The use of silane-coated silica particles for density gradient centrifugation in in-vitro fertilization

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Silane-coated silica particles (PureSperm®) were evaluated as an alternative to Percoll for gradient separation of spermatozoa, for use in assisted reproduction. Recovery of motile and morphologically normal spermatozoa after using a four-layer Percoll and a two- and four-layer PureSperm® gradient respectively was recorded. In-vitro fertilization (IVF) results after using PureSperm® for the sperm preparation were also evaluated. No difference in sperm recovery or sperm motility was found when comparing the use of Percoll and the four-layer gradient of PureSperm®. When using a two-layer PureSperm® gradient, motility was significantly decreased ($P < 0.05$) compared to Percoll. Normal sperm morphology increased from 8–17.2% after using Percoll and to 12.7% and 11.4% after using a four-layer and a two-layer PureSperm® gradient respectively. All gradient preparations showed a significant decrease in the teratozoospermia index compared to the ejaculate ($P < 0.01$). No significant differences in IVF results regarding fertilization and pregnancy rates were found when PureSperm® or the swim-up technique were used for the sperm preparation. PureSperm® seems to be an acceptable alternative to Percoll but although the percentage of sperm recovery was higher after PureSperm® we still recommend the swim-up technique to be the first choice, as a higher percentage of progressive motile spermatozoa is obtained without using other chemicals than IVF culture medium.

Key words: IVF/Percoll/PureSperm®/sperm morphology/swim-up

Introduction

Several methods have been used to select motile spermatozoa prior to assisted fertilization, e.g. the swim-up technique (Lopata et al., 1976), discontinuous Percoll density gradient centrifugation (Pertoft et al., 1977; Pertoft et al., 1978; Gorus and Pipeleers, 1981), glass wool filtration (Perry et al., 1977; Paulson et al., 1979) and centrifugation through albumin gradients (Ericsson, 1977; Koper et al., 1979). The most common methods used today are the swim-up and gradient separation techniques. In several studies it has been shown that Percoll separation is more efficient than swim-up for selecting motile spermatozoa with good fertilization ability (McClure et al., 1989; Englert et al., 1992; Ng et al., 1992). It has also been suggested that Percoll can act as a protective medium against production of reactive oxygen species (ROS) in the semen (Aitken and Clarksson, 1988). On the other hand it is known that Percoll contains high levels of endotoxins, between 10 and 100 times the Food and Drug Administration (FDA) cut-off level for human injectables (Svalander et al., 1995) which might be associated with increased fragmentation of human embryos and reduce the pregnancy rates (Fishel et al., 1988), also the product is no longer available for clinical use. In our laboratory the routine is to use the swim-up technique whenever possible, but in some cases there is a need for gradient centrifugation, e.g. when very few spermatozoa are available in the ejaculate and/or the motility is very poor. When intrauterine inseminations are performed high numbers of motile spermatozoa are required, therefore it can be valuable to use gradient centrifugation in those cases. Now new products with reduced endotoxin levels are available in the market, e.g. PureSperm® (Nidacon, Göteborg, Sweden) and IxaPrep® (Medi-Cult, Copenhagen, Denmark).

The aim of this study was to evaluate PureSperm® as an alternative to Percoll for gradient separation of spermatozoa for use in assisted reproduction. Since different numbers of layers can be used in gradient centrifugation, and the routine in our laboratory has been to use a four-layer Percoll, this was compared to a four-layer and also to the more standard use of a two-layer PureSperm® gradient. Progressive motility, normal morphology before and after preparation and yield from the initial semen samples were evaluated. In the second part of the study fertilization and pregnancy rates in our in-vitro fertilization (IVF) programme after using PureSperm® gradient separation versus swim-up procedure during a 6 month period were compared.

Material and methods

Semen samples

Semen samples were obtained from patients attending the Unit for Reproductive Medicine at Sahlgrenska University Hospital, Göteborg, Sweden.

Gradent centrifugation with Percoll and PureSperm® respectively.

A total of 22 semen samples were included in the study. Ejaculates were collected after 3–5 days sexual abstinence and were left to liquefy for 20–30 min. Semen analysis was performed according to World Health Organization guidelines (WHO, 1992) except for the morphology analysis which will be described separately. After this basic evaluation the samples were divided into three equal parts and put onto the different gradients; four-layer Percoll and four- and two-layer PureSperm® respectively.

Percoll gradient centrifugation technique

Percoll, 100% (Pharmacia, Uppsala, Sweden) was supplemented with Earle’s balanced salt (Sigma-Aldrich, Stockholm, Sweden), sodium
bicarbonate, NaHCO₃ (Sigma S-4019), penicillin (Sigma) and HSA (human serum albumin 200 mg/ml, Pharmacia & Upjohn). Osmolarity was adjusted to 290–295 mOsm/kg. This stock solution (100% Percoll) was diluted with IVF medium IVF<sup>TM</sup>·50 (Scandinavian IVF Science AB, Göteborg, Sweden) to 90, 80, 70 and 45% Percoll solutions. The density gradients were performed layering 1 ml of each concentration into a 15 ml, conical Falcon tube. Gradients were pre-incubated overnight to obtain 37<sup>°</sup>C, and after liquefaction 0.5–1.0 ml of the ejaculate was layered on the top of each gradient and centrifuged for 30 min at 300 g.

After centrifugation the upper layer (seminal plasma plus 45% Percoll) was aspirated and the 90% layer was collected from the bottom of the tube using a clean pipette and was transferred to a new, clean tube and washed twice in IVF medium (300 g, 10 min). The pellet was re-suspended in exact volumes of IVF medium before concentration and motility were evaluated. Recovery rates of motile, progressive (grade A and B) spermatozoa from the initial semen samples were calculated by dividing the retrieved number of motile spermatozoa by the initial number of available motile spermatozoa.

**PureSperm® gradients**
A two-layer gradient was prepared by using ready-to-use solutions 90% and 45% PureSperm<sup>®</sup>. The four-layer gradient was made by diluting 90% PureSperm<sup>®</sup> with IVF medium to 80% and 70% respectively. The preparation and evaluation technique was the same as for Percoll preparation.

**Morphology slide preparation and morphology evaluation**
Smears for morphology evaluation according to strict criteria (Kruger et al., 1986, 1988) were prepared before and after Percoll and PureSperm® centrifugation respectively. Slides were washed in 70% ethanol before use. For each smear 5–20 µl of the sample were used. The slides were air-dried before fixation and staining was performed with a modified Papanicolaou technique as previously described (Menkveld et al., 1991).

Sperm morphology evaluation was always performed by the same trained technician. A total of 200 spermatozoa were evaluated on each slide under oil immersion at a magnification ×1000. Head, neck and tail defects or combinations of these were recorded separately. Teratozoospermia index (TZI) (WHO, 1992), i.e. the average number of defects per abnormal spermatozoon, which is equivalent to the multiple anomalies index (MAI) (Jouannet et al., 1988), was also calculated.

**Fertilization and pregnancy rates after using PureSperm® or swim-up procedure**
This part of the study included 88 conventional IVF cycles where swim-up was performed and 63 IVF cycles where PureSperm® was used for the sperm preparation. Handling of semen samples and preparation techniques was the same as described above. A four-layer PureSperm® gradient was used for this part of the study. Due to practical reasons in the laboratory, a randomization was performed by alternating between swim-up procedure and gradient separation every other day.

The women were down-regulated with gonadotrophin releasing hormone (GnRH) agonist buserelin (Suprefact® or Suprecu®; Hoechst, Frankfurt am Main, Germany) for 3–5 weeks, starting on cycle day 21 or 1 and then stimulated with daily s.c. injections of human menopausal gonadotrophin (HMG; Pergonal®; Serono Laboratories, Geneva, Switzerland), or with daily s.c. injections of purified or recombinant follicle stimulating hormone (FSH; Fertinorm® or Gonaf-P®; Serono), followed by human chorionic gonadotrophin (HCG; Profasi® 10 000 IU i.m.; Serono). Oocyte retrieval was performed 36–38 h later via transvaginal ultrasound-guided retrieval. The oocytes were inseminated with a sperm concentration of 200 000/ml 4–5 h after oocyte aspiration. Embryos were cultured in IVF<sup>TM</sup>·50 medium 2–3 days until embryo transfer with one to three embryos (mean 2.0) was carried out.

Fertilization and pregnancy rates were calculated for each group. The percentage of good quality embryos was also calculated for the two groups (number of embryos good enough for embryo transfer or freezing/number of fertilized embryos).

**Swim-up technique**
Swim-up was performed by adding 0.5–1.0 ml of the ejaculate to the bottom of a Falcon tube (15 ml) containing 1.5 ml of IVF medium. The tube was placed in a 45<sup>°</sup> angle and incubated at 37<sup>°</sup>C in 5% CO₂ for 45 min. After incubation 1.0 ml of the supernatant was collected, IVF medium added and the supernatant was washed once at 300 g for 10 min. Evaluation was performed in the same way as after gradient centrifugation.

**Statistics**
In the comparisons of Percoll and PureSperm® gradients the Mann–Whitney U test with the Bonferroni correction was used. Student’s t-test was used when comparing IVF results between the different sperm preparation methods. χ² test was used for categorical data. A P value < 0.05 was considered statistically significant.

**Results**

**Gradient centrifugation with Percoll and PureSperm® respectively**
As can be seen in Table I there was no difference in sperm recovery or progressive sperm motility when comparing the use of Percoll and four-layer PureSperm<sup>®</sup>. But when using a two-layer PureSperm<sup>®</sup> gradient progressive motility was significantly decreased (P < 0.05) compared to Percoll. Normal sperm morphology increased from 8.3–17.2% (P < 0.001) after using Percoll, while after separation with PureSperm<sup>®</sup> normal morphology was 12.7% (four-layer gradient) and 11.4% (two-layer gradient). TZI decreased from 1.70–1.28 in the 90% layer when Percoll was used and to 1.43 and 1.46 after using PureSperm<sup>®</sup> with four or two layers respectively. All gradient preparations showed a statistically significant decrease in the TZI compared to the ejaculate (P < 0.001). The TZI of the Percoll preparation was also significantly lower than that of the PureSperm<sup>®</sup> gradients (P < 0.01).

**Fertilization and pregnancy rates after using PureSperm® gradient centrifugation and swim-up**
In Table II semen characteristics for ejaculates included in the different preparation groups used for the second part of the study are presented. The initial sperm motility was higher in the swim-up group (52.8%) than in the PureSperm<sup>®</sup> group (49.0%). Although this difference was statistically significant it was not considered to be of any clinical importance. As can be seen in Table III there was no difference between the two groups concerning female age, mean number of aspirated oocytes, fertilization rate or embryo transfer rate. Pregnancy rate was slightly higher in the swim-up group but this was not statistically significant. The percentage of good quality embryos was equivalent in the two groups; 48.9% in the PureSperm<sup>®</sup> group versus 46.0% after using the swim-up procedure.
Discussion

Improvements of the laboratory conditions in assisted reproduction are continually being made, and one factor of vital importance is the use of ‘clean’ products. However, before switching to a novel product it is important to ensure that it is efficient/suitable for its intended use. Since Percoll is no longer sold for clinical purposes it has been necessary for many IVF laboratories to find an equivalent product for gradient separation of sperm samples. Only a few studies have compared the efficiency of Percoll and the new products that have been introduced to replace it (Centola et al., 1998; Claassens et al., 1998; Chen and Bongso, 1999). In this present study it was found that PureSperm® is similar to Percoll regarding percentage of sperm recovery and percentage of progressive sperm motility when using four-layer gradients. This is in agreement with results shown by Centola et al. (1998) and Claassens et al. (1998) while in the study by Chen and Bongso (1999) Percoll was found to be superior to PureSperm® regarding motility and yield but no differences in sperm morphology were found. In the study by Claassens et al. (1998) sperm morphology was slightly better after preparation with PureSperm®, Menkveld et al. (1990, 1998), have shown that the overall sperm morphology after Percoll was reduced compared to the initial sperm morphology, while in this present study a better morphological selection was obtained by Percoll. This may be due to differences in the centrifugation techniques. Chen and Bongso (1999) also found that three-layer Pure-Sperm® gradients produced significantly greater percentages of motile spermatozoa and normal forms than two-layer gradients. Our results also indicate that more than two layers are preferable when gradient centrifugation with PureSperm® is performed, since this resulted in a numerically higher recovery of motile and morphologically normal spermatozoa, although not statistically significant.

Since it was not possible to compare Percoll and PureSperm® in a clinical situation, i.e. using the prepared semen samples for fertilization, the outcome after using spermatozoa obtained by gradient separation with PureSperm® was instead compared to the outcome after using the swim-up technique for the semen preparation. No differences were found between the two groups in fertilization rate, pregnancy rate, term pregnancy rate or percentage of good quality embryos (Table III). A higher pregnancy rate has been reported in an IVF programme (Vanderzwalmens et al., 1991) after using gradient separation with Percoll than after swim-up, which could not be confirmed.

### Table I. Sperm characteristics after gradient centrifugation with Percoll and PureSperm® (n = 22); mean ± SD (range)

<table>
<thead>
<tr>
<th></th>
<th>Ejaculate</th>
<th>Percoll (4-layer)</th>
<th>PureSperm® (4-layer)</th>
<th>PureSperm® (2-layer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery (% of total motile spermatozoa)</td>
<td>16.5 ± 12.8a</td>
<td>15.1 ± 10.1b</td>
<td>16.5 ± 9.4c</td>
<td></td>
</tr>
<tr>
<td>Motility (% forward progressive)</td>
<td>52.3 ± 10.2a</td>
<td>74.8 ± 13.4a</td>
<td>72.5 ± 13.2a</td>
<td>65.5 ± 15.1a</td>
</tr>
<tr>
<td>Morphology (% normal forms)</td>
<td>8.3 ± 4.8h</td>
<td>17.2 ± 8.9h</td>
<td>12.7 ± 6.4h</td>
<td>11.4 ± 5.9h</td>
</tr>
<tr>
<td>Teratozoospermia index</td>
<td>1.70 ± 0.19g</td>
<td>1.28 ± 0.13m</td>
<td>1.43 ± 0.16o</td>
<td>1.46 ± 0.14n</td>
</tr>
</tbody>
</table>

Table II. Basic semen characteristics for the different groups when swim-up (group A) and PureSperm® (group B) preparations are compared; mean ± SD (range)

Table III. Results of in-vitro fertilization after utilizing swim-up and PureSperm® preparation

<table>
<thead>
<tr>
<th></th>
<th>Swim up (group A)</th>
<th>PureSperm® (group B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cycles</td>
<td>88</td>
<td>63</td>
</tr>
<tr>
<td>Male age, years</td>
<td>34.4 ± 5.3</td>
<td>35.6 ± 5.4</td>
</tr>
<tr>
<td>Initial motility</td>
<td>52.8 ± 8.8</td>
<td>49.0 ± 10.1</td>
</tr>
<tr>
<td>Concentration (×10^9/ml)</td>
<td>71.4 ± 46.4 (14–250)</td>
<td>79.5 ± 60.4 (14–320)</td>
</tr>
<tr>
<td>Initial morphology</td>
<td>9.3 ± 3.5 (1–20)</td>
<td>9.6 ± 3.4 (3–16)</td>
</tr>
<tr>
<td>Recovery (% of total motile spermatozoa)</td>
<td>10.1 ± 6.2 (1.3–34.0)</td>
<td>20.4 ± 11.2 &lt; 0.001</td>
</tr>
<tr>
<td>Motility post-preparation (% forward progressive)</td>
<td>97.7 ± 2.8 (90–100)</td>
<td>94.1 ± 11.2 (40–100)</td>
</tr>
</tbody>
</table>

### Table III. Results of in-vitro fertilization after utilizing swim-up and PureSperm® preparation

<table>
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<tbody>
<tr>
<td>Number of cycles</td>
<td>88</td>
<td>63</td>
</tr>
<tr>
<td>Female age (mean ± SD)</td>
<td>32.8 ± 3.8 (25–40)</td>
<td>33.9 ± 4.0 (22–40)</td>
</tr>
<tr>
<td>Mean number of oocytes (±SD) (range)</td>
<td>10.9 ± 4.9 (1–27)</td>
<td>11.0 ± 5.1 (3–26)</td>
</tr>
<tr>
<td>Fertilization rate (% of fertilized)</td>
<td>68.1 ± 24.4 (0–100)</td>
<td>64.3 ± 25.1 (0–100)</td>
</tr>
<tr>
<td>Mean no. of embryos transferred (±SD)</td>
<td>1.98 ± 0.23</td>
<td>2.00 ± 0.23</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>45.3</td>
<td>38.2</td>
</tr>
<tr>
<td>Ongoing pregnancy (%)</td>
<td>11.8</td>
<td>9.5</td>
</tr>
<tr>
<td>Good quality embryos (%)</td>
<td>46.0</td>
<td>48.9</td>
</tr>
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</table>

*One extrauterine pregnancy.

There were no significant differences between the groups.
in the present study using PureSperm®. However, the fact that Vanderzwalmen washed the semen twice by centrifugation before swim-up may have caused damage to the spermatozoa previously discussed (Aitken and Clarkson, 1988; Mortimer, 1991; Alvarez et al., 1993), resulting in a lower fertilization rate after swim-up than after gradient centrifugation of samples with moderate sperm parameters.

As has been shown earlier (Englert et al., 1992; Ng et al., 1992), we also found that the swim-up procedure yielded a higher percentage of motile spermatozoa than density gradient separation with PureSperm®, P = 0.01 (Table II). Ng et al. (1992) also showed that the swim-up method resulted in the recovery of spermatozoa with significant higher straight linear velocity (VSL) and average path velocity (VAP). It was reported recently that the fertilization rate was significantly increased when the fastest moving spermatozoa were used for intracytoplasmic sperm injection (ICSI) (Van den Bergh et al., 1998). These findings may support the use of the swim-up technique apart from the fact that no ‘additional substances’ are introduced when swim-up is performed. Other findings (Mortimer, 1991; Alvarez et al., 1993), suggesting that centrifugation of semen samples may cause damage to the membranes of the spermatozoa, also support the use of the direct swim-up technique where usually only one centrifugation is performed.

In conclusion, the results from this study show that PureSperm® can be used to replace Percoll when density gradient separation is performed, although a higher percentage of morphologically abnormal spermatozoa are obtained than after using Percoll. The results also indicate that it may be of importance to use at least a three layer gradient when PureSperm® gradient separation is used, since this resulted in a slightly better motility and morphology than the two-step gradient. It was also found that using PureSperm®-treated spermatozoa resulted in similar fertilization and pregnancy rates as using spermatozoa obtained after the swim-up procedure. However, the direct swim-up technique has the advantage of selecting a higher percentage of motile spermatozoa without using any chemicals other than the IVF culture medium, and therefore we still recommend the swim-up technique to be the method of choice when separation of spermatozoa for IVF and ICSI is performed, particularly for cases likely to yield sufficient sperm numbers after preparation.

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References

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