Immature germ cell separation using a modified discontinuous Percoll gradient technique in human semen

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The difficulty of identifying immature germ cells in unstained, fresh semen has led most laboratories to use the broad definition ‘round cells’ to indicate cells other than spermatozoa, thus grouping together both leukocytes and immature germ cells. This is also the case in research andrology, where very little attention has been given to immature germ cells in the semen apart from some rare exceptions, such as the attempts to study meiosis. Here we report on the use of a discontinuous Percoll gradient method modified to enable the best separation possible of immature germ cells from the other cells found in the ejaculate, in order to obtain a cellular suspension free of spermatozoa. Our technique (intra-assay variation in duplicates <10%) demonstrated a high immature germ cell concentration in gradient fractions with 30% to 45% Percoll with a small contamination (1.5–6%) of leukocytes, confirmed by May–Grunwald–Giemsa staining, immuno-fluorescence and cytofluorimetry. The concentrations of immature germ cells ranged from zero in obstructive azoospermia to 2.0 × 106/ml in oligozoospermia and genital tract infection. The purified immature germ cell suspensions obtained can be useful for diagnostic and research purposes.

Key words: human semen/immature germ cells/Percoll gradient

Introduction

Until very recently, immature germ cells in human semen have received little real consideration or evaluation in routine semen analysis. This has been the case both in the diagnostic examination of human semen and in the clinical interpretation of seminal data where immature germ cells are recorded. Undoubtedly, the difficulty of their identification in fresh unstained semen has been the main reason responsible for this lack of attention. Many laboratories simply use the broad definition ‘round cells’ to indicate forms other than spermatozoa, thus grouping together both leukocytes and immature germ cells (Fedder, 1996). The World Health Organization Manual (1992) dedicated only a short paragraph to these cells, concerning itself principally with the differentiation of various immature germ cell types using specific staining. This has also been true in research andrology where very little consideration has been given to immature germ cells in semen, except for some rare exceptions such as attempts to study meiosis (Templado et al., 1980). In clinical andrology this has meant that techniques for selecting pure pools of immature germ cells have received scant attention to date. In fact, the various separation techniques that employ Percoll gradients seek to eliminate round cells in order to obtain a suspension of only spermatozoa (Berger et al., 1985; Ord et al., 1990).

Immature germ cells are always present in human semen in variable numbers not necessarily related to spermatozoa concentration, although some authors have reported an inverse correlation (Sperling and Kaden, 1971). Recently, various attempts have been made in order to identify specific sub-populations of immature germ cells for clinical purposes. In particular, spermatids from human semen in patients with non-obstructive azoospermia have been collected and used in intracytoplasmic sperm injection (ICSI) programmes, resulting in normal births (Tesarik et al., 1995). For the same clinical purpose, a simple Percoll gradient has allowed other authors to collect spermatids identified both by staining and by evaluating haploidy using fluorescence in-situ hybridization (Angelopoulos et al., 1997). More recently, a purified population of immature germ cells was obtained from the testicular tissue of azoospermic men employing a fluorescent activated cell sorter (Aslam et al., 1998).

The aim of this paper is to report on the use of a discontinuous Percoll gradient method, modified to enable the best separation possible of immature germ cells from the other cells found in the ejaculate, in order to obtain a cellular suspension free of spermatozoa for diagnostic and experimental purposes.

Materials and methods

Ejaculates

To test the efficacy of the method, we selected ejaculates from normal subjects and from patients with various andrological pathologies. All those selected had not been treated medically in the 3 months prior to the study. The samples were collected by masturbation, after a 3-day period of sexual abstinence. The seminal parameters are reported in Table I. The ejaculates were as follows:

(i) Five ejaculates were from fertile subjects (cases 1, 2 and 3: subjects whose partners were pregnant at the time; and cases 4 and 5: sperm bank donors with proven fertility in artificial insemination by donor programmes). These samples were selected to evaluate the validity of the method in identifying the presence of immature germ cells in normal ejaculates.

(ii) Five ejaculates were from azoospermic...
patients [case 6: acquired inflammatory obstruction of the ejaculatory ducts; case 7: cystic fibrosis with congenital absence of the vas deferens; case 8: post-vasectomy; cases 9 and 10: various degrees of spermatogenesis arrest studied with fine needle cytology and testicular biopsy (one case of primary spermatocyte arrest and one case of spermatid arrest)]. The cases of obstructive azoospermia were chosen as negative controls for the immature germ cells to test for the presence of leukocytes in the gradients from 30 to 45%. The two cases of primitive testicular azoospermia were chosen to evaluate the correspondence between the immature germ cells found in the samples separated by our technique and those taken by fine needle cytology and testicular biopsy. Owing to the low volume of the ejaculate in cases 6 and 7, we prepared a gradient employing 0.5 ml for each dilution instead of 1.0 ml. (iii) Five ejaculates were from patients suffering from dyspermy with clinical diagnosis of cryptorchidism (cases 11 and 12) and varicocele (cases 13, 14 and 15). These samples showed various concentrations of leukocytes and immature germ cells in the ejaculate. (iv) Five ejaculates (cases 16–20), showing various concentrations of leukocytes and immature germ cells (cases 11 and 12) and varicocele (cases 13, 14 and 15). These samples were selected to evaluate if the method was able to select immature germ cells from leukocytes.

The ejaculates were divided into two aliquots to evaluate intra-assay variation by duplicates, except cases 6 and 7, owing to the low volume of the semen.

**Discontinuous Percoll gradient**

Isotonic 100% Percoll (Sigma Chemical Co, St Louis, MO, USA) was obtained by adding nine parts of Percoll to one part of Earle’s salt solution 10X (Imperial, UK). The Percoll 100% was diluted with Isotonic 100% Percoll (Sigma Chemical Co, St Louis, MO, USA) to obtain the following dilutions: 30%, 35%, 40%, 45%, 50%, 55%, 60%, 70%, 80%, 100%. The gradient column was prepared in a 15-ml Falcon tube by gently layering 1 ml of each of the above-mentioned solutions, starting from the 100% fraction at the bottom (0.5 ml for each dilution was used when the semen volume available was \( \leq 0.5\) ml). One millilitre of the semen (or the whole semen in cases with less than 1 ml of ejaculate) was diluted with Earle’s solution (1:2) and centrifuged at 400 g for 15 min at 18°C. The semen cell pellet was resuspended in 0.5 ml of Earle’s solution. The semen cell suspension was gently stratified on top of the discontinuous Percoll gradient and centrifuged for 25 min at 800 g at 18°C. The single Percoll fractions were separated and each was put into a single test tube. The single fractions were analysed in order to select the ones with the greatest concentration of immature germ cells. The fractions which contained the majority of the immature germ cells (30%, 35%, 40% and 45%) were mixed with Earle’s solution (1:2) and centrifuged at 150 g for 10 min at 18°C. The pellet was resuspended in 1 ml of Earle’s solution and the cell concentration was evaluated.

**Cell identification**

Immature germ cells were counted using a Thoma counting chamber, evaluating at least 100 cells. The May-Grünwald-Giemsa staining technique was used to identify various kind of germ cells (spermatogonia, spermatocytes I and II, spermatids) and leukocytes (Schenck and Schill, 1988; Foresta et al., 1992). To further define the leukocyte contamination, the pool of the cell fractions containing the major quantity of germ cells (from 30% to 45%) was tested with an immunofluorescence microscopic technique using anti-CD45 fluorescein isothiocyanate (FITC) monoclonal antibodies (mAb) (El-Demiry et al., 1986). A sample of 100 µl of the cell suspension obtained by pool of the fractions from 30% to 45% was mixed with 10 µl of mAb (CD45 anti-Hle-1; Becton Dickinson, Mountain View, CA, USA) conjugated to FITC. After a 30-min incubation at room temperature in the dark, the cells were washed twice with PBS, resuspended in 100 µl of PBS and analysed (at least 100 germ cells) using fluorescence microscopy (Leica Dialux 22, 50× and 100×) with the following filter combination: 490 nm excitation and 530 nm barrier filters.

With the same aim, leukocyte identification by dual-colour immunophenotyping was performed using the following Becton Dickinson matched murine monoclonal antibody reagents directly conjugated to phycoerythrin (PE) or fluorescein isothiocyanate (FITC): anti-Leu
M3 PE (CD14)/anti-Hle-1 FITC (CD45) (monocytes and leukocytes); anti-Leu 15/CR3 PE (CD11b) (granulocytes); and anti-Leu 12 FITC (CD19)/anti-Leu-4 PE (CD3) (B and T lymphocytes). A sample of 100 µl of the cell suspension obtained by pooling of the fractions from 30% to 45% was stained using 5 µl of monoclonal antibody reagents. This suspension was incubated for 20 min, at room temperature, in the dark. After incubation the cells were washed three times with PBS and analysed (evaluating 25 000 cells) using an Ortho Cytoron Absolute 4 flow cytometer (Ortho Instruments).

Data obtained from side scatter against forward scatter allowed us to study cellular population. Debris noise was eliminated with the discriminator. The data were reported as the percentage positivity of the total population.

**Statistical analysis**

Mean values of duplicates of the immature germ cell concentrations were performed in the four groups of the total population.

**Results**

The results are reported in Table I. The concentration of immature germ cells obtained after the Percoll gradient procedures in the 20 ejaculates tested is summarized in the right hand column of Table I. The other columns of Table I show the sperm parameters and diagnosis. The immature germ cell concentrations are expressed as means of the values of the duplicates for each sample (except cases 6 and 7). The intra-assay variation of the duplicates was lower than 10% for all the samples. The evaluation with May–Grunwald–Giemsa staining of the various gradients showed that immature germ cells were found in the Percoll fractions 30%, 35%, 40%, and 45% (Figure 1).

In the fractions 50%–55%, containing leukocytes, we found a contamination of 2 to 5% of immature germ cells (mostly spermatids) using the May–Grunwald–Giemsa staining technique. A contamination of 1.5 to 6% of leukocytes (mostly polymorphonuclear granulocytes) was found in the fractions from 30% to 45% using both May–Grunwald–Giemsa staining and anti-CD45 FITC mAb immunofluorescence microscopy. The highest values of contamination were found in patients suffering from genital tract infection, in which leukocytes were highly concentrated in the semen (>2×10^6/ml ejaculate). These data were confirmed by cytfluorimetry. In fact, in the cell suspension obtained by pooling fractions from 30 to 45%, using CD14 PE/CD45-FITC mAb, we found a leukocyte contamination of 0.8–5.7% and a monocyte contamination of 0.1–1.5%. Furthermore, using CD11b PE mAb, we found a contamination of granulocytes of 0.4–5.5%. An example of this is given in Figure 2. Finally, using CD19 FITC/CD3 PE mAb, we found a contamination of B and T lymphocytes of 0 and 0.4%.

No immature germ cells were found in the fractions from 60 to 100%. The immature germ cell concentration in fertile subjects and donors ranged from 0.9 to 1.5×10^6/ml with a mean and SD of 1.2 ± 0.3. Immature germ cells were not found in obstructive azoospermic patients. In patients 9 and 10, only a few immature germ cells were found (0.4 and 0.3×10^6/ml). In patients with dyspermia, immature germ cells ranged from 0.6 to 1.7×10^6/ml with mean and SD of 1.1 ± 0.5. In patients with genital tract infection, immature germ cells ranged from 1.6 to 2.0×10^6/ml with a mean and SD of 1.8 ± 0.2.

The immature germ cell types most frequently found were primary spermatocytes and spermatids. In the ejaculates of patients suffering from spermatogenesis arrest (cases 9 and 10), we found spermatocytes, but an absence of spermatids in the one case of primary spermatocyte arrest. However, in the case of spermatid arrest, we found all the germ cells, including spermatids. This agrees perfectly with the results of testicular cytology and histology.

**Discussion**

It has been shown in the literature that in normal subjects immature germ cells represent about 90% of the non-sperm cells present in semen (Auroux et al., 1985; Jassim and Festenstein, 1987; Smith et al., 1989; Fedder et al., 1993). In normal subjects, the concentration is on average 10–15% of the overall sperm concentration and is sometimes inversely correlated with the sperm number (Fedder et al., 1993). Of the germ cells found in seminal fluid, spermatogonia seem to be the least represented, even though a few studies have found them in very high concentrations (Auroux et al., 1985).

Primary spermatocytes are more numerous than secondary spermatocytes, perhaps owing to the shorter life of the latter. Spermatids are the most frequent cells seen in the ejaculate both in fertile and infertile subjects, obviously excluding cases of spermatogenic arrest (Fedder et al., 1993). Spermatogonia have a scanty cytoplasm with a round nucleus 6–7 µm width and 1 or 2 nucleoli. Primary spermatocytes are broad cells with a large central nucleus 8–9 µm in diameter in which it is possible to recognize the chromosomal spindle and a large central nucleolus 1–2 µm in diameter. They are very fragile cells, easily damaged by preparation techniques. Secondary spermatocytes are small round cells, with a central condensed nucleus 6–7 µm in diameter (sometimes two nuclei). Initial spermatids are small round cells with an condensed nucleus (sometimes two nuclei). Elongated spermatids have characteristics that, depending on their maturity, can make them similar to spermatooza.

As mentioned in the Introduction, to date very little attention has been given to the evaluation of immature germ cells in the semen and yet the separation of these cells could be extremely useful for research and diagnostic purposes. Two simple examples can be given to support this. The study of immature germ cells in the ejaculate would help to increase our knowledge of the normal and pathological chromosomal arrangements of the spermatogenic line. In fact, it is now possible to employ molecular probes to identify specific DNA fragments of interphasic cells (interphasic cytogenetics), using in-situ hybridization. This technique allows us to study single chromosomes and their numeric (aneuploidia) and structural alterations (deletions, translocations). Fertile and infertile subjects with various andrological pathologies (cryptorchidism, testicular cancer, etc.) can be evaluated. For example, data
Immature germ cell separation

Figure 1. Immature germ cells isolated by semen using Percoll gradient, May–Grunwald–Giemsa staining: (a) spermatogonia; (b) primary spermatocyte; (c) spermatid; (d) primary spermatocyte, primary spermatocyte in meiotic division, spermatid; (e) group of spermatocytes and spermatids. Scale bars = 1 µm.

Figure 2. Example of flow cytometric analysis of granulocytes stained with anti-Leu 15/CR3 PE (CD11b) found in the fractions from 30 to 45% of the Percoll gradient. Axes: RD-FL (orange fluorescence); GR-FL (green fluorescence); CD11b/PE (antiCD11b monoclonal antibody conjugated to phycoerythrin).

Regarding the hyperploidia of chromosome 1 in seminoma and in carcinoma in situ of the testis could permit early diagnosis by studying the semen instead of requiring testicular biopsy (Giwercman et al., 1987; Skakkebæk et al., 1987; Meng et al., 1996; Salanova et al., 1996). These complex cytogenetic techniques must employ cellular samples, as much as possible free not only of debris and leukocytes but also of spermatozoa, in order to avoid cellular overlapping that can make microscopic evaluation difficult.

Moreover, recent progress in the field of assisted reproduction has substantially modified the management of azoospermic patients as well as the concept of azoospermia itself. In fact, it is now possible to use spermatozoa obtained from the epididymis and from the testis (Craft et al., 1993; Silber, 1994). In addition, immature haploid germ cells (spermatids) can now be taken from the testis and from semen for micro-injection techniques (Edwards et al., 1994; Silber and Lenahan, 1995; Tesarik et al., 1995, 1996, Tesarik and Mendoza, 1996). Also with this technique, it is important to have as pure a
sample as possible, in order to identify easily the spermatids necessary for the microinjection procedure. Various methods have been proposed in order to select different cellular populations; discontinous gradient separation is one of the most frequently used techniques. This method employs the centrifugation of a gradient column constituted by a viscous liquid whose density increases gradually from top to bottom of the test tube. Such procedures are based on the differences of cell dimensions and on the speed of centrifugation. In fact, a sample, stratified on the gradient column and centrifuged for a sufficient time, passes through the gradients forming zones of sedimentation at the gradient interfaces, each one containing cells characterized by a specific velocity of sedimentation. This velocity depends on the density and dimension of the cell, on the density and viscosity of the medium and how much the cell differs from the spheric form. For these reasons we used a gradient comprising numerous fractions of Percoll with minimum differences of density. This allowed us to separate not only immature germ cells from spermatozoa, which are very different in form and density, but also to separate immature germ cells from leukocytes which are sometimes very similar.

In seminology, the Percoll gradient is the most widely used separation technique. In order to obtain the best population of spermatozoa, Berger et al. (1985) proposed the use of 40, 55, 70, 80, 90 and 100% gradients. However, round cells, leukocytes and immature germ cells were packed together in the upper fractions. Recently, a Percoll gradient with only three fractions (50–70–100%) was proposed by Angelopoulos et al. (1997). In this paper, a good separation of round spermatids was obtained in fraction 70%. Difficulties were seen when the spermatid concentration was low. Germ cells were then obtained by pooling the 70% fraction and the interface of fraction 50%. Moreover, this was possible only if the leukocyte concentration was low; in all the other cases, leukocyte contamination made the cell identification difficult.

In this paper we show that, in the patients analysed, the number of immature germ cells isolated from the samples varied considerably both in fertile and infertile subjects and between the various groups of andrological pathologies. In particular, the highest concentrations were found in subjects with genital tract infection. In this case, we postulate that the inflammation induced a greater exfoliation of immature germ cells from seminiferous tubules. However, also in these samples, our method achieved a good separation of immature germ cells with scanty leukocyte contamination. Our study demonstrates that the fractions from 30 to 45% showed the highest concentration of immature germ cells and the lowest leukocyte contamination. These data are supported by the use of permanent staining techniques that give a morphological definition even if they require a high level of experience from the analyst. In fact, spermatids and spermatoocytes can seem, in some cases, very similar to leukocytes and, due to the presence of more than one nucleus, they can seem like polymorphonuclear neutrophils with a multilobulated nucleus. To overcome these possible mistakes, we also used immunofluorescence microscopy and flow cytometry analysis: rapid and simple methods that were particularly useful to discriminate the various subpopulations of leukocytes in the Percoll fractions. These data indicate that our method can separate most of the immature germ cells, not only spermatids, from the other cells with a low level of leukocyte contamination (≤6%). The method also allows the successive identification of the different spermatogenic cellular types and, consequently, the confirmation of cytotogic- and histologic-diagnosed maturative arrest (cases 6 and 7).

In conclusion, many situations require the separation of immature germ cells from spermatozoa, leukocytes and debris in order to enable the best unstained identification of immature germ cells. This separation could also serve to improve our understanding of spermatogenetic disorders. The modified Percoll gradient set up and described above can provide the purified immature germ cell suspensions necessary for certain diagnostic and research purposes.

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References


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