J. Reprod. Fert. (1988) **84**, 551–556

Printed in Great Britain

Nuclear maturity and morphology of human spermatozoa selected by Percoll density gradient centrifugation or swim-up procedure

D. Le Lannou and Y. Blanchard

Unite de Biologie de la Reproduction, Cecos de l'ouest, Hotel Dieu, 35000 Rennes, France

Summary. The selection of motile human spermatozoa, from fertile and infertile semen samples was compared by using Percoll density gradient centrifugation or the swim-up procedure. Selected spermatozoa were evaluated according to their motility, % normal forms, nuclear maturity (aniline blue staining, acridine orange staining, ethidium bromide uptake and SDS nuclear decondensation). These methods showed differences between fertile and infertile men. The swim-up procedure, based on motility, resulted in greater proportions of motile spermatozoa and eliminated mainly tail abnormalities. Percoll gradient separation, based on density, selected oval-headed spermatozoa with good motility. Nuclear maturity level was improved by both methods but Percoll gradient separation generally resulted in spermatozoa with better nuclear maturity than those selected by the swim-up procedure.

Keywords: man; spermatozoa; nuclear maturity; selection; Percoll gradient; swim-up

Introduction

Human spermatozoa, compared to those of other mammal species, are characterized by a great heterogeneity in morphology, motility and nuclear maturity. The first stage to oocyte fertilization is the migration through the cervical mucus, which selects spermatozoa having appropriate morphology, motility and higher nuclear stability (Katz *et al.*, 1978; Gonzales & Jezequel, 1985; Le Lannou *et al.*, 1985). For in-vitro fertilization, a fraction of motile spermatozoa is usually selected by swim-up migration (Drevius, 1972; Lopata *et al.*, 1976; Aitken, 1988). Other techniques have been developed for the separation of motile spermatozoa, e.g. Percoll density gradient (Gorus & Pipeleers, 1981; Lessley & Garner, 1983; Bolton & Braude, 1984; Kaneko *et al.*, 1986). This method appears to improve the percentage of oocyte fertilization (Forster *et al.*, 1983; Hyne *et al.*, 1983; Berger *et al.*, 1985).

In this study we have compared various characteristics of human spermatozoa selected by the swim-up procedure or by Percoll density gradient centrifugation.

Materials and Methods

Samples. Semen samples, obtained from patients at our Center, were collected by masturbation after 3 days of sexual abstinence and allowed to liquefy for 30 min at 37°C. The samples were placed in one of two groups. Group 1 contained normozoospermic samples from fertile men (candidates for vasectomy) with $>20 \times 10^6$ spermatozoa/ml, $>50\%$ motility and $>50\%$ normal forms. Group 2 contained asthenozoospermic samples from infertile men with $>20 \times 10^6$ spermatozoa/ml, $<50\%$ motility and $<50\%$ normal forms.

Percoll density gradient centrifugation. Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) was made isotonic by adding 0·8 volumes of 0·4 M-Hepes-buffered Hank's 10-fold concentrated solution (Flow Laboratories, Puteaux, France) to 9·2 volumes of Percoll. The resulting solution (considered to be 100% Percoll) was further diluted with

Hank's solution to give 70%, 80% and 90% Percoll solutions. The pH was equilibrated to 7·4. The gradient was prepared by placing 2 ml of each of the three solutions in a conical plastic tube 30 mm in diameter.

A 1·5-ml semen sample was loaded at the top of the gradient and centrifuged at 700 *g* for 15 min for the normospermic samples and at 400 *g* for 30 min for the asthenospermic samples. After centrifugation, each fraction was collected by aspiration, beginning from the upper layer. The spermatozoa were washed once with 5 ml washing medium for in-vitro fertilization (I.M.V., 61300 L'aigle, France), to remove the Percoll.

Swim-up technique. A semen sample of 150 µl was layered on the bottom of a Falcon tube containing 1·5 ml washing medium (I.M.V.). After 30 min for normospermic samples and 60 min for asthenospermic samples, the upper part (1 ml) of the supernatant was aspirated.

Semen analysis. The different fractions were evaluated according to spermiogram characteristics. Motility was evaluated under phase-contrast microscopy (× 160) at 37°C. The spermocytogram was analysed according to the classification of David *et al.* (1975) after staining with haematoxylin–Shorr's stain.

Nuclear maturity. The nuclear maturity of spermatozoa was evaluated by various techniques. Lysine-rich nuclear proteins, revealing an immaturity of the spermatozoon, were assessed by aniline blue staining. After washing in Tyrode's solution (Difco laboratories, Detroit, MI, U.S.A.) (2 × 10 min, 700 *g*), smears were made on precleaned slides and allowed to dry in air. Smears were then fixed for 30 min in 2·5% glutaraldehyde in phosphate buffer (0·2 M, pH 7·2). The slides were stained with aniline blue solution (pH 3·5) as described by Terquem & Dadoune (1983) and measurements were taken in white light at 490 nm (Reichert-Jung microphotometer). Spermatozoa were classified as being coloured (< 50% transmission) or not coloured (> 50% transmission).

Evaluation of the DNA–DNP complex was studied by ethidium bromide uptake. For each fraction, 2×10^6 spermatozoa were washed twice in Tyrode's solution (700 *g*, 10 min), fixed for 30 min in methanol (4°C), washed again and treated by RNase (30 min). After the last centrifugation the pellet was resuspended in 200 µl 0·01% ethidium bromide solution. Samples were kept in the dark at 4°C until measurement. For measurement, 10 µl of the solution were smeared on the slide and the coverslip was sealed with nail polish. Measurements were made at 620 nm with an excitation filter at 546 nm. The fluorescence was expressed in arbitrary units. The excitation lamp was a mercury lamp (HBO-OSRAM).

Chromatin stability was evaluated by the nuclear swelling test with sodium dodecyl sulphate (SDS). After washing in Tyrode's solution, fractions were adjusted to 50×10^6 spermatozoa/ml and 50 µl of the solution were diluted in 50 µl, of a 2% SDS solution in borate buffer (50 mM, pH 9) (Bedford *et al.*, 1973; Kvist, 1980). The reaction was stopped after 10 min by addition of 200 µl 2·5% glutaraldehyde in cacodylate buffer. Assessment of the reaction was made by examining a drop of the reaction mixture with a phase-contrast microscope and the nuclei were classified as stable or unstable.

Acridine orange staining allowed evaluation of the integrity of the DNA. After washing in Tyrode's solution, fractions were adjusted to 50×10^6 spermatozoa/ml, one drop was smeared, allowed to dry in air and fixed in Carnoy's solution overnight. Slides were stained as described by Tejada *et al.* (1985).

Statistical analysis. The mean values are expressed ±s.e.m. Data analysis was performed according to the χ^2 method. Average comparisons were performed by Student's paired *t* tests.

Results

Figure 1 represents the distribution of motility, normal morphology and aniline blue staining of spermatozoa in the initial samples, Percoll density gradient fractions and after the swim-up procedure. The characteristics of spermatozoa were better in the 90% Percoll fraction and in the swim-up supernatant than in the ejaculate. However, spermatozoa recovered from the lower density fractions (Percoll semen supernatant; 70% Percoll) showed a significant decrease in the characteristics measured. Profiles from asthenospermic and normospermic semen were similar.

Both techniques selected spermatozoa with a higher motility ($P < 0{\cdot}01$) than in the initial semen. There was no statistical difference between them.

The Percoll gradient centrifugation and the swim-up procedure selected sub-populations of spermatozoa with good morphology. The percentage of normal forms was higher after Percoll selection than after the swim-up procedure. The difference was significant with normospermic samples ($P < 0{\cdot}01$). When considering the different types of abnormalities (Fig. 2), the results show that Percoll separation was more efficient in relation to head morphology, and the swim-up technique was more efficient in relation to the flagellar morphology.

The percentage of aniline blue-stained spermatozoa was significantly decreased in the Percoll higher density fraction (90%) and in the swim-up supernatant compared to the ejaculate. Percoll separation selected more mature spermatozoa (unstained) than did the swim-up procedure,

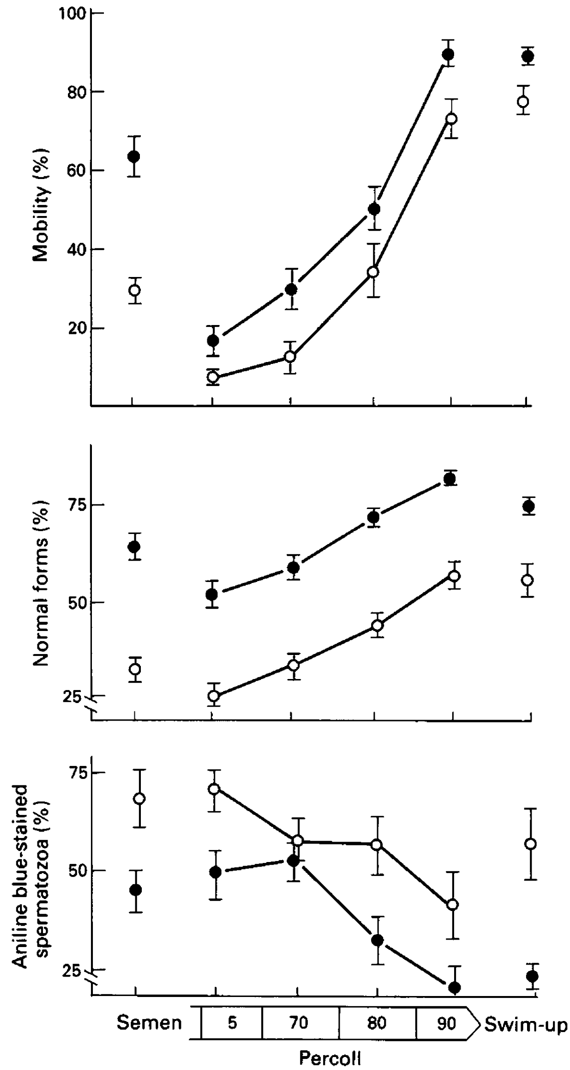

Fig. 1. Distribution of motility, normal morphology and aniline blue-stained spermatozoa in semen, Percoll density gradient fractions and swim-up supernatant in normospermic samples (—●—) and in asthenozoospermic samples (—○—). Values are mean ± s.e.

especially in asthenospermic samples ($P < 0{\cdot}01$). The percentage of immature spermatozoa was significantly higher in the asthenospermic semen samples than in the normospermic semen samples but the profiles of distribution were similar for both.

As shown in Table 1, fertile and infertile semen samples were significantly different with respect to nuclear maturity assessed by aniline blue staining, acridine orange staining and SDS nuclear decondensation. Compared to the initial samples, selected spermatozoa had a higher percentage of SDS-stable nuclei, acridine-orange green nuclei, a lower ethidium bromide fluorescence intensity and a lower percentage of aniline blue-stained spermatozoa ($P < 0{\cdot}01$). When a significant difference between the two separation methods was noted (Table 2) it was always the Percoll gradient separation that gave the better result.

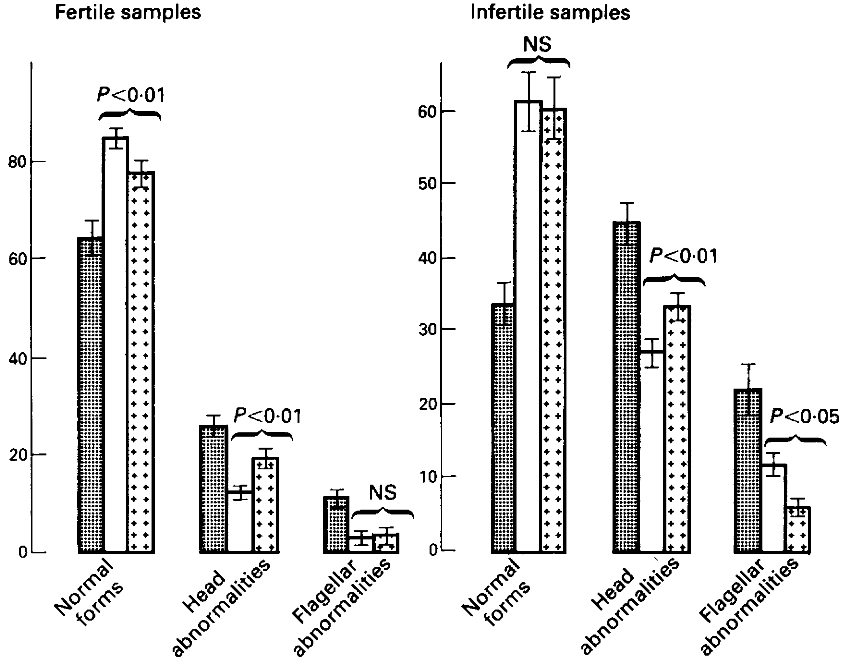

Fig. 2. Distribution of normal forms, head abnormalities and flagellar abnormalities in initial samples ; the Percoll 90% fraction and the swim-up supernatant . Values are mean ± s.e. The differences between initial samples and both selected samples were all significant ($P < 0{\cdot}01$).

Table 1. Nuclear maturity evaluation of semen samples in fertile and infertile men

	Fertile samples ($n = 10$)	Infertile samples ($n = 10$)
Ethidium bromide fluorescence at 620 nm (arbitrary units)	41·92 ± 3·04	49·32 ± 2·70
Acridine orange staining (% green spermatozoa)	83·0 ± 3·28	62·4 ± 3·80*
Nuclear swelling (% stable nuclei with SDS)	79·75 ± 1·35	63·0 ± 3·63*
Aniline blue staining (% of stained spermatozoa)	46·32 ± 4·98	69·2 ± 6·74*

Values are mean ± s.e.
*$P < 0{\cdot}01$.

Discussion

Numerous studies have shown that sperm separation, by the swim-up technique or by Percoll gradient centrifugation, selects spermatozoa with higher motility and better morphology (Lopata *et al.*, 1976; Gorus & Pipeleers, 1981; Arcidiacono *et al.*, 1983; Lessley & Garner, 1983; Bolton & Braude, 1984; Kaneko *et al.*, 1986; Aitken, 1988). In this study, the percentage of motile spermatozoa was increased with each method and there was no difference between them. However,

Table 2. Nuclear maturity evaluation after selection by Percoll density gradient centrifugation or swim-up procedure in fertile (n = 10) and infertile (n = 10) semen samples

	Fertile samples		Infertile samples	
	Percoll density gradient separation	Swim-up separation	Percoll density gradient separation	Swim-up separation
Ethidium bromide fluorescence at 620 nm (arbitrary units)	35·75 ± 3·00	36·97 ± 3·39*	45·79 ± 2·33	47·10 ± 2·54†
Acridine orange staining (% green spermatozoa)	95·12 ± 1·64	95·75 ± 0·81	78·60 ± 4·86	80·50 ± 5·90
Nuclear swelling (% stable nuclei with SDS)	89·05 ± 1·08	88·62 ± 1·45	73·56 ± 4·55	74·80 ± 3·13
Aniline blue staining (% of stained spermatozoa)	26·72 ± 5·37	28·5 ± 3·86	44·86 ± 8·22	63·6 ± 10·6†

Values are mean ± s.e.
*P < 0·02.
†P < 0·01.

Guerin *et al.* (1987) have shown that Percoll selection produces a higher survival rate of spermatozoa in B2 medium, while Akerlof *et al.* (1987) have shown that the Percoll technique selects spermatozoa with significantly higher specific progressive motility and ATP content than does the swim-up method.

Morphological abnormalities were better eliminated by Percoll gradient than by swim-up separation; moreover, the study of the different types of abnormalities shows that selection is not identical by the two methods: Percoll gradient separation eliminated mainly head abnormalities while tail abnormalities were better eliminated by the swim-up technique. These results illustrate that selection of spermatozoa by these two methods is based on different sperm characteristics. The swim-up technique is based on the intrinsic motility of spermatozoa; the more motile spermatozoa selectively accumulate in the overlying medium with a concomitant elimination of spermatozoa presenting tail abnormalities in the pellet.

In Percoll density gradients, spermatozoa are separated by equilibrium, when sperm density equals that of the gradient. In this way spermatozoa will be separated principally on the basis of differences in density (Bolton & Braude, 1984). Spermatozoa with oval heads present a dense and homogeneous nucleus when viewed in an electron microscope, whereas chromatin from atypical heads has a coarsely granular appearance with nuclear vacuoles of various sizes (Fawcett, 1958). Thus spermatozoa with good nuclear morphology are more dense and selected in the higher density fraction of the Percoll gradient.

Human spermatozoa are characterized by a remarkable morphological heterogeneity and also by the presence of various degrees of nuclear maturation (Bedford *et al.*, 1973; Le Lannou *et al.*, 1986). In this study, several methods were used for the evaluation of nuclear condensation, nuclear stability or DNA–DNP complex. All these methods showed differences between the two groups of fertile and infertile men, and confirmed previous reports (Johanisson & Eliasson, 1981) that defects in spermatogenesis correlate with alterations in nuclear maturity.

Sperm populations prepared by the Percoll gradient or swim-up techniques contained more homogeneous sub-populations, with a higher degree of nuclear maturity, but the Percoll gradient technique generally results in spermatozoa with a greater nuclear maturity than those prepared by the swim-up technique. Our results show that Percoll gradient separation can be used for normospermic and asthenospermic ejaculates and, although the recovery rate is low (7% for asthenospermic samples and 13% for normospermic samples in our study), the number of spermatozoa recovered will be adequate for their use in in-vitro fertilization.

Further work is needed to evaluate the importance of using Percoll-gradient separation of spermatozoa for human in-vitro fertilization programmes.

This work was supported by a grant from the Institut National de la Santé et de la Recherche Medicale (INSERM, contrat No. 84-4011).

References

Aitken, R.J. (1988) Assessment of sperm function for IVF. *Human Reprod.* 3 (1), 89–95.

Akerlof, E., Fredricson, B., Gustafsson, O., Lundin, A., Lunell, N.O., Nylund, L., Rosenborg, L. & Pousette, A. (1987) Comparison between a swim-up and a Percoll gradient for the separation of human spermatozoa. *Int. J. Androl.* **10,** 663–669.

Arcidiacono, A., Walt, H., Campana, A. & Balerna, M. (1983) The use of Percoll gradients for the preparation of subpopulations of human spermatozoa. *Int. J. Androl.* **6,** 433–445.

Bedford, J.M., Bent, M.J. & Calvin, H. (1973) Variations in the structural character and stability of the nuclear chromatin in morphologically normal human spermatozoa. *J. Reprod. Fert.* **33,** 19–39.

Berger, T., Marrs, R. & Moyer, D. (1985) Comparison of techniques for selection of motile spermatozoa. *Fert. Steril.* **40,** 268–273.

Bolton, V.N. & Braude, P.R. (1984) Preparation of human spermatozoa for in vitro fertilization by isopycnic centrifugation on self generating density gradient. *Archs Androl.* **13,** 167–176.

David, G., Bisson, J.P., Czyglik, F., Jouannet, P. & Gernigon, C. (1975) Anomalies morphologiques du spermatozoide humain. *Eur. J. Gyn. Obst. Biol. Reprod.* **4,** 17–36.

Drevius, L.O. (1972) The 'sperm-rise' test. *J. Reprod. Fert.* **24,** 427–432.

Fawcett, D.W. (1958) The structure of the mammalian spermatozoon. *Int. Rev. Cytol.* **7,** 195–234.

Forster, M.A., Smih, W.D., Lee, W.L., Berger, R.E., Karp, L.E. & Stenchever, M.A. (1983) Selection of human spermatozoa according to their relative motility and their interaction with zona-free hamster eggs. *Fert. Steril.* **40,** 655–660.

Gonzales, J. & Jézéquel, F. (1985) Influence of the quality of the cervical mucus on sperm penetration: comparison of the morphologic features of spermatozoa in 101 postcoital with those in the semen of the husband. *Fert. Steril.* **44,** 796–799.

Gorus, F.K. & Pipeleers, D.G. (1981) A rapid method for the fractionation of human spermatozoa according to their progressive motility. *Fert. Steril.* **35,** 662–665.

Guerin, J.F., Mathieu, C., Pinatel, M.C., Lornage, J. & Boulieu, D. (1987) Indication masculine de la FIV. Intéret du traitement du sperme par centrifugation sur gradient de Percoll. *Contra. Fert. Sex.* **15,** 704–705.

Hyne, R.V., Stojanoff, A., Clarke, G.N., Lopata, A. & Johnston, W.I.H. (1983) Pregnancy from in vitro fertilization of human eggs after separation of motile spermatozoa by density gradient centrifugation. *Fert. Steril.* **45,** 93–96.

Johannisson, E. & Eliasson, R. (1981) Ethidium bromide uptake by spermatozoa from fertile and infertile men. *Int. J. Androl.* Suppl. **3,** 72, abstr.

Kaneko, S., Oshio, S., Kobanawa, K., Kobayashi, T., Mohri, H. & Iizuka, R. (1986) Purification of human sperm by a discontinuous Percoll density gradient with an inner column. *Biol. Reprod.* **35,** 1059–1063.

Katz, D.F., Mills, R.N. & Pritchett, T.R. (1978) The movement of human spermatozoa in cervical mucus. *J. Reprod. Fert.* **53,** 259–265.

Kvist, U. (1980) Sperm nuclear chromatin decondensation ability. *Acta physiol. scand., Suppl.* **486,** 5–25.

Le Lannou, D., Colleu, D., Boujard, D. & Segalen, J. (1985) Stability of nuclear chromatin in human spermatozoa during capacitation in vitro. *In Human In Vitro Fertilization: Actual Problems and Prospects,* pp. 149–152. Eds J. Testart & R. Frydman. Elsevier Science Publishers, Amsterdam.

Le Lannou, D., Colleu, D., Boujard, D., Le Couteux, A., Lescoat, D. & Segalen, J. (1986) Effect of duration of abstinence on maturity of human spermatozoa nucleus. *Archs Androl.* **17,** 35–38.

Lessley, B.A. & Garner, D.L. (1983) Isolation of motile spermatozoa by density gradient centrifugation in Percoll. *Gamete Res.* **7,** 49–61.

Lopata, A., Patullo, M.J., Chang, A. & James, B. (1976) A method for collecting motile spermatozoa from human semen. *Fert. Steril.* **27,** 677–684.

Tejada, R.I., Mitchell, J.C., Norman, A., Marik, J.J. & Friedman, S. (1985) A test for practical evaluation of male fertility by acridine orange (ao) fluorescence. *Fert. Steril.* **42,** 87–91.

Terquem, A. & Dadoune, J.P. (1983) Aniline blue staining of human spermatozoa chromatin. Evaluation of nuclear maturation. In *The Sperm Cell,* pp. 249–252. Ed. J. André. Martinus Nijhoff, The Hague.

Received 10 March 1988