimulation and that other factors may also play a role in regulation of inhibin in mature Sertoli cells.

It appears from the round spermatid binding data that FSH alone plays a role in the binding of round spermatids to the mature Sertoli cell, whereas FSH alone did not stimulate binding to immature Sertoli cells. This suggests that the control of round spermatid binding may differ between the two Sertoli cell ages for reasons that are not known. Since N-cadherin levels were not stimulated by FSH and/or T, while round spermatid binding was stimulated by FSH, this suggests that the increasing round spermatid binding is not a consequence of increased cadherin levels, and thus other FSH-sensitive factors must be involved.

The availability of an appropriate adult Sertoli cell isolation-and-culture method enables the investigation of the interactions of germ cells with Sertoli cells. Traditionally, such studies have had to rely on the use of Sertoli cells from immature rats, in which the most advanced germ cell subtype present are late pachytene spermatocytes. Hence, it is conceivable that the hormonal regulation of the interaction of later germ cells (e.g., round and elongating spermatids) is suboptimal with these immature cells. The present study supports this contention. Studies investigating the hormonal regulation of the junctional apparatus between mature Sertoli cells and spermatids are in progress.

In conclusion, these studies demonstrate a method for the isolation and culture of mature Sertoli cells from adult (>70 days old) rats with resultant high yield and high viability. Mature Sertoli cells cultured for up to 10 days were greater than 76% pure, with cellular contaminants being degenerating cells and peritubular cells with residual (<7%) pachytene spermatocytes and round and elongated spermatids. Mature Sertoli cells were responsive to FSH in terms of inhibin production, with ED_{50} values approximately eightfold less than for immature Sertoli cells cultured under identical conditions. Significant differences between mature and immature Sertoli cells were noted in the hormonal regulation of the cell adhesion protein N-cadherin and in the binding of purified round spermatids in co-culture, indicating changes in the hormonal regulation of these functions between Sertoli cells isolated from these two age groups.

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