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Assessment of the degree of contamination of rat germ cell preparations using specific cDNA probes

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Abstract

Recent reports showing a decrease in sperm count in men have brought new concerns about male infertility. Animal models have been widely used to provide some relevant information about the human male gamete, and extrapolations are made to men and to the clinical context. The present study assesses one of the methods used for separation of germ cells of the adult rat testis, namely centrifugal elutriation followed by density gradients (Percoll®). This method was chosen since it presents the best results for cell purity in separating germ cells from the rat testis. A comparison between continuous and discontinuous Percoll® gradients was performed in order to identify the best type of gradient to separate the cells. Maximal cell purity was obtained for spermatocytes (81 ± 8.2%, mean ± SEM) and spermatids (84 ± 2.6%) using centrifugal elutriation followed by continuous Percoll® gradients. A significant difference in purity was observed between elongating spermatids harvested from continuous Percoll® gradients and from discontinuous gradients. Molecular analysis was used to assess cell contamination by employing specific probes, namely transition protein 2 (TP2), mitochondrial cytochrome C oxidase II (COX II), and sulfated glycoprotein 1 (SGP1). Molecular analysis of the samples demonstrated that morphological criteria are efficient in characterizing the main composition of the cell suspension, but are not reliable for identifying minimal contamination from other cells. Reliable cell purity data should be established using molecular analysis.

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- Mitochondrial cytochrome C oxidase II (COX II)
- Sulfatedglycoprotein 1(SGP1)

Introduction

Many hypotheses for the various processes leading to conception *in vivo* are extrapolated from studies on other eutherian mammals. The methods used in animal models have to be under constant revision and development due to limitations that should not be overlooked. The highest purity of germ cells described in the literature was obtained by Meistrich et al. (1) who used a combination of mechanical and trypsin methods, centrifugal elutriation, and further separation on continuous Percoll® gradients. As a result, 96% of early spermatids, 98% of pachytene spermatocytes, and 98% of cytoplasts were obtained.

The objective of the present study was to investigate the accuracy of identifying morphological cell contamination of rat germ cells separated by a combination of methods (mechanical and enzymatic digestion, fol-

lowed by centrifugal elutriation and a further step of Percoll® purification). Transitional protein 2 (TP2), mitochondrial cytochrome C oxidase II (COX II), and sulfated glycoprotein 1 (SGP1) are cDNA (complementary DNA) probes which recognize mRNA encoding specific proteins expressed in different germ cells. TP2 is only present in elongating spermatids (steps 7-13) (2). SGP1 is a specific marker for rat Sertoli cells (3). COX II is present in mitochondria of all germ cells and Sertoli cells, but its highest expression occurs in pachytene spermatocytes (4). These cDNA probes were employed to determine the purity of samples stained with Periodic Acid Schiff and hematoxylin (PAS + H), and viewed under the light microscope. Labeling of extracted mRNA with a ^{32}P cDNA probe was detected using a PhosphorImager and the data were analyzed using computer software, thus reducing subjective bias.

The molecular approach for assessing contamination of germ cell preparations is novel and should be more rigorous than morphological identification which has been used routinely in the literature.

Material and Methods

Material for enzymatic digestion for elutriation and Percoll® separation

The material for enzymatic digestion of the testis was prepared as previously described (5).

Preparation of the cell suspension

Eight adult male Wistar rats weighing 350-550 g and aged 90-180 days were used for each experiment. The rats were killed by CO_2 asphyxiation and cervical dislocation. The abdomen was washed with 70% ethanol and the testes were dissected out, decapsulated, chopped up into 2-mm^3 pieces, and divided into two equal parts. Each part was placed in a different 50-ml Falcon tube (Becton Dickinson & Co., Lincoln Park, NJ) containing 15 ml of enzyme solution A, and the tubes were incubated for 30 min at 37°C in a shaking water bath at 100 strokes per min.

Following the first enzymatic digestion, 15 ml of dissection medium was added. The tubes were inverted 20 times with rotational motion and centrifuged for 1 min at 150 *g* in an Omnispin R centrifuge (Sorvall Instruments, DuPont Company Biotechnology Systems, Chandler Wilmington, DE). The supernatant containing interstitial cells was removed and discarded. Enzyme solution B was added to the pelleted tubules (7.5 ml of enzyme solution B), and the pellet was resuspended and incubated for an additional 30 min at 37°C in a shaking water bath at 100 strokes per min. Following the second enzymatic digestion, a further 10 ml of dissection medium was added to the suspensions. The tubule suspensions were filtered through a double 60-µm nylon gauze mesh, and pelleted at 150 *g* for 10 min at room temperature (22°C) in an Omnispin R. The supernatant was discarded, and the pellet was resuspended in 1 ml of dissection medium and aspirated with a 20-ml syringe.

Separation by centrifugal elutriation

A suspension of approximately 2 x 10^9 cells was loaded onto a JE-6B elutriator rotor (Beckman Instruments Inc., Palo Alto, CA). The conditions used to optimize both purity and yield of cells according to the diameter of the tubes used in the present study are as outlined below:

Fraction	Rotor speed (rpm)	Flow rate (ml/min)	Pump setting	Cell size (µm)
Load	3000	20	1.2	<6.5
1	2500	25	2.0	<10
2	2000	25	2.0	<14
3	2000	40	3.2	<16
4	0	70	6.0	>16

Fraction 2 was discarded and the others

were pelleted in elutriation medium at 150 *g* for 10 min in an Omnispin R.

Percoll® gradients were performed in both a continuous and discontinuous manner. The continuous gradients were performed in a Gradient Former (model 395, BIO-RAD Laboratories, Inc., Milan, Italy). The first chamber was filled only with dissection medium at a density of 1.007 g/ml. The other chamber was filled with diluted Percoll® (Pharmacia, Uppsala, Sweden, No. 225334, density of 1.134 g/ml). A density of 1.084 g/ml was obtained with 25 ml of Percoll®, 1 ml 1 mM HEPES (Gibco BRL-Life Technologies Ltd., Paisley, Scotland), and 15 ml of dissection medium. A 40-ml continuous linear gradient was poured into a 50-ml Falcon tube. For the discontinuous gradients, the different densities were obtained by diluting Percoll® with dissection medium and 1 mM HEPES (25:1). The gradients formed had four steps for the load fraction (1.084, 1.043, 1.031, 1.019 g/ml) and three for the other fractions from the elutriation (1.084, 1.043, 1.019 g/ml). These gradients were designed to separate the spermatids and pachytene spermatocytes based on the buoyant density of these cells as described in the literature (1).

The pelleted cell fractions from the centrifugal elutriator were gently layered above the gradients using a plastic Pasteur pipette. The cells were centrifuged at 1470 *g* for 30 min at room temperature in an Omnispin R.

The bands formed in the continuous gradients were collected as follows. The second and third bands from the top of the gradient were collected from the load gradients and in the other fractions the band formed in the middle of the gradient was aspirated. In the discontinuous gradients, the band at a density of 1.043 g/ml was collected. These fractions were removed using a Pasteur pipette. The top part of the gradient was removed until the desired band was reached. An unused Pasteur pipette was used to aspirate the band. Special care was taken to avoid contamination from different fractions in the continuous gradients. The purified fractions were washed from Percoll® by diluting them with four volumes of 1 x PBS (Sigma Chemical Co., Poole, Dorset, UK) (1:4) in a 14-ml Falcon tube and pelleted at 150 *g* for 5 min. Only 1 ml, containing the pelleted cells, was left in the bottom of the tube.

Slide preparation and staining

The slides were pretreated with 3-aminopropyltriethoxy silane (Sigma) in acetone (Sigma) (2% w/v) in order to make the cells adhere to them. A drop of 1-4 µl (approximately 50,000 cells counted in a hemocytometer) from each fraction was laid upon the slide, spread and allowed to air dry for 2 to 4 min. The slides were incubated in Bouin's fluid for 10 min and the cells stained with PAS + H according to established methods (6,7).

Cell identification

The cells were identified according to their morphology based on cell size and nuclear appearance and by the presence of the acrosome. The classical description of Meistrich et al. (8) adapted by Platz and Grimes Jr. (9), and the photomicrography of histological sections from rat testis described by Hess (10) were used as a reference for cell identification. Figure 1 shows photomicrographs of some of the germ cells. The criteria used for cell identification in smears was as follows:

Spermatogonia (Figure 1H). The nucleus of these cells was variable in size and contained deeply stained chromatin. The nucleus was surrounded by a thin rim of cytoplasm.

Spermatocytes (Figure 1E,F). The spermatocytes are formed by a continuous series of cells that differ in size, but have a similar nuclear appearance and consist of cells from leptotene to secondary spermatocyte stages. The nucleus of leptotene and zygotene cells

was deeply stained with woolly masses of chromatin. The nucleus of pachytene spermatocytes (Figure 1E) was large and contained separate clumps or thick strands of chromatin corresponding to the chromosomes. The cytoplasm was more abundant than in previous stages and increased proportionally in subsequent stages. The secondary spermatocytes were identified by the presence of dusty and fine chromatin inside the nucleus. The cytoplasm was also abundant. Divisions of primary to secondary spermatocytes were often seen.

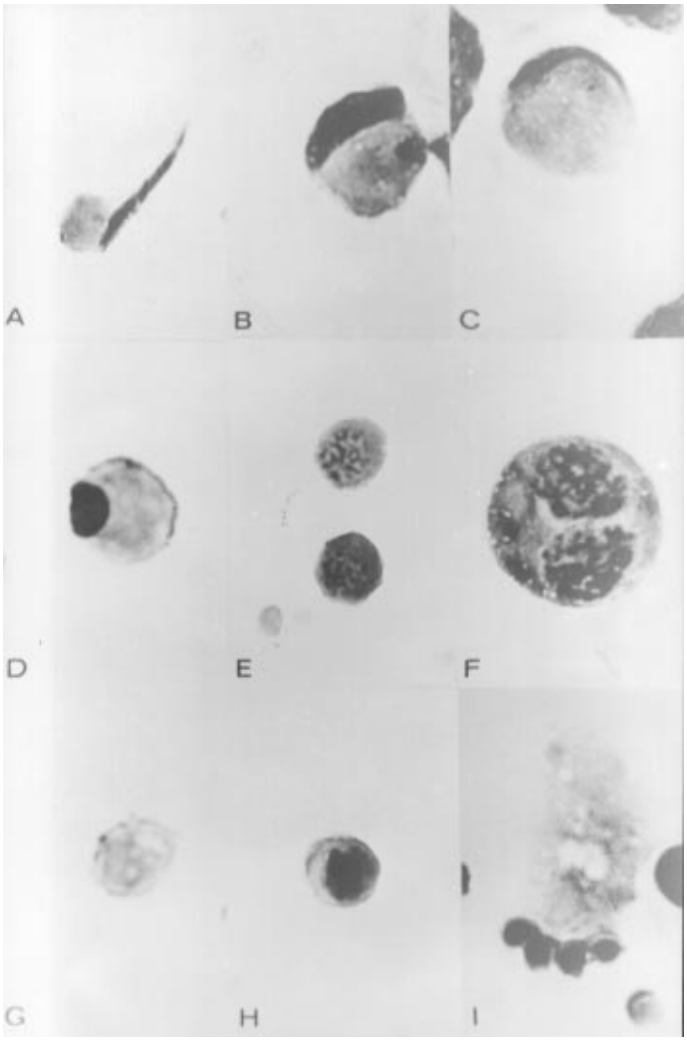

Figure 1 - Photomicrographs of rat testis cells on smears stained with PAS + hematoxylin. *A,B* and *C*, Late spermatids; *D*, early spermatids (step 8); *E*, pachytene spermatocytes; *F*, binuclear spermatocyte; *G*, residual body; *H*, spermatogonium; *I*, Sertoli cell. In the basal portion, four spermatogonia are attached. Magnification, 400X.

Spermatids (Figure 1A-D). These cells were identified by their nuclear shape and cytoplasmic/nuclear ratio higher than 1. The classical division of the steps from 1 to 19 given by Meistrich et al. (8) was not used here. Instead, the classification adopted was early and late spermatids. Early spermatids (Figure 1D) were characterized by abundant cytoplasm with a small and deeply stained nucleus. In preparations of good quality, the Golgi zone and the acrosomic system could be seen. The early spermatids were equivalent to steps 1 to 8 of spermiogenesis. Late spermatids (Figure 1A-C) were determined by the discrete amounts of cytoplasm and by the dark stained and elongated nucleus. The amount of cytoplasm attached to the nucleus was a small conical droplet of about 30% or less of the area of the full complement at the base of the nucleus. The late spermatids were equivalent to steps 9 to 19 of spermiogenesis.

Sertoli cells (Figure 1I). These cells were characterized by the presence of an irregular and elongated cytoplasm and the large nucleus contained a fine pale-staining chromatin and were often seen attached to other cells or in clumps.

Cytoplasmic fragments and residual bodies (Figure 1G). Pale-stained cytoplasmic fragments without any nucleus were seen and identified as residual bodies. Large fragments with late spermatid nuclei were identified as Sertoli cell cytoplasm.

The slides were coded randomly and examined by an experienced cytologist for cell identification. Four fields at 400X magnification were counted. Following this procedure, cell counts were performed by an operator who did not know the results obtained by the previous cytologist. The results were then compared to check the ability for cell recognition.

RNA extraction

Total RNA was extracted from pools of

cells prepared by the techniques described above, using RNAzol® B (CINNA/BIOTECX Laboratories Inc., Houston, TX). The methodology followed was a slight modification of the manufacturer instructions. Briefly, the maximum volume of RNAzol® B added to the sample was 5 ml. The cells were lysed by vortexing. RNA was separated by electrophoresis according to standard methods (11) on gels containing 2.7% (w/v) SeaKem GTG agarose (FMC BioProducts, Rockland, ME), 0.66 M formaldehyde (Sigma), 9.8% (w/v) 10 x running buffer (final concentration containing 200 mM MOPS, 10 mM EDTA and 50 mM sodium acetate, pH 7.0). RNA separated on a denaturing gel was transferred by capillary blotting onto Hybond N membranes (Amersham, Buckinghamshire, UK) using 20 x saline-sodium citrate [20 x SSC (3 M NaCl, 0.3 M Na citrate, pH 7.0)] and fixed with ultraviolet light.

Northern blot analysis

Double-stranded DNA for labeling was usually prepared by amplification of the cloned cDNA insert from a plasmid vector using polymerase chain reaction (PCR) (12) according to methods described elsewhere (13). Three probes were used: TP2, SGP1 and COX II. The double-stranded DNA probes were radiolabeled with 30 µCi of radioisotope [α^{32}P]dCTP (Amersham) by the random primer method (14) using a Random Primed DNA labeling kit (Boehringer, Mannheim, Germany) according to manufacturer instructions.

Membranes were prehybridized at 65ºC for 3 h in buffer solution containing 0.05% (w/v) bovine serum albumin Fraction V (Sigma), 0.05% (w/v) polyvinylpyrrolidone (PVP) (Sigma), 0.05% (w/v) Ficoll® (Pharmacia), 0.1% (w/v) sodium dodecylsulfate (SDS) (Sigma), 0.1% sodium pyrophosphate (Sigma), and 5 x SSC (from a 20 x SSC stock solution). The radiolabeled probe was added to the hybridization mixture at a final concentration of 1 x 10^6 cpm/ml buffer. Hybridization was allowed to continue for 18 h. In the next stage, the membranes were washed with 1 x SSC at 65ºC for 2 x 30 min and exposed to a PhosphorImager (Molecular Dynamics, Sunny Vale, CA) in order to measure the signal. The membranes were also placed in autoradiography cassettes with DuPont® enhancing screens and exposed to X-OMAT AR-5 Film or X-OMAT LS X-ray film (Kodak Co., Cambridge, UK) at -70ºC for 1 h to 12 h.

Membrane hybridization for labeled oligonucleotides

Antisense oligonucleotides for 18S ribosomal RNA (14) were used to check for the balanced transfer of RNA by Northern blot. Oligonucleotides (17-24 mers) were synthesized according to methods described elsewhere (13). Following hybridization and detection of the signals from the labeled double-stranded DNA, the membranes were washed with solution containing 40 mM sodium phosphate, pH 7.2, 1 mM EDTA and 1% (w/v) SDS at 65ºC for 2 x 30 min. The membranes were checked in the PhosphorImager to confirm the removal of the radiolabeled probe and then prehybridized with SSC plus Denhardt's solution for oligonucleotide probes at 65ºC for 4 h. The oligonucleotide probe (18S) was labeled with [γ^{32}P]ATP (Amersham) using 5' end labeling with polynucleotide kinase (PNK, Amersham). DNA was incubated with 30 µCi [γ^{32}P]ATP in a reaction mixture containing 50 ng DNA, 1 x kinase buffer (10 x buffer contains 0.5 M Tris-HCl, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine and 1 mM EDTA) and 8 U T4 PNK. The reaction was allowed to continue for 30 min at 37ºC in a dry block heater. The radiolabeled probe was mixed with the prehybridized membrane and incubated for 18 h at 65ºC in a rotational oven (Hybaid). The membranes were washed with 1 x SSC

at 65ºC for 1 h, and exposed again to the PhosphorImager for analysis of the quantity of sample and to X-ray film according to the technique described above. The specific signal from the three probes was divided by the signal from the 18S. The result was considered to be the adjusted signal and plotted according to the sample.

Statistical analysis

The existence of a difference between continuous and discontinuous Percoll® gradients was tested. Data are reported as mean ± SEM. All experiments were performed in triplicate and the *t*-test was used for statistical analysis. $P<0.05$ was considered to be significant.

Table 1 - Comparison of discontinuous and continuous gradients for the separation of rat testis cells.

Data are reported as mean ± SEM (N = 3). Shaded data indicate the most homogeneous cell populations. [a]Includes all the stages of spermatocytes; [b]includes all spermatids from step 1 to 19; [c]spermatids from step 1 to 8; [d]spermatids from step 9 to 19.

Fraction	Load		1	3	4
	A	B			
Density (g/ml)	1.019	1.031	1.043	1.043	1.043
Cells x 10^6/ml	61	92	265	35	8.4
Discontinuous					
Spermatogonia	0.5 ± 0.06	2.26 ± 0.8	2 ± 0.8	5 ± 1	18 ± 2
Spermatocytes[a]	12 ± 7	2.2 ± 1	13 ± 1	69 ± 3	40 ± 16
Pachytene	6 ± 3	0.7 ± 0.3	4 ± 0.7	58 ± 7	19 ± 10
Residual bodies	17 ± 8	8.73 ± 4	2 ± 2	0.1 ± 0.1	1.2 ± 1
Spermatids[b]	56 ± 8	77 ± 2	73 ± 4	26 ± 4	29 ± 3
Early spermatids[c]	15 ± 4.2	12 ± 10	28 ± 9	17 ± 3	10 ± 2
Late spermatids[d]	39 ± 9.5	64 ± 12	44 ± 10	5 ± 1	18 ± 4
Sertoli cells	0.2 ± 0.1	0	0.5 ± 0.2	0.5 ± 0.2	25 ± 5
Unknown	11 ± 4	9 ± 3	6 ± 2	1.2 ± 1	2 ± 0.7
Continuous					
Spermatogonia	0.3 ± 0.3	0.83 ± 0.6	0.83 ± 0.6	0.83 ± 0.6	22 ± 6
Spermatocytes[a]	1.3 ± 1	0.8 ± 1	11 ± 1	81.56 ± 8.26	40 ± 16
Pachytene	0.16 ± 0.1	0.8 ± 0.6	9.7 ± 1	75 ± 8	37 ± 16
Residual bodies	23 ± 7	18 ± 4	1 ± 0.9	0.33 ± 0.33	0
Spermatids[b]	71 ± 3	79.5 ± 6	84.16 ± 2.61	16 ± 8	25 ± 10
Early spermatids[c]	36 ± 15	18 ± 6	63 ± 9	0.3 ± 0.3	11 ± 2
Late spermatids[d]	35 ± 12	61 ± 18	20.33 ± 7.91	13 ± 7	14 ± 8
Sertoli cells	0.16 ± 0.16	0.16 ± 0.16	0.3 ± 0.3	2.7 ± 0.3	10 ± 3
Unknown	0.83 ± 0.33	0.66 ± 0.16	1 ± 0.2	0.7 ± 0.1	0.5 ± 0.2

Results

Cytological analysis

Eight rats were killed and their testes were divided into two groups to be used in continuous or discontinuous gradients. Cell dispersion was performed simultaneously for each treatment. The samples were subjected to centrifugal elutriation following cell dispersion and five fractions were collected. From these, fraction 2 was discarded and further purification was performed using Percoll® gradients. The continuous gradient was composed of a linear density of 1.007 to 1.084 g/ml, while the discontinuous one consisted of three or four steps of densities ranging from 1.084 g/ml to 1.043 g/ml, 1.031 g/ml and 1.019 g/ml.

The mean values for each aggregate fraction of germ cells (i.e., spermatogonia, spermatocytes, spermatids and Sertoli cells) in the specific Percoll® fractions are reported in Table 1. The data in Table 1 show that the highest purity was obtained for spermatids (from step 1~19) (84 ± 2.6%) from fraction 1 of continuous Percoll® gradient, and for spermatocytes (81 ± 8.2%) from fraction 3 of continuous Percoll® gradient. A significant difference was found when early spermatids were harvested from continuous Percoll® gradients from the cell fraction originating from fraction 1 of centrifugal elutriation. No other significant difference between the methods of continuous and discontinuous Percoll® gradients was observed. The contamination of Sertoli cells was zero or less than 1% in many fractions, except for fraction 4 where 25 ± 5% was obtained.

The highest purity of germ cells (96% for spermatocytes and 89% for spermatids) was obtained from the first experiment using continuous Percoll® gradients.

Molecular analysis of the samples

The results in Tables 2 and 3 show that in

the continuous Percoll® gradient the TP2 probe (present in elongating spermatids) presented highest expression in the fraction load from centrifugal elutriation collected from a density of 1.043 g/ml (load B).

COX II (present in pachytene spermatocytes) was expressed mainly in fraction 3 from centrifugal elutriation and collected from 1.043 g/ml density in both continuous and discontinuous Percoll® gradients. SGP1 (present in Sertoli cells) showed enhanced expression in fraction 4. Here again, no statistical difference was found between the continuous and discontinuous Percoll® gradients.

Correlation between the cytological and molecular data

Table 4 shows the combination of cytological and molecular results of each fraction obtained from continuous and discontinuous Percoll®. The main germ cell composition, its contamination according to the cytology, and the highest and lowest signals from the specific probes for the same fractions are presented in this table. Each probe corresponds to a specific type of cell as mentioned above. Briefly, TP2 is present in late (or elongating) spermatids, COX II mainly in pachytene spermatocytes, and SGP1 in Sertoli cells. Several features are apparent from this table. A correlation of the characterization of the enriched germ cell composition of each fraction using cytological analysis and the probes, except for fraction load A from discontinuous Percoll®, was observed. Discrepancies between the cytological and molecular findings occurred when determining the nature of the contaminating cells in the fractions. In the load A, 1 and 3 fractions of the discontinuous Percoll®, the almost complete absence of Sertoli cells on the slides was not confirmed by the presence of the signal of the SGP1 probe, a specific marker for Sertoli cells. These findings are even more striking when the results for load B of discontinuous Percoll® gradients and fraction 3 of the continuous Percoll® gradient are analyzed. Cytological analysis of these fractions revealed no Sertoli cells, although the signal of the SGP1 probe was present in all of them.

Considering the individual values of fraction 3 of the first continuous Percoll® gradient, for which 96% purity of spermatocytes was obtained and no Sertoli cells were visualized, a clear signal of the SGP1 probe (8.09 cpm of the adjusted signal) was demonstrated by molecular analysis as shown in Figure 2.

The poor reproducibility of the experiments is reflected by the high values of the

Table 2 - Specific probe analysis of fractions of rat testis obtained by continuous Percoll® gradients.

Data are reported as mean ± SEM (N = 3). *Percentage of the control of the adjusted signal (specific signal/18S). Percentages are related to the control, which was considered to be 100%. See text for details. TP2, Transition protein 2; COX II, mitochondrial cytochrome C oxidase II; SGP1, sulfated glycoprotein 1.

Fraction	Highest signal*	Lowest signal*
Load A	TP2 (35 ± 22%)	SGP1 (4.9 ± 3%)
Load B	TP2 (116 ± 82%)	SGP1 (6 ± 3%)
Fraction 1	TP2 (83 ± 48%)	SGP1 (17.9 ± 8%)
Fraction 3	COX II (257 ± 171%)	TP2 (7 ± 4%)
Fraction 4	COX II (183 ± 58%)	TP2 (19 ± 11%)

Table 3 - Levels of specific probes labeled with radioisotope ([α^{32}P]dCTP) obtained by discontinuous Percoll® gradients.

Data are reported as mean ± SEM (N = 3). *Percentage of the control of the adjusted signal (specific signal/18S). Percentages are related to the control, which was considered to be 100%. See text for details. Probe abbreviations are identified in the legend to Table 2.

Fraction	Highest signal*	Lowest signal*
Load A	COX II (48 ± 45%)	SGP1 (24 ± 8%)
Load B	TP2 (77 ± 16%)	SGP1 (6 ± 3%)
Fraction 1	TP2 (83 ± 48%)	SGP1 (24 ± 9%)
Fraction 3	COX II (38 ± 20%)	TP2 (6 ± 4%)
Fraction 4	SGP1 (172 ± 21%)	TP2 (6 ± 2%)

standard errors, mainly observed in the molecular analysis (Tables 2 and 3). Although wide variance was observed, data for the same main germ cell population were consistent.

Discussion

Recent reports showing a decrease in sperm count in men (15,16) brought new concerns about male infertility and its physiology. To understand the physiology of cells and their relationship, cell purity is a paramount issue. No definitive conclusion can be reached about a contaminated system. Methods have been developed to increase the purity of cells in order to draw reliable conclusions about a biochemical aspect of a particular cell population.

Rats have been used as an animal model for studying testicular cell biology by many investigators (17-23). Among the methods described in the literature for the separation of rat germ cells, the combination of centrifugal elutriation and isopycnic density (Percoll®) has produced the highest degree of purity of spermatogenic cells from the adult rat testis (1). This method has been used by other investigators to study molecular aspects of rat germ cells (2,5) and therefore conclusions have been made about molecular and biochemical aspects of the cells, assuming that the purity of the cells was as high as described in the original study.

The present study was carried out using the standard techniques employed in this department, which are based on the work of Meistrich et al. (1). The decision to use triplicates was based on previous studies in the literature in which the methods were repeated 2 to 8 times (1,18). Considering these references, triplicates seemed to be a reasonable number of replications. The expectation of cell purity for spermatocytes and spermatids from the rat germ cell suspension was 98% using Meistrich's method. In the first experiment, using a continuous Percoll® gradient, a result of 96% purity of spermatocytes suggested that the method was reliable and reproducible. However, after performing the experiments in triplicate, the reproducibility was found not to be as high as expected. The second experiment using continuous Percoll® gradients obtained only 67.2% spermatocytes, a poorly enriched population. A possible reason for this discrepancy may be related to animal age. The animals used were adult rats aged 90 to 180 days. The 90-day old rats were used in the first experiment and older animals were used in the subsequent ones. The best results were obtained for the younger rats. Such variability for adult rats has not been reported in the literature. In fact, Meenakumari and Duraiswami (24) have worked with animals aged 8 to 30 days. Their results for separating cells from rat testis with Ficoll® started to decrease from 14-day old animals. They found an increased and steady degree of contamination in germ cell fractions (22~26%) in animals 18 days old or older. However, in the original study of Meistrich et al. (1),

Table 4 - Comparison between the results of cytological and molecular analyses of the germ cell types in each fraction of continuous and discontinuous Percoll® gradients.

Shaded areas represent inconsistency between the cytological and molecular findings. *Considered as the lowest percentage of a cell population in the cytology examination and the lowest signal from hybridization.

Fraction	Main cell fraction		Contamination*	
	Continuous	Discontinuous	Continuous	Discontinuous
Load A				
Cytology	Early spermatids	Late spermatids	Sertoli	Sertoli (<1%)
Probe	TP2	COX II	SGP1	SGP1
Load B				
Cytology	Late spermatids	Late spermatids	Sertoli	Sertoli (absent)
Probe	TP2	TP2	SGP1	SGP1
Fraction 1				
Cytology	Early spermatids	Late spermatids	Sertoli	Sertoli (<1%)
Probe	TP2	TP2	SGP1	SGP1
Fraction 3				
Cytology	Pachytene	Pachytene	Sertoli (absent)	Sertoli (<1%)
Probe	COX II	COX II	SGP1 (present)	TP2
Fraction 4				
Cytology	Pachytene	Sertoli	Residual bodies	Residual bodies
Probe	COX II	SGP1	TP2	TP2

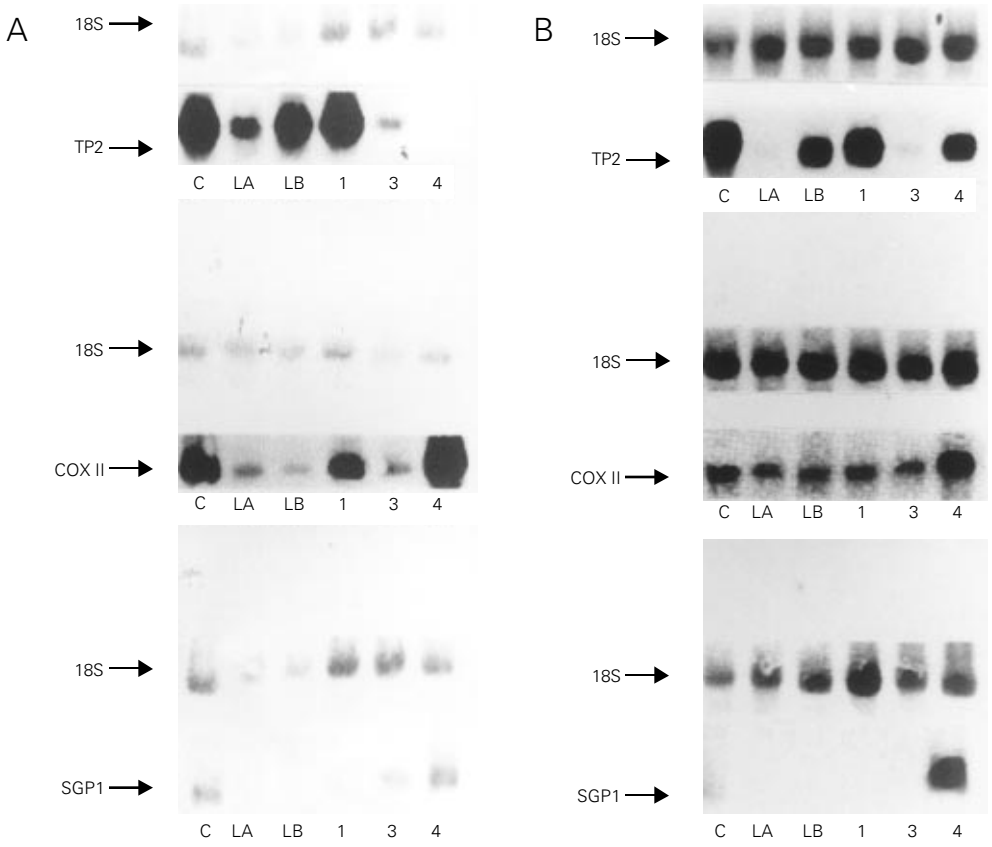

Figure 2 - Northern blot analysis of TP2, COX II and SGP1 mRNA expression in germ cell-enriched fractions. RNA samples were obtained from isolated cell fractions from centrifugal elutriation followed by continuous (A) and discontinuous (B) Percoll® gradients. Enriched cells suspension of spermatids (lanes LA, LB and 1), pachytene spermatocytes (lane 3) and Sertoli cells (lane 4). RNA extracted from the whole testis was used as control (C). Lane 3 of the continuous Percoll® gradient shows a SGP1 signal, demonstrating contamination. The image for COX II from discontinuous gradients was generated from the PhosphorImager computer.

animals much older than 30 days were used. Based on these studies, the variance in adult rat age should not have been a compromising variable, but this was the case in the present study. To confirm these data, another study comparing the degree of purity according to adult rat age should be performed. Furthermore, work in the literature with duplicates (18) should be viewed with caution.

In addition, some considerations about centrifugal elutriation are pertinent. It is recommended that rotor speed be kept constant and flow rate variable, because the rotor speed tends to overshoot the set speed. Furthermore, the strobe control must then be readjusted to view the chamber at each new lower speed (25). The highest purity was obtained from fraction 3 of the centrifugal elutriator, when the rotor speed was set at 2000 rpm for fraction 2 and during the collection of fraction 3. The flow rate was the only variable. This statement supports the data of Onoda et al. (26), who achieved better results by changing the settings of the centrifugal elutriation reaching more than 90% purity of pachytene spermatocytes and round spermatids, while Meistrich et al. (1) achieved only 75% and 80%, respectively.

Another possibility accounting for the difference between methods may be the harvesting of cells from the Percoll® gradients. The continuous gradients do not always form a sharp band, except when an almost pure population obtained from the centrifugal elutriation is achieved. This malformed band can be 1 cm high and cell collection can be problematic. However, this is a limitation of the method itself. Meistrich et al. (1) tried to overcome this problem by collecting the cells by puncturing the side of the centrifuge tubes using a greased 25 gauge needle and aspirat-

ing a predetermined volume. The discontinuous Percoll® overcomes this problem since a sharp band is formed between the gradients. Thus, a balance between risk and benefit exists in this part of the method. On the one hand, the discontinuous gradients give a sharp band for collecting the cells, but the density is fixed, and on the other, continuous gradients can give the small differences in densities, but no sharp band. Small changes in cell density due to enzyme digestion, temperature, age of the animal or even preparation of the medium can change the final density of the gradients and cells and consequently modify the purity of the fractions. The working window for cell density is very narrow (1.044~1.051 g/ml), and therefore rigorous conditions should be applied.

The continuous Percoll® gradients are advantageous for overcoming the problems which are a result of small variations in the medium or cell density. However, sharp bands are not always obtained. According to the results obtained in this study, the use of continuous gradients seems to be recommended.

Sertoli cells were not observed in fraction 3 of continuous and discontinuous Percoll® gradients, although later molecular analysis suggested their presence. One possibility is that the cells were lost from the slides during the fixation and staining process. This loss may be responsible for an inaccurate analysis of the cell suspension from a non-representative sample. For this reason, the slides were treated with 3-aminopropyltriethoxy silane in acetone, reducing this possibility. Furthermore, as already demonstrated mathematically in the literature (8), the loss factors are independent of the actual frequencies of cell types in the sample, but are a function of the smearing technique. Comparison between the cell count of the smears and of the cell suspension was within 20% of unity (8).

Considering cell identification, phase contrast microscopy does not seem to be an accurate method to identify cells when compared with staining methods. It is possible to identify some cells based on the cell size and nucleus. Nevertheless, some differences between spermatocytes and spermatids are difficult to distinguish, since the cell size is approximately the same and the nuclear contents are not clearly visible. In addition, publications showing photomicroscopy of the purified cell suspension (20,26) using a phase contrast microscope should be viewed with caution because the nucleus cannot be seen properly and cell size is the only possible parameter for identifying the cell. Thus, cytological analysis of stained smears was considered the method of choice for cytological cell identification since the parameters are clearer, a permanent record is obtained, and morphological cell standards are available.

Molecular analysis of the samples did not reveal uniform standard errors probably due to differences in the intensity of labeling between experiments caused by differences in the specific activity of the probes. Therefore, the results should be seen as a qualitative and not as a quantitative value. Molecular analysis identifies whether the fraction was contaminated by another cell type and this is the reason why molecular analysis was used in the present study. However, molecular analysis is a very expensive method to be used for identifying the main cell composition and should be avoided for this purpose. Furthermore, in the present molecular analysis only three specific probes were used, one of which (COX II) is not cell-specific, since it is more frequent in pachytene spermatocytes but is also present in the other cells. Therefore, this probe should not be used for assessing contamination.

In conclusion, the present study has raised new questions about the reliability of morphological criteria based only on basic staining techniques for checking the purity of the sample. Even though the cell purity achieved was not as expected, the results showed that morphological criteria are not reliable for

defining sample contamination as a single method. The use of specific probes demonstrated that fractions in which cytological analysis revealed the absence of certain cell types actually had these cells. SGP1 should be used as a gold standard to check the presence of Sertoli cell contamination in germ cell preparation. Therefore, the use of more specific techniques to define contamination, such as immunocytochemistry, or molecular analysis is recommended, as they reduce the bias in identifying and misinterpreting similar cells.

A multi-step method for cell separation may present good results, but may also enhance the chances of technical mistakes due to the number of steps involved. Results from previous experiments should not be taken for granted.

It is therefore possible to achieve a higher degree of purity of cell populations using the techniques described in the present study, provided that animal age, elutriation settings and Percoll® densities are taken into consideration. In order to identify sample contamination, the use of molecular analysis proved to be more rigorous than cytological analysis. In the absence of new efficient and low-cost techniques, the combination of the present methods seems to be a current trend for pure cell preparation. Double centrifugal elutriation and double Percoll® centrifugation are variations that could be used to enhance the purity of the cells.

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