J. Biosci., Vol. 10, Number 3, September 1986, pp. 413-422. © Printed in India.

A method for the isolation of intact Sertoli cell-germ cell associations from rat seminiferous tubules and their further partition into Sertoli cell and germ cell fractions

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MS received 30 June 1986

Abstract. A technique is described for obtaining a Sertoli cell-enriched and a germ cell-enriched fraction from immature rat testes. Sertoli cell-germ cell associations were obtained by incubating washed seminiferous tubule fragments with Collagenase and Pancreatin. They were then manually dissociated into a suspension comprising Sertoli cells as well as the various germ cell types characteristic for a given day of ontogeny. Fractionation into a Sertoli cell-enriched fraction and a germ cell-enriched fraction was effected by centrifugation following layering over a stepwise gradient of Ficoll-400. While the time-span compares favourably with other procedures reported in the literature, it is believed this is the first time a method is described that enables the simultaneous recovery of both the Sertoli cells and the germ cells.

Keywords. Rat seminiferous tubules; Sertoli cells; germ cells; isolation.

Introduction

It is now generally accepted that the Sertoli cells have an important role to play in germ cell development and differentiation (Fritz, 1978; Russell, 1980). Furth ermore, the view that the orderly progression of spermatogenesis is dependent upon a continuous interplay between the 'Sertoli cell compartment' and the constituents of the 'germ cell compartment' has attracted much attention (Ritzen, 1983). Paradoxically enough, the presumed nexus between these two compart ments appears to have had little impact, until recently (Ritzen *et al.*, 1982), on the design of investigations aimed at unraveling the molecular basis of spermatogenesis and its regulation (Meenakumari, 1984). For the most part, *in vitro* biochemical studies have utilized either cultured Sertoli cells or isolated germ cell types.

We were interested in an investigation of possible Sertoli cell-germ cell interactions, specifically in relation to protein synthesis, during the ontogeny of spermatogenesis in the colony-bred rat. Consequently, our experimental design (Meenakumari, 1984) called for incubation *in vitro* of either seminiferous tubules *per se* or of intact 'Sertoli cell-germ cell associations' (SGAs) isolated therefrom, to be followed, at the appropriate time, by separation into a Sertoli cell-enriched and a germ cell-enriched fraction for purposes of analyses or further fractionation. In the absence of a suitable published procedure meeting such a requirement, it became necessary to develop one.

Abbreviations used: SGAs, Sertoli cell-germ cell associations; SCS, single cell suspension.

Materials and methods

Animals

The male rats used in this study were from our Holtzman-derived colony maintained under standard housing conditions (24±1°C; 14 h light-10 h dark schedule; free access to water and commercial pelleted rat diet). For reasons dealt with elsewhere (Meenakumari, 1984), attention was focussed on immature animals of postnatal age, days 8, 14, 18, 23 and 30, respectively. Only rats born on the same day were used for a given experiment. Testes (6–30) were employed depending on the selected age group.

Chemicals and reagents

Collagenase (Type 1) and Pancreatin (porcine, Grade VI) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Ficoll-400 was bought from Pharmacia, Uppsala, Sweden. All other reagents were purchased locally and were of analytical grade purity.

Buffers

Buffer A contained: sodium chloride-97·6 mM; potassium chloride-25 mM; glucose-8·3 mM; L-glutamine-5 mM; Tris (Tris hydroxymethyl amino methane)-10 mM; penicillin-50 units/ml; streptomycin-50 μg/ml and phenol red-0·008 mM. The pH was adjusted to 7·3 at 10°C using 2 N HCl.

Buffers B, C and D had the same composition as Buffer A but were adjusted to pH 7·3 at different temperatures (B-28°C; C-32°C; D-25°C). In addition, B was made 26 mM with respect to $CaCl_2$ and D, 1 mM with respect to EDTA. Buffer C was used whenever tissue-seminiferous tubule segments/SGAs-had to be incu bated.

Isolation of SGAs

The following procedure modified from Welsh and Wiebe (1975) was used Animals were anesthetized and the testes removed and kept in Buffer A till end of collection. Decapsulated testes were suspended in Buffer B and passed gently through a 1 ml hypodermic syringe 10–12 times. With the rapid settling of the seminiferous tubules, the supernatant was decanted. This step was repeated once more. The washed tubules were cut into 0·5–1·0 mm long fragments, suspended in Buffer B containing Collagenase and Pancreatin (1 g tissue/10 mg of each enzyme/20 ml Buffer B) and incubated at 28°C in a Dubnoff metabolic shaker water-bath at 140 oscillations per min for an appropriate period of time. The Leydig cell-peritubular cell aggregates formed in this step were removed by aspiration to yield a preparation consisting of SGAs. These were allowed to settle and the supernatant containing the enzymes, single cells and small-sized SGAs was removed by aspiration. The SGAs were washed several times with Buffer D and suspended in a known volume of the same buffer.

Preparation of single cell suspension

The washed suspensions of SGA, derived from testes of defined age, were processed as follows to obtain, in each case, a single cell suspension (SCS): (i) 8 and 14–passed 30 times through a Pasteur pipette; (ii) 18-passed 30 times through Pasteur pipette and twice through a hypodermic syringe fitted with a 22-gauge needle, at the rate of 1 ml per second; (iii) 23 and 30-passed 30 times through Pasteur pipette and 8–10 times through the hypodermic syringe. Frothing was avoided at all times.

The cells in SCS were counted using a haemocytometer.

Separation of cell types

The SCS containing both Sertoli cells and the various germ cell types was mixed with an equal volume of a 6% Ficoll-400 solution made up in Buffer D. Aliquots containing 3 to 4 × 10^6 cells were layered over 30 ml of a stepwise Ficoll gradient (5%–10%–15% or 5%–10%–15%–20%) and centrifuged in a Spinco SW-25 rotor for 10 min at 25°C. The cells formed discrete bands based on their densities. Each of the resultant fractions was removed by aspiration, mixed with an equal volume of Buffer D and centrifuged to recover the cells. Each fraction was washed twice to remove the Ficoll and finally suspended in 0·5 ml of Buffer D.

Identification of cell types

A small drop from each fraction was spread over a clean glass slide and fixed immediately in formaldehyde vapour, at room temperature. The cells were stained by the Feulgen technique. The various cell types in each fraction were identified according to Meistrich *et al.* (1973) and counted.

Cell viability

The viability of cells was monitored at every step by the Trypan blue exclusion test.

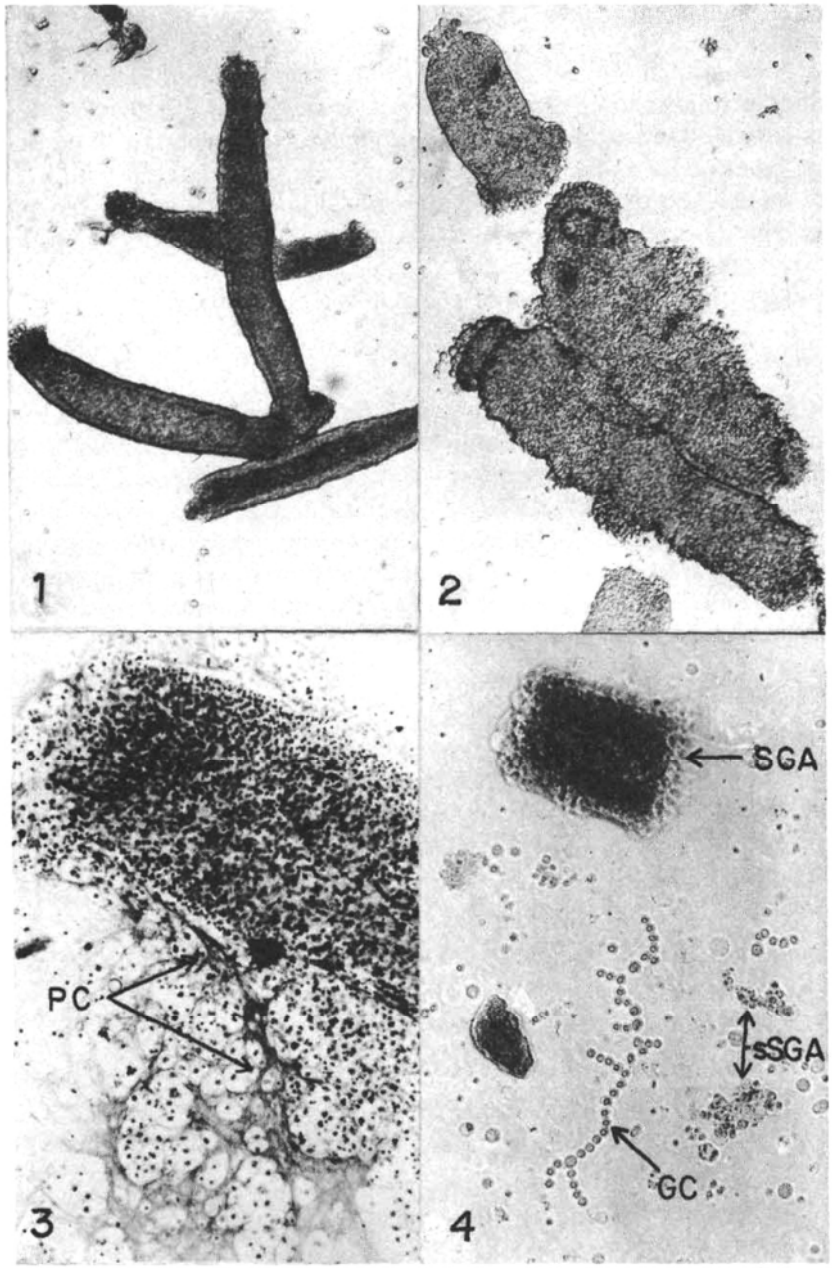
Purity of SGAs

Leydig cell contamination was checked by the histochemical method described by Welsh and Wiebe (1975). The peritubular cells were identified by periodic acid-Schiff staining as well as by their characteristic spindle shaped appearance (Tung and Fritz, 1977).

Results

Isolation of SGAs

The steps described above enabled recovery of intact, viable SGAs free of peritubular and Leydig cells. The sequential removal of these two cell types is shown in figures 1–4. Passage through the hypodermic syringe freed the seminiferous tubule fragments of perivascular-Leydig cells (figure 1). The peritubular cells as well as the peritubular-Leydig cells were removed during the treatment with the enzymes (figures 2 and 3). The duration of exposure to enzymes



Figures 1–4 . Sequence in the conversion of seminiferous tubule fragments into a pure preparation of SGAs, as monitored by microscopy. (1 -3), Transmission micrographs; **(4)** phase contrast micrograph. **1.** Tubular fragments freed of perivascular-Leydig cells by initial washing and mincing (× 125). **2.** Appearance of tubule fragments half-way through incubation with Collagenase and Pancreatin (× 125). **3.** Tubular fragment during the last phase of enzyme digestion. Material was fixed in formaldehyde vapour and stained by the Feulgen method. Aggregated peritubular cells are seen in the areas marked PC (× 77). **4.** End of enzyme digestion: The preparation is free of Leydig cells as well as peritubular cells. SGA: a typical Sertoli cell-germ cell association. Note the presence of small SGA clusters (sSGA) and strings of germ cells (GC). Isolated single cells are also seen (×123).

was dictated by the age of the donor animals, being 15–20 min for day 8, 25 min for day 14, 35 min for day 18, 45 min for day 23 and 60 min for day 30.

SCS

Conversion of the SGAs from 8- and 14-day old testes to single cell suspensions was readily effected, in as much as, at this early stage of ontogeny, the association of the limited number of germ cells with the Sertoli cells was tenuous. This was in contrast to the later stages—especially following their migration to the adluminal compartment.

Cell separation

The pattern of cell separations obtained on centrifugation of the SCSs on the Ficoll gradients is shown in figures 5-9 (insets). The distribution of the various cell types in the different fractions is shown for each age group (figures 5-9). The purity of the Sertoli cell and the germ cell fractions.for the specified age groups as well as the extent of cross-contamination are presented in table 1.

Table 1. Purity of fractions.

Age in days	Sertoli cell fraction (%)	Germ cell in Sertoli cell fraction (%)	Germ cell fraction (%)	Sertoli cell in germ cell fraction (%)	Peritubular cells (%)	Leydig cells
8	96	4	91	1	Nil	—
14	92	8	77·27	21·53	1·19	—
18	85	15	84·84	14·39	0·9	—
23	42	58	76·77	22·42	0·8	—
30	78	22	88·39	10·84	0·76	—

Discussion

In the past few years, several methods have been described for the isolation and culture of Sertoli cells from rats (Dorrington *et al.,* 1975; Steinberger *et al.,* 1975 a,b; Welsh and Wiebe, 1975; Galdieri *et al.,* 1981). As for germ cells, methods have been described for obtaining preparations enriched with respect to one or more cell types from immature as well as sexually mature rats (Go *et al.,* 1971; Davis and Schuetz, 1975; Grimes *et al.,* 1975; Platz *et al.,* 1975; Aleman *et al.,* 1978; Beckman *et al.,* 1978; Nakamura *et al.,* 1978). Since our investigations (Meenakumari, 1984) required recovering both the Sertoli cell fraction and the germ cell fraction from a given source, preferably in quantitative yields, it became necessary to devise a fresh procedure.

The present method was modified from the technique originally described by Welsh and Wiebe (1975) for obtaining Sertoli cells from immature rat testes. Not surprisingly, they discard a germ cell-rich fraction (fraction 2) during the initial washing. Since such heavy loss of germ cells had to be obviated for our purposes, the seminiferous tubule suspension, obtained by stripping off the tunica albuginea,

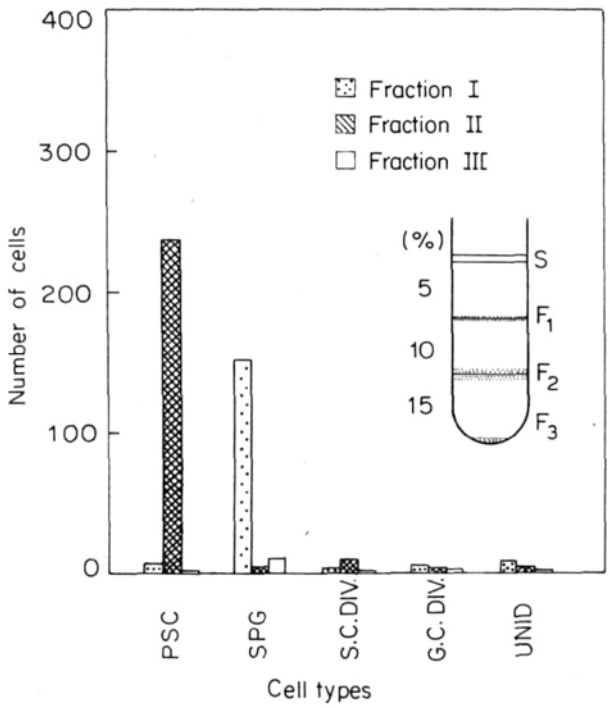


Figure 5. For caption, see page 420.

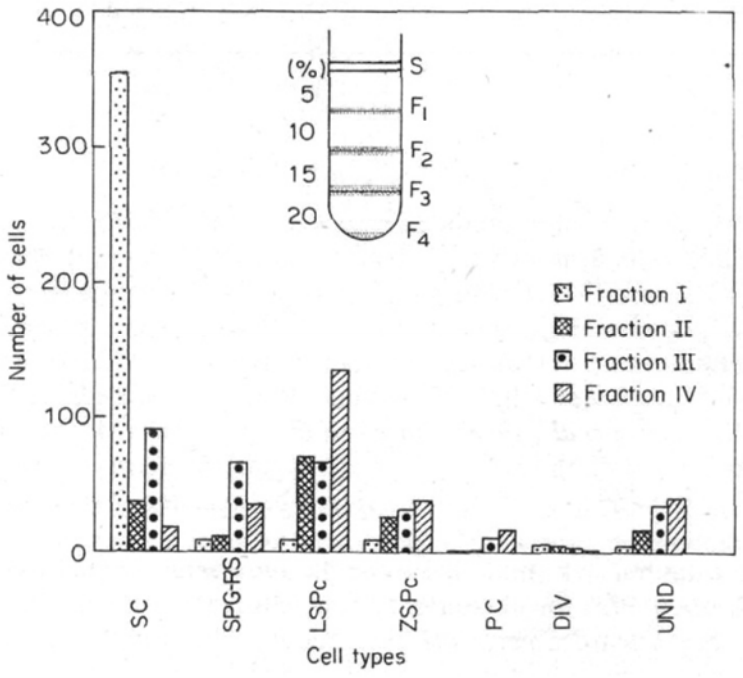
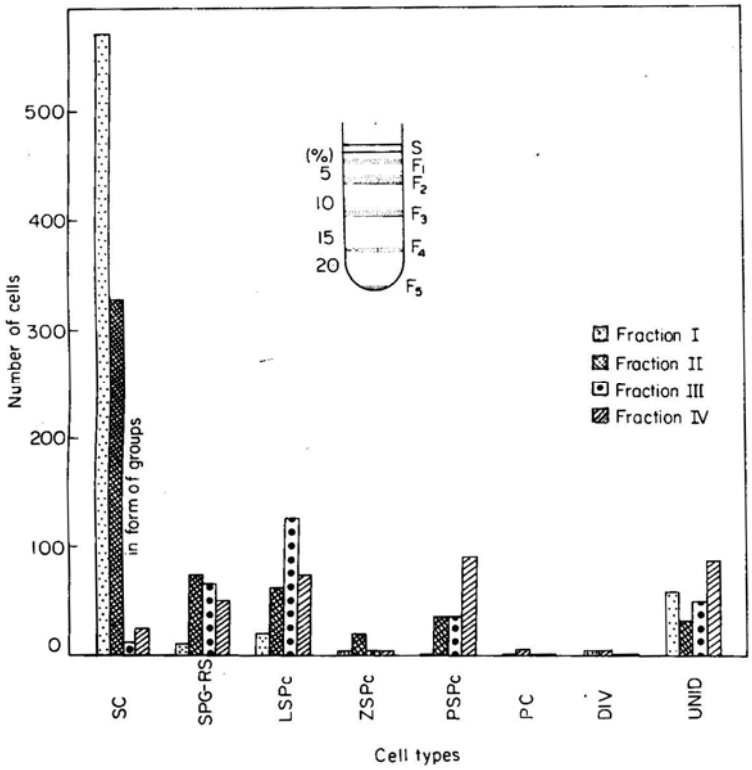


Figure 6. For caption, see page 420.



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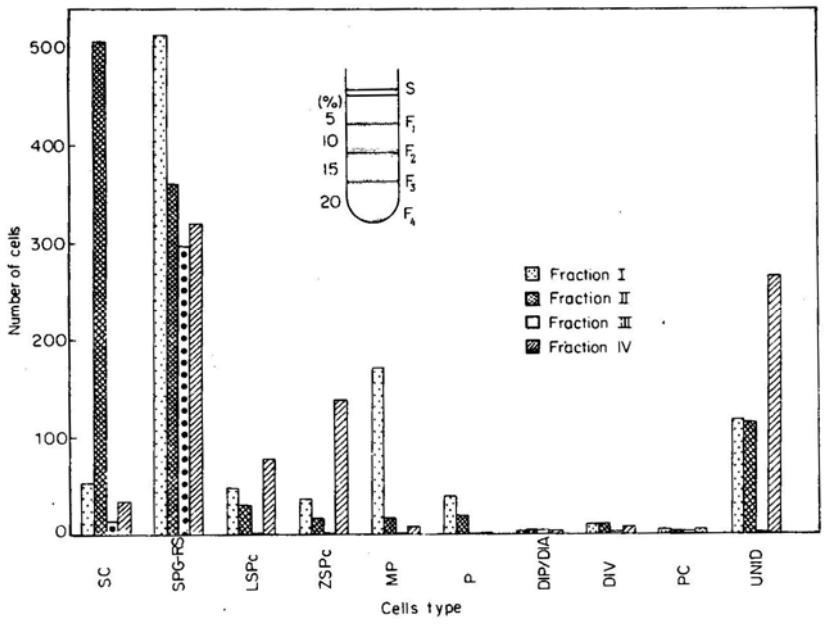

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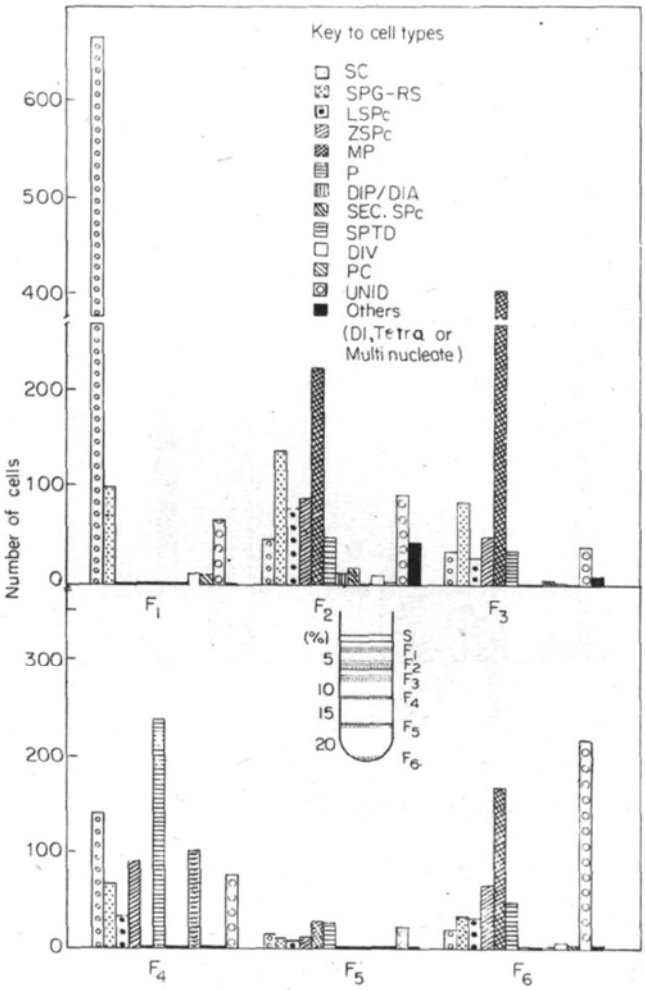

Figure 9.
Figures 5–9. Histograms of the distribution of specific cell types in the bands (F_1, F_2 etc) obtained by Ficoll gradient centrifugation of single cell suspensions. Details in the text. Inset in each figure is a diagrammatic representation of the banding pattern obtained. **5.** Day 8. **6.** Day 14. **7.** Day 18. **8.** Day 23. **9.** Day 30.

S, Sample layer on the gradient at the time of loading; PSC, presumptive Sertoli cells; SC, Sertoli cells; SPG, spermatogonia; SPG-RS, spermatogonia and preleptotene sperm atocytes; LSPc, leptotene spermatocytes; ZSPc, zygotene spermatocytes; PSPc, pachytene spermatocytes; MP, mid-pachytene spermatocytes; P, late-pachytene sper matocytes; DIP/DIA, diplotene/diakinesis spermatocytes; SEC SPc, secondary spermato cytes; SPTD, spermatids; PC, peritubular cells; S.C.DIV., presumptive Sertoli cells in division; G.C.DIV./DIV., germ cells in division; UNID, unidentified cells; Others, di-, tetra- or multi-nucleate germ cells.

was rapidly passed through the hypodermic syringe. The resultant supernatant contained mostly blood cells and the perivascular-Leydig cells and was discarded. It was found that incubating the seminiferous tubule fragments simultaneously with Collagenase and Pancreatin, rather than sequentially, was quite effective in freeing the SGAs of peritubular and Leydig cells and had the obvious advantage of saving time and an additional step.

Another significant departure from the Welsh-Wiebe procedure was the substitution by Tris of the phosphate buffer used in the enzyme incubation step so as to avoid precipitation of the added Ca^{2+} required for Collagenase activity (as the phosphate salt). Due note was taken of the temperature-dependence of the pH of Tris-HCl buffers.

The SGAs recovered following the enzyme treatment could be fairly readily converted to single cell suspensions. However, with material from the older donors —days 18, 23 and 30—a small population of SGAs (less than 5% of the total cell mass) persisted as aggregates, possibly due to the presence of tight junctions between adjacent Sertoli cells, known to appear in the rat between days 15 and 18 (Vitale *et al.,* 1973). Vortexing proved effective in disaggregating such SGAs, but with a drastic reduction in the viability of the single cells thus obtained.

In our hands, the technique of separating the cell types in the single cell suspensions on stepwise gradients of Ficoll-400 proved reliable and satisfactory (table 1). For reasons not readily apparent, the SC-fraction from day 23 animals was heavily contaminated with germ cells—mostly spermatogonial cells and early spermatocytes.

It is pertinent to point out that unlike the earlier published procedures which require a few hours/few days to effect the needed separation, the method described here requires no more than 1·5 h to recover both Sertoli cells and germ cells from single cell suspensions, irrespective of the age of donor rats used in this study. Based on the criterion of exclusion of the vital dye, Trypan blue, the isolated single cell populations showed better than 95% viability. Furthermore, the isolated Sertoli cells could be successfully cultured to form monolayers and maintain viability for atleast 72 h.

Acknowledgement

This study received generous financial support from the Indian Council of Medical Research, New Delhi.

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