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Evaluation of three substitutes for Percoll in sperm isolation by density gradient centrifugation

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Silane-coated silica particle solutions (ISolate™ and PureSperm™) and iodixanol (OptiPrep™) were compared to polyvinylpyrrolidone (PVP)-coated silica particles (Percoll™) in their efficacy to recover spermatozoa by gradient centrifugation for use in assisted reproductive procedures. Efficacy was assessed in terms of percentages of sperm recovery, sperm vitality and motility, normal sperm morphology and normal sperm chromatin condensation. No significant difference was found in the recovery of spermatozoa for men with both normal sperm counts and oligozoospermia, between PVP-coated and silane-coated particle solutions. Iodixanol had significantly lower sperm recovery compared to the other products. Sperm vitality, progressive motility, normal morphology and normal chromatin condensation did not differ significantly between any of the sperm isolation products.

Key words: iodixanol/Percoll/silane silica colloids/sperm preparation

Introduction

Increasing the proportion of motile and morphologically normal spermatozoa from semen is an important objective in assisted reproductive technology. Several gradient centrifugation materials have been introduced to improve sperm quality and fertilization outcome, including albumin (Ericsson, 1977), Ficoll (Bongso *et al*., 1989), Sephadex (Steeno *et al*., 1975), iohexol (Gellert-Mortimer *et al*., 1988), Accudenz (Sbacia *et al*., 1996), polysaccharide beads (Ohashi *et al*., 1992) and Percoll (Gorus *et al*., 1981). The beneficial effect of Percoll gradient centrifugation on sperm count and motility has been well documented (Berger *et al*., 1985; Hyne *et al*., 1986) and it became the predominant product used for sperm preparation in assisted reproductive technology. The average recovery of motile spermatozoa from Percoll gradients is 49% (Berger *et al*., 1985) with a lesser and varying degree from oligozoospermic and asthenozoospermic samples (Hyne *et al*., 1986). Increases as well as decreases in the recovery of morphologically normal spermatozoa have been documented after Percoll-gradient centrifugation (Hyne *et al*., 1986; Menkveld *et al*., 1990a; Van der Zwalmen *et al*., 1991). Percoll is also effective in removing bacteria (Bolton *et al*., 1986) and in reducing reactive oxygen species formation (Aitken *et al*., 1988) during gradient centrifugation of spermatozoa. High pregnancy rates have been reported after sperm preparation using Percoll (Hyne *et al*., 1986; Ord *et al*., 1990; Van der Zwalmen *et al*., 1991). The withdrawal of Percoll for use in assisted reproductive technology in humans (Pharmacia Biotech, October 1996) has led to the introduction of several alternative products including iodixanol (Harrison, 1997; Smith *et al*., 1997) and silane-coated silica colloid solutions (Stone *et al*., 1997).

In comparison with Percoll [colloidal polyvinylpyrrolidone (PVP)-coated silica particles], two of the substitute products recently introduced for sperm preparation, namely ISolate and PureSperm, consist of colloidal silane-coated silica particles. Another replacement product, OptiPrep, is a solution of iodixanol, {5,5′-[(2-hydroxy-1–3-propanediyl)-bis(acetylamino)]-bis-[*N*,*N*'-bis(2,3-dihydroxypropyl-2,4,6-triiodo-1,3-benzene carboxamide]} designed for the in-vitro isolation of organelles and a wide range of cell types. The purpose of this study was to evaluate the performance of these products as a replacement for Percoll. The efficacy of these products was assessed by evaluation of the percentages of sperm recovery, sperm vitality and motility, normal sperm morphology and normal sperm chromatin condensation since it has been shown that sperm motility (Mahadevan and Trounson, 1984), normal sperm morphology (Kruger *et al*., 1986, 1988) and percentage normal chromatin condensation (Foresta *et al*., 1992) are significantly related to oocyte fertilization rates *in vitro*. It is, therefore, of importance to find a substitute product for Percoll which will give equal or better sperm preparation results in order to continue achieving good results with assisted reproductive technology.

Materials and methods

Semen preparation

Semen samples of 20 patients having assisted reproductive technology treatment with Queensland Fertility Group were included in this study. Specimens were produced by masturbation into sterile plastic containers and prepared within 4 h of ejaculation. Ten oligozoospermic samples (sperm concentration $<20\times10^6$/ml) and 10 samples with normal sperm counts ($\geqslant20\times10^6$/ml) were randomly selected. An aliquot of 1.6 ml or 2 ml semen was removed from 18 of the original samples and divided into four equal parts and used for ISolate, OptiPrep, Percoll and PureSperm density centrifugation techniques. Aliquots of 1.2 ml and 0.6 ml were removed from the remaining two samples and were treated similarly to the other samples by dividing them into four equal parts for the different gradient centrifugation techniques.

ISolate, OptiPrep, Percoll and PureSperm density gradients were prepared as described below, in 15 ml plastic conical centrifuge tubes

(Falcon 2095, Becton Dickinson, NJ, USA) and warmed in a 5% CO_2 incubator at 37°C for at least 3 h before use.

ISolate gradient
The gradient for sperm selection was prepared by pipetting 1 ml of the commercially supplied 'lower layer' ISolate solution (Irvine Scientific, Santa Ana, CA, USA) into the bottom of the centrifuge tube and layering 1 ml of the supplied 'upper layer' ISolate carefully on top of the 'lower layer'.

OptiPrep gradient
OptiPrep solutions and gradients were prepared as described by Harrison (1997). Stock OptiPrep was prepared by adding 9 ml OptiPrep (Nycomed Pharma AS, Majorstua, Oslo, Norway) to 1 ml of ×10 concentrated Ham's F10 solution (Sigma N6635). The bottom fraction of the OptiPrep gradient (40%) was prepared by mixing 4 ml of the OptiPrep stock with 6 ml of 'in-house'-produced human tubal fluid (HTF) culture medium (Quinn *et al*., 1985). The upper fraction (25% OptiPrep) consisted of 2.5 ml of OptiPrep stock solution mixed with 7.5 ml HTF culture medium. Solutions were stored at 4°C until used. To prepare the gradient for sperm purification, 1 ml of 40% OptiPrep was pipetted into the bottom of the centrifuge tube and 1 ml of 25% OptiPrep was carefully layered over the bottom fraction.

Percoll gradient
Stock isotonic Percoll solution was prepared by adding 9 ml Percoll (Pharmacia AB, Uppsala, Sweden) to 1 ml of 10× concentrated Ham's F10 solution (Irvine Scientific). The bottom fraction of the Percoll gradient (90%) was prepared by mixing 9 ml of the Percoll stock with 1 ml of HTF culture medium. The upper fraction (45%) consisted of 4.5 ml of Percoll stock solution mixed with 5.5 ml HTF culture medium. Solutions were stored at 4°C until used. To prepare the gradient, 1 ml of 90% Percoll was pipetted into the bottom of the centrifuge tube and 1 ml of 45% Percoll was carefully layered over the bottom fraction.

PureSperm gradient
The bottom fraction of the PureSperm gradient (80%) was prepared by mixing 12 ml of commercially supplied PureSperm solution (Nidacon Laboratories AB, Goteborg, Sweden) with 3 ml of HTF culture medium. The upper fraction (40%) was obtained by dilution of the 80% solution with an equal volume of HTF culture medium and stored at 4°C until used. To prepare the gradient for sperm purification, 1 ml of 80% PureSperm was pipetted into the bottom of the centrifuge tube and 1 ml of 40% PureSperm was carefully layered over the bottom fraction.

After semen liquefaction, a 0.4 or 0.5 ml aliquot of the sample was layered on top of the upper layers of the Percoll, ISolate, PureSperm and OptiPrep gradients respectively. The tubes were centrifuged at 400 *g* for 20 min and the resulting pellets aspirated and centrifugally washed once in 3 ml of HTF (supplemented with 10% HSA) at 400 *g* for 10 min. The final pellet was diluted in 0.1–0.5 ml HTF medium (supplemented with 10% HSA) and used for evaluation of the characteristics of recovered spermatozoa.

Sperm concentration was measured with a haemocytometer. The percentage progressive motile spermatozoa was estimated to the nearest 10% on a wet preparation (400× magnification) before and after gradient centrifugation.

Supra-vital staining
A modified eosin–nigrosin method, as described by Harrison and Campbell (1976), was performed to evaluate sperm vitality (living and dead). Two drops of semen were mixed with seven drops of stain and a smear was prepared after 5 min and allowed to dry in air. One hundred spermatozoa were counted at ×1000 magnification. Red, or any sperm cells not totally white, were regarded as dead and the results expressed as the percentage of live (white) spermatozoa.

Finally, smears were prepared for the evaluation of sperm morphology and chromatin condensation.

Morphology evaluation

Thin smears were prepared of the original semen samples and the resultant suspensions after completion of the different isolation procedures. The smears were air-dried and fixed in 95% alcohol and stained by a modified Papanicolaou method (Menkveld *et al*., 1991). Coverslips were mounted with DPX (BDH 36029). The slides were coded and at least 100 spermatozoa per slide were evaluated according to strict criteria (Menkveld *et al*., 1990b) at ×1250 oil magnification in a blind fashion. Spermatozoa were classified as morphologically normal or abnormal.

Teratozoospermia index
Spermatozoa were regarded as abnormal if a head, neck/midpiece or tail defect or a cytoplasmic droplet was present. It is therefore possible for each abnormal spermatozoon to have one or up to four defects. Each abnormality per abnormal spermatozoon was tallied separately so that the teratozoospermia index (TZI) could be calculated [World Health Organization (WHO) 1992; Mortimer, 1994]. This was done by dividing the total number of abnormalities per 100 spermatozoa counted by the number of abnormal spermatozoa. The number of abnormal spermatozoa can be obtained by subtracting the number of normal spermatozoa from 100 (WHO, 1992; Mortimer, 1994).

Acrosome index
Acrosome morphology was evaluated as described previously (Menkveld *et al*., 1996). The following differential classification was used for the evaluation of the acrosomes: normal, staining defect, too large, too small and other/amorphous. From this evaluation the acrosome index (AI) was calculated and expressed as the percentage of morphological normal acrosomes (Menkveld *et al*., 1996; Menkveld and Kruger, 1996).

Chromatin condensation

Air-dried smears were fixed for 30 min in 3% glutaraldehyde (Merck, Darmstadt, Germany) and stained for 7 min in acidic aniline blue (Sigma M5528), pH 3.5, and washed in phosphate-buffered saline, as described by Dadoune *et al*. (1988). Slides were carefully blotted dry with filter paper. Examination was performed at ×1000 magnification within 2 days of preparation since the dye fades with time. Two staining intensities of spermatozoa were distinguished: (i) absolutely unstained plus negatively and partially stained spermatozoa with slight impregnation of the outlines (normal) and (ii) completely stained (abnormal).

Statistical analysis

Sperm recovery was calculated as follows:
[(final volume×final concentration)/(initial volume×initial concentration)] ×100.
To determine vital and progressive motile sperm recovery the final and initial percentages of vital (% supra-vital negative) and progressive motile (visual estimation) spermatozoa were incorporated in the formula.

Results were analysed as a two-factor, partially nested analysis of variance to determine the effect of initial sperm concentration and the four different gradient centrifugation techniques on sperm recovery and the various sperm parameters. Results were expressed as mean ± SD.

Results

Table I shows the percentage recovery of vital and progressive motile spermatozoa using ISolate, OptiPrep, Percoll and PureSperm gradient centrifugation techniques in two sperm populations, oligozoospermia and samples with normal counts. There was no significant difference ($P > 0.05$) in the recoveries between ISolate, Percoll and PureSperm for oligozoospermia and the normal count group. The recovery of vital and progressive motile spermatozoa were significantly lower ($P < 0.05$) with OptiPrep compared to the other products for both sperm populations. Vital and progressive motile sperm recovery after iodixanol treatment were 10.7 and 13.2% for oligozoospermia and 19.7 and 27% for the normal count group respectively (Table I). Sperm recoveries were significantly higher ($P < 0.05$) in the group of patients with normal sperm counts compared to oligozoospermic samples, as would be expected.

Table II summarizes the sperm characteristics of the untreated, and Percoll-, ISolate-, PureSperm- and OptiPrep-treated samples. The concentration, vitality and progressive motility for the untreated oligozoospermic samples were 12.6 ± 6.3 $\times 10^6$/ml, 59.8 ± 16.9%, 42 ± 9.2% ($n = 10$) and for untreated men with normal sperm concentrations, 72.8 ± 38.3 $\times 10^6$/ml, 74.0 ± 16.5%, 58 ± 18.1% ($n = 10$), respectively. Since the concentration obtained after each treatment depended on the volume of HTF medium added, no statistical significance in the changes of sperm concentrations was determined; however, the final sperm number (concentration/volume) is unaffected by the volume added. The percentages of sperm vitality and progressive motility obtained with all of the materials tested (ISolate, OptiPrep, Percoll and PureSperm) increased significantly ($P < 0.01$) over those of the untreated samples for both groups of men. There was no significant difference in sperm vitality and progressive motility between the four treatment groups. The percentage of progressive motile spermatozoa exceeded the percentage of vital spermatozoa except in the iodixanol-treated oligozoospermia group (Table II).

No change occurred in the percentage of morphological normal spermatozoa and the acrosome index in both the oligozoospermia and normal count groups. However, the TZI showed a statistical significant decrease ($P < 0.001$) after all four treatments, compared to the baseline values, in both groups (Table II).

Table I. Sperm recoveries on ISolate, OptiPrep, Percoll and PureSperm density gradients (values are mean ± SD)

	Recovery (%)			
	ISolate	OptiPrep	Percoll	PureSperm
Oligozoospermia ($n = 10$)				
Vitality	23.5 ± 26.2	10.7 ± 7.9[a]	18.9 ± 16.9	23.2 ± 23.2
Progressive motile	32.9 ± 24.4	13.2 ± 7.5[a]	28.1 ± 15.6	30.9 ± 16.1
Normal sperm counts ($n = 10$)				
Vitality	32.0 ± 11.2	19.7 ± 13.3[a]	32.0 ± 12.7	33.2 ± 13.9
Progressive motile	44.0 ± 15.9	27.0 ± 18.5[a]	44.8 ± 15.1	45.8 ± 18.4

[a]Significantly lower than other values in the same row ($P < 0.05$).

Table II. Comparison between baseline semen parameters and resultant values after ISolate, OptiPrep, Percoll and PureSperm gradient centrifugation for the oligozoospermia and normal count groups (values are mean ± SD)

Sperm parameters	Untreated	ISolate	OptiPrep	Percoll	PureSperm
Oligozoospermia ($n = 10$)					
Concentration[a] ($\times 10^6$/ml)	12.6 ± 6.3	2.8 ± 1.4	1.9 ± 1.6	2.7 ± 2.0	3.6 ± 2.6
Vitality (%)	59.8 ± 16.9[b]	69.7 ± 16.1	73.4 ± 13.7	73.3 ± 14.9	67.0 ± 15.4
Progressive motility (%)	42.0 ± 9.2[b]	85.8 ± 16.8	71.5 ± 18.9	87.4 ± 9.1	77.5 ± 18.6
Morphology normal[c] (%)	3.2 ± 1.9	4.6 ± 3.9	4.5 ± 2.5	3.6 ± 2.5	4.3 ± 2.5
Teratozoospermia index	1.7 ± 0.3[d]	1.42 ± 0.2	1.5 ± 0.2	1.5 ± 0.3	1.5 ± 0.2
Acrosome index[c] (%)	4.3 ± 2.1	5.5 ± 3.2	6.1 ± 2.2	4.4 ± 2.5	5.4 ± 2.7
Normal chromatin condensation (%)	64.3 ± 13.2[e]	90.3 ± 9.7	79.1 ± 10.8	90.5 ± 9.3	88.2 ± 10.8
Normal sperm counts ($n = 10$)					
Concentration[a] ($\times 10^6$/ml)	72.8 ± 38.3	20.5 ± 13.6	12.3 ± 8.8	20.5 ± 15.6	23.3 ± 19.9
Vitality (%)	74.0 ± 16.5[b]	84.8 ± 6.7	80.9 ± 9.6	85.2 ± 6.9	82.6 ± 8.0
Progressive motility (%)	58.0 ± 18.1[b]	87.4 ± 8.4	82.4 ± 13.5	92.7 ± 6.4	87.4 ± 10.5
Morphology normal[c] (%)	6.9 ± 4.3	7.1 ± 4.1	5.9 ± 2.9	5.4 ± 3.6	8.7 ± 4.5
Teratozoospermia index	1.5 ± 0.2[d]	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
Acrosome index[c] (%)	8.1 ± 4.9	7.4 ± 3.6	7.7 ± 4.4	7.6 ± 5.2	9.7 ± 4.6
Normal chromatin condensation (%)	80.1 ± 12.5	86.9 ± 14.9	88.0 ± 14.9[f]	85.1 ± 22.4	82.5 ± 18.7

[a]Statistical differences not determined.
[b,e]Significantly lower than other values in same row ($P < 0.01$).
[c]No significant difference between groups.
[d]Significantly higher than other values in same row ($P < 0.001$).
[e]Significantly higher than untreated sample ($P < 0.05$).

In oligozoospermic samples, all treatments (ISolate, OptiPrep, Percoll and PureSperm) significantly increased ($P < 0.01$) the percentage of spermatozoa with normal chromatin condensation, compared to baseline values. The increase with iodixanol was less than with silica-based solutions (Table II). In the group of men with normal sperm counts the percentage of spermatozoa with normal condensed chromatin also increased but only OptiPrep showed statistical significance. No significant differences were found between the four different sperm preparation treatments (Table II). Only OptiPrep-purified samples showed statistically significant increases for both groups of men (Table II).

Discussion

In the present study two silane-coated silica particle solutions (PureSperm and ISolate) were compared with iodixanol (OptiPrep) as replacement products for Percoll in the separation of spermatozoa by density gradient centrifugation. Our results showed that vital and progressive motile sperm recovery, using the two silane-coated silica solutions, were equally effective compared to Percoll (Table I) for both sperm populations, oligozoospermia and the normal count samples. This is in agreement with the findings of Stone *et al*. (1997) who found no differences in motile sperm recovery between Percoll and two silane-coated particle solutions (ISolate and Enhance-S Plus).

Our study indicated that iodixanol resulted in significantly lower vital and progressive motile sperm recovery compared to silica-based colloid solutions (ISolate, Percoll, PureSperm) for both groups of men (Table I).

Significantly fewer ($P < 0.05$) spermatozoa were recovered from oligozoospermic samples compared to samples with normal sperm counts, for all of the gradient materials tested (Table I). This result could be expected since low initial sperm concentrations have been shown to result in lower sperm recovery using Percoll (Arcidiacono *et al*., 1983).

The percentages of vital and progressive motile spermatozoa increased significantly for all the gradient materials tested (ISolate, OptiPrep, Percoll and PureSperm) for both sperm populations, oligozoospermia and the normal count group (Table II). The higher percentage of progressive motile spermatozoa compared to vital (percentage alive) spermatozoa, after silica-particle gradient centrifugation procedures, was unexpected (Table II). This could be caused by sperm membrane damage occurring during centrifugation using silica-coated particles. Damage to sperm membranes would allow an increased influx of eosin stain with a subsequent high 'false dead' interpretation, although the spermatozoa may still have been progressively motile. Prominent swelling of the sperm membrane (Arcidiacono *et al*., 1983) and sperm abnormalities, such as big vacuoles in the acrosomal region (Menkveld *et al*., 1990a), following Percoll treatment have been described. However, the use of hyperosmotic Percoll gradients prevents osmotic swelling of spermatozoa during centrifugation (Chan *et al*., 1994; Carbone *et al*., 1997).

Fears of sperm damage by silica-coated particles during centrifugation have long been expressed (Arcidiacono *et al*., 1983; Leventhal *et al*., 1987), particularly at higher centrifugation speeds (Fredricsson *et al*., 1977; Alvarez *et al*., 1993). It was suggested that the PVP coating of Percoll may cause harm to human spermatozoa and/or may affect subsequent fertilization events (De Vos *et al*., 1997). However, it was shown that the double washing procedure, after gradient centrifugation, successfully removes any residual PVP-silica particles from the sperm preparation (Pickering *et al*., 1989).

Damage can also be caused to spermatozoa by the formation of the dense pellet during the centrifugation process (Aitken and Clarkson, 1988; Iwasaki *et al*., 1992). Pellet formation during centrifugation can be avoided if the density of the bottom layer of the gradient is higher than the density of human spermatozoa. Such a condition is met with one approach to iodixanol centrifugation (Smith *et al*., 1997). No significant difference was found in the recovery of morphologically normal, motile spermatozoa between iodixanol and Percoll (Smith *et al*., 1997). Iodixanol is a non-silica-based medium and, since pellet formation can be eliminated during centrifugation, sperm damage can theoretically be minimized. Pellet formation, however, has advantages over harvesting from a gradient interface (or layer) in samples with extremely low numbers of spermatozoa as one can be more certain of concentrating all available spermatozoa. Also, iodixanol is non-toxic and inert and allowed for IVF use by the US Federal Drug Administration.

The results of both sperm populations, oligozoospermia and the normal count samples, showed no change in the percentages of morphologically normal spermatozoa and acrosome index compared to the baseline values. This is in agreement with the results of Smith *et al*. (1997) and Menkveld *et al*. (1990a). However, it was interesting to note that there was a statistically significant reduction in the TZI for both groups with all four treatment methods compared to the baseline values. The reduction in the TZI values was in all cases due to a lower percentage of neck/midpiece, tail and cytoplasmic droplet defects and not due to a decrease in the percentage of morphologically abnormal heads. This is also in agreement with the results of Menkveld *et al*. (1990a) of the effect of Percoll treatment and a wash and swim-up method compared to the original baseline values. This may indicate that sperm motility plays an important role in the selection of motile spermatozoa. Selection of motile spermatozoa seems to be independent of the normal head morphology of spermatozoa involved, but rather due to the presence of structural abnormalities of the neck/midpiece and tail of the spermatozoa.

Both sperm populations, oligozoospermia and the normal count group, showed increased percentages of normal sperm chromatin condensation after all treatments (ISolate, OptiPrep, Percoll and PureSperm) compared to baseline values (Table II), although in normal count samples the increase was only significant for OptiPrep. This is in agreement with the results of Herruzo *et al*. (1997) who found that spermatozoa isolated from PureSperm contained a high degree of nuclear maturity.

In conclusion, silane-coated silica particle solutions appear to be suitable replacement products for Percoll regarding sperm recovery in the two sperm populations, oligozoospermia and normal count group. Sperm yield after iodixanol preparation

was significantly lower. No significant differences occurred in sperm motility, normal morphology and normal chromatin condensation between the isolation products for both groups of men. Clinical fertilization and pregnancy outcomes are required for final evaluation of these products.

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References

Aitken, R.J. and Clarkson, J.S. (1988) Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J. Androl*., **9**, 367–376.

Alvarez, J.G., Lasso, J.L., Blasco, L. *et al*. (1993) Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile lifetime. *Hum. Reprod*., **8**, 1087–1092.

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

Berger, T., Marrs, R.P. and Moyer, D.L. (1985) Comparison of techniques for selection of motile spermatozoa. *Fertil. Steril*., **43**, 268–273.

Bolton, V.N., Warren, R.E. and Braude, P.R. (1986) Removal of bacterial contaminants from semen for *in vitro* fertilization or artificial insemination by the use of buoyant density centrifugation. *Fertil. Steril*., **46**, 1128–1132.

Bongso, A., Ng, S., Mok, H. *et al*. (1989) Improved sperm concentration, motility, and fertilization rates following Ficoll treatment of sperm in a human *in vitro* fertilization program. *Fertil. Steril*., **51**, 850–854.

Carbone, D.J., Seifarth, K., Thomas, A.J. *et al*. (1997) Improved motile sperm recovery by a hyperosmotic Percoll gradient. *J. Assist. Reprod. Genet*., **14**, 394–397.

Chan, Y., Mostafa, I., Malmomnson, J.H., *et al*., (1994) Selection of human spermatozoa by hyperosmotic two-layer Percoll gradient. *Fertil. Steril*., **61**, 1097–1102.

Dadoune, J.P., Mayaux, M.J. and Guihard-Moscato, M.L. (1988) Correlation between defects in chromatin condensation of human spermatozoa stained by aniline blue and semen characteristics. *Andrologia*, **20**, 211–217.

De Vos, A., Nagy, Z.P., Van de Velde, H. *et al*. (1997) Percoll gradient centrifugation can be omitted in sperm preparation for intracytoplasmic sperm injection. *Hum. Reprod*., **12**, 1980–1984.

Ericsson, R.J. (1977) Isolation and storage of progressively motile human sperm. *Andrologia*, **9**, 111–114.

Foresta, C., Zorzi, M., Rossato, M. *et al*. (1992) Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. *Int. J. Androl*., **15**, 330–337.

Fredricsson, B., Waxegard, G., Brege, S. *et al*. (1977) On the morphology of live spermatozoa of human semen. *Fertil. Steril*., **28**, 168–174.

Gellert-Mortimer, S.T., Clarke, G.N., Gordon Baker, H.W. *et al*. (1988) Evaluation of Nycodenz and Percoll density gradients for the selection of motile human spermatozoa. *Fertil. Steril*., **49**, 335–341.

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

Harrison, K.L. (1997) Iodixanol as a density gradient medium for the isolation of motile spermatozoa. *J. Assist. Reprod. Genet*., **14**, 385–387.

Harrison, K.L. and Campbell, A.M. (1976) Sperm vitality. *Aust. J. Med. Technol*., **7**, 182–184.

Herruzo, A., Molina, J., Hortas, M.L. *et al*. (1997) Sperm preparation techniques and chromatin condensation. Abstracts from 10th world congress on IVF and assisted reproduction. *J. Assist. Reprod. Genet*., **14** (Suppl.), PP-17-385, 191S.

Hyne, R.V., Stojanoff, A., Clarke, G.N. *et al*. (1986) Pregnancy from *in vitro* fertilization of human eggs after separation of motile spermatozoa by density gradient centrifugation. *Fertil. Steril*., **45**, 93–96.

Iwasaki, A. and Gagnon, C. (1992) Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil. Steril*., **57**, 409–415.

Kruger, T.F., Menkveld, R., Stander, F.S.H. *et al*. (1986) Sperm morphologic features as a prognostic factor in *in vitro* fertilization. *Fertil. Steril*., **46**, 1118–1123.

Kruger, T.F., Acosta, A.A., Simmons, K.F. *et al*. (1988) Predictive value of abnormal sperm morphology in *in vitro* fertilization. *Fertil. Steril*., **49**, 112–117.

Leventhal, A., Margalioth, E.J. and Schenker, J.G. (1987) Testing the fertilizing ability of motile spermatozoa separated by Percoll in patients with abnormal sperm analysis or sperm penetration. *Int. J. Fertil*., **32**, 302–305.

Mahadevan, M.M. and Trounson, A.O. (1984) The influence of seminal characteristics on the success rate of human *in vitro* fertilization. *Fertil. Steril*., **42**, 400.

Menkveld, R. and Kruger, T.F. (1996) Evaluation of sperm morphology by light microscopy. In Acosta, A.A. and Kruger, T.F. (eds), *Human Spermatozoa in Assisted Reproduction*, Parthenon Publishing, Carnforth, UK, pp. 89–107.

Menkveld, R., Swanson, R.J., Kotze, T.W.vW. *et al*. (1990a) Comparison of a discontinuous Percoll gradient method versus a swim-up method: effects on sperm morphology and other semen parameters. *Andrologia*, **22**, 152–158.

Menkveld, R., Stander, F.S.H., Kotze, T.J.vW. *et al*. (1990b) The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum. Reprod*., **5**, 586–592.

Menkveld, R., Oettle, E.E. and Kruger, T.F. (eds) (1991) *Atlas of Human Sperm Morphology*. Williams & Wilkins, Baltimore, 117 pp.

Menkveld, R., Rhemrev, J.P.J., Franken, D.R. *et al*. (1996) Acrosomal morphology as a novel criterion for male fertility diagnoses: Relation with acrosin activity, morphology (strict criteria) and fertilization *in vitro*. *Fertil. Steril*., **65**, 637–644.

Mortimer, D. (ed.) (1994) *Practical Laboratory Andrology*. Oxford University Press, Oxford, pp. 79–83.

Ohashi, K., Saji, F., Wakimoto, A. *et al*. (1992) Preparation of oligozoospermic and/or asthenozoospermic semen for intrauterine insemination using the SpermPrep semen filtration column. *Fertil. Steril*., **57**, 866–870.

Ord, T., Patrizio, P., Marello, E. *et al*. (1990) Mini-Percoll: a new method of semen preparation for IVF in severe male factor infertility. *Hum. Reprod*., **5**, 987–989.

Pharmacia Biotech (1996) Important notice: Percoll® NOT to be used in Assisted Reproduction Technologies in Humans. Pharmacia Biotech Inc, December 12, 1996.

Pickering, S.J., Fleming, T.P., Braude, P.R. *et al*. (1989) Are human spermatozoa separated on a Percoll density gradient safe for therapeutic use? *Fertil. Steril*., **51**, 1024–1029.

Quinn, P., Kerin, J.F. and Warnes, G.M. (1985) Improved pregnancy rate in human *in vitro* fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil. Steril*., **44**, 493.

Sbacia, M., Sayme, N., Grasso, J. *et al*. (1996) Sperm function and choice of preparation media: comparison of *Percoll* and Accudenz discontinuous density gradients. *J. Androl*., **17**, 61–67.

Smith, T.T., Byers, M., Kaftani, D. *et al*. (1997) The use of iodixanol as a density gradient material for separating human sperm from semen. *Arch. Androl*., **38**, 223–230.

Steeno, O., Adimoelja, A. and Steeno, J. (1975) Separation of X- and Y-bearing human spermatozoa with the sephadex gel-filtration method. *Andrologia*, **7**, 95–97.

Stone, B.A., Ong, E., Faridi, R. *et al*. (1997) Effects of alternate silica-based sperm separation materials on motile sperm recoveries from human semen, on sperm function, sperm survival, and on early murine embryonic development. Abstracts of the 53rd Annual Meeting of the American Society for Reproductive Medicine. *Am. Soc. Reprod. Med*., P-210, S192.

Van der Zwalmen, P., Bertin-Segal, G., Geerts, L. *et al*. (1991) Sperm morphology and IVF pregnancy rate: comparison between Percoll gradient centrifugation and swim-up procedures. *Hum. Reprod*., **6**, 581–588.

World Health Organization (1992) *Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction*, 3rd edn. Cambridge University Press, Cambridge.

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