Fertilizability and structural properties of boar spermatozoa prepared by Percoll gradient centrifugation

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Two techniques for the preparation of boar spermatozoa for *in vitro* fertilization were studied: a simple washing procedure and centrifugation on a discontinuous Percoll gradient. Their respective effects on motility of spermatozoa were analysed by computer-assisted sperm analysis. The Percoll density gradient technique selected spermatozoa with significantly ($P < 0.0001$) enhanced motility and movement characteristics. *In vitro* matured oocytes inseminated with spermatozoa prepared by Percoll gradient centrifugation had significantly ($P < 0.0001$) greater cleavage rates than did oocytes inseminated with washed spermatozoa. This increased penetration ability was not due to an increased proportion of acrosome-reacted spermatozoa. Transmission electron microscopy revealed no unique ultrastructural differences between the spermatozoa from either preparation. Spermatozoa prepared by Percoll gradient centrifugation are recommended for insemination and studies of porcine *in vitro* fertilization.

Introduction

Pig oocytes have been shown to mature, fertilize and develop *in vitro* (Iritani *et al.*, 1978; Cheng, 1985; Cheng *et al.*, 1986; Mattioli *et al.*, 1989; reviewed by Hunter, 1990; Nagai *et al.*, 1990; Yoshida *et al.*, 1990; Wang *et al.*, 1991). However, compared with human *in vitro* fertilization (IVF) programmes, consistent rates of normal fertilization and embryo cleavage remain disappointingly low. The unpredictability of boar spermatozoa undergoing an acrosome reaction *in vitro* concomitant with inadequate sperm motility contribute significantly to these poor results. Hence, a simple method to improve these factors may enhance fertilization and cleavage rates.

During the past decade, many human infertility research groups have reported that spermatozoa washed on a gradient of silica coated with polyvinylpyrrolidone (Percoll: Sigma Chemical Co., Poole) demonstrate improved motility and *in vitro* fertilizing capacity (Gorus and Pipeleers, 1981; Arcidiacono *et al.*, 1983; Forster *et al.*, 1983; Kaneko *et al.*, 1983, 1986, 1987; Lessley and Garner, 1983; Berger *et al.*, 1985; Hyne *et al.*, 1986; McClure *et al.*, 1989; Nice *et al.*, 1991). However, only a few workers have used Percoll to assist IVF in farm animals (Lessley and Garner, 1983; de Curtis *et al.*, 1986; Berger and Horton, 1988; Berger and Parker, 1989; Mermillod *et al.*, 1992) and most of these experiments specifically evaluated the motility of spermatozoa and the penetration of zona-free hamster oocytes. Consequently, no comparison has been made between the direct influence of sperm preparation technique and subsequent fertilization and development of matured oocytes *in vitro*.

This study was performed to determine whether boar spermatozoa washed on a Percoll gradient would enhance cleavage rates and embryo development in porcine oocytes matured *in vitro*. In addition, the morphological homogeneity of these spermatozoa was assessed.

Materials and Methods

Culture and maturation of follicular oocytes

Ovaries were collected from prepubertal gilts (Landrace × Large White, body mass about 85 kg) at slaughter and returned to the laboratory in saline (0.9% NaCl (w/v), 100 iu benzyl penicillin ml⁻¹ (Glaxo, Greenford)) at 37°C within 1 h. Follicles, 2–5 mm in diameter, were aspirated with a 1 ml syringe equipped with a 25 gauge needle, and the follicular fluid was diluted with TCM 199 (containing Earle's salts, 25 mmol Hepes l⁻¹ and L-glutamic acid (Gibco, Life Technologies Ltd, Paisley)). Only oocytes possessing a compact cumulus mass and evenly granulated cytoplasm were selected for the experiments. The compact oocyte complexes were cultured at 39°C under humidified 5% CO₂ in maturation medium TCM 199 containing 10% (v/v) heat-inactivated fetal calf serum (FCS: Imperial, Andover), 75 iu hCG ml⁻¹ (Chorulon: Intervet, Cambridge), 50 iu Penicillin ml⁻¹ (Gibco), 50 µg Streptomycin ml⁻¹ (Gibco) and 1 µg oestradiol ml⁻¹ (Sigma Chemical Co., Poole). After incubation for 28–34 h, maturation of cumulus–oocyte complexes was evaluated under a stereomicroscope and those complexes with an expanded cumulus mass were selected for IVF.

Received 28 May 1993.

Preparation of a discontinuous Percoll column

Nine parts of Percoll (Sigma Chemical Co.) were diluted (v/v) with one part of concentrated ($\times 10$) modified Tyrode's solution originally designated TALP (Tyrode–albumin–lactate–pyruvate, Bavister and Yanagimachi, 1977; Bavister, 1989) to make isotonic Percoll. TALP medium ($\times 1$) supplemented with 2 mg BSA ml⁻¹ (Fraction V, Globulin Free, Sigma Chemical Co.), 0.25 mmol sodium pyruvate l⁻¹ (Gibco Life Technologies Ltd), 50 iu penicillin ml⁻¹ and 50 µg Streptomycin ml⁻¹ (TALP–BSA) was then mixed with isotonic Percoll in appropriate proportions to make 40% and 90% isotonic Percoll. A discontinuous Percoll gradient was prepared by carefully layering 4 ml of 90% isotonic Percoll beneath 4 ml of 40% isotonic Percoll in a sterile 15 ml centrifuge tube (Falcon, Becton Dickinson UK Ltd, Oxford) such that the interface between the layers was visible.

Preparation and incubation of spermatozoa

Boar semen, commercially prepared for artificial insemination (JSR Healthbred, Selby), was delivered to the laboratory within 24 h of collection. Throughout the experiment, semen from the same two boars was used to minimize inter-boar variation. Two methods of preparing the spermatozoa were evaluated.

Method 1. To remove the commercial extender and concentrate spermatozoa, spermatozoa were initially washed twice in TALP–BSA by centrifugation at 250 g for 4 min. The final pellet was resuspended in approximately 1 ml of medium to give a final concentration of 3×10^9 spermatozoa ml⁻¹. To maintain the pH, the sperm suspension was gently gassed with 5% CO₂, then incubated at 37°C for 5 h in a tightly capped test-tube (washed spermatozoa).

Method 2. Spermatozoa were initially washed as described above. The final pellet was resuspended to about 1.5 ml, which was layered on top of a 40:90 discontinuous Percoll gradient and centrifuged at 300 g for 35 min. The resultant pellet was carefully removed from the bottom of the column and washed twice more in TALP–BSA. After aspiration of the final supernatant, the pellet was resuspended to a concentration of 3×10^9 spermatozoa ml⁻¹, the tube was gassed and incubated at 37°C for 5 h (spermatozoa prepared by Percoll gradient centrifugation).

In vitro fertilization, embryo culture and examination of oocytes

Mature oocytes (15–20) were transferred to a droplet (40 µl) of TALP–BSA medium under paraffin oil. The IVF dishes (35 mm, Falcon) were prepared and incubated under humidified 5% CO₂ at 39°C sufficiently in advance of the experiment to allow equilibration of the oil and the culture medium droplets. A portion of the appropriate preincubated sperm suspension was introduced into the TALP–BSA droplets to give a final concentration of between 5×10^5 and 1×10^6 spermatozoa ml⁻¹. The rate of polyspermic fertilization was examined by randomly removing some oocytes 12–15 h after insemination, and fixing them with acetic–alcohol (methanol:acetic acid, 3:1 v:v) for at least 24 h and staining with 1% aqueous aceto-orcin.

The remaining oocytes were cultured for a further 65 h to examine their developmental capacity. The assessment of cleavage was performed at intervals of 24 h.

Analysis of sperm motility

Sperm motility parameters were measured with a Hamilton Thorne Motility Analyser Version 7 (HTM; Hamilton Thorne Res. Inc, Danvers). A 20 µl sample of each sperm suspension was placed in a disposable Micro-cell counting chamber (Microcell, Cyto-Fluidics, Silver-Spring, MD) and the sample analysed immediately as delays in the procedure enable the spermatozoa to agglutinate or adhere to the glass surface. Motility was measured using the internal microscope of the analyser, with the parameter settings adjusted as follows from the manufacturer's recommended values for analysing bovine spermatozoa: temperature, 37°C; diluent:sample, 0:1 (v:v); chamber, Micro-cell (20 µm); image type, phase contrast; field selection, select; calculate ALH, yes; morphology, live; beat frequency, yes; automatic sort, no; allow static override, no; main gates, frames, 20; frame rate, 25 s⁻¹; minimum contrast, 4; minimum size, 3; LO/HI size gates, 0.5, 1.5; LO/HI intensity gates, 0.5, 1.5; non-motile head size, 10; non-motile intensity, 190; medium mean path velocity (VAP) value, 25; low VAP value, 10; low cells motile, no; threshold STR (straight track ratio), 75. Preliminary trials using the play-back facility of the machine confirmed that the size and contrast settings enabled the computer to differentiate accurately between motile and immotile spermatozoa.

The variables of sperm movement characteristics evaluated in this study included mean path velocity (VAP; µm s⁻¹), percentage of motile spermatozoa (VAP > 10 µm s⁻¹), percentage of spermatozoa with rapid motility (VAP > 25 µm s⁻¹), progressive velocity (VSL; µm s⁻¹), track velocity (VCL; µm s⁻¹), amplitude of lateral head displacement (LHD; µm) and beat cross frequency (BCF; Hz).

Determination of the acrosomal status of spermatozoa

After incubation for 0, 1, 3 and 5 h, aliquots of spermatozoa prepared by both methods were stained with Naphthol Yellow and Erythrosin B and assayed for the presence or loss of acrosomes according to the method of Bryan and Akruk (1977). A minimum of 800 cells were analysed from each aliquot.

Transmission electron microscopy

After incubation for 0, 1, 3 and 5 h, aliquots of spermatozoa prepared by Percoll gradient centrifugation and washed spermatozoa were fixed in 2.5% glutaraldehyde in 0.1 mol sodium cacodylate buffer l⁻¹ at 4°C for a minimum of 4 h. Spermatozoa were then postfixed in 2% osmium tetroxide in buffer (0.2 mol l⁻¹) for 1 h, rinsed twice in buffer and serially dehydrated in 70%, 90% and 100% ethanol. Samples were processed with propylene oxide and propylene:epoxy resin (50:50 v:v), embedded and polymerized at 60°C for 48 h. Thin sections of the spermatozoa were then stained with saturated uranyl acetate in 50% methanol for 20 min followed by Reynold's lead citrate for 5 min, and viewed using a Philips 300 transmission electron microscope.

Fig. 1. The distribution of progressive velocity (VAP; $\mu\text{m s}^{-1}$) and lateral head displacement (LHD; μm) as percentages of the motile sperm population prepared by washing (▨) or Percoll centrifugation (■) after (a) 0, (b) 1, (c) 3 and (d) 5 h incubation.

Statistical analysis

Significant relationships between sperm motility characteristics within sperm treatments were determined by subjecting the relevant data to linear correlation analysis. Significant differences of individual variables between treatments were tested by standard analysis of variance and least significant differences. Differences between polyspermic fertilization and cleavage rates were tested according to the χ^2 method.

Results

Sperm motility

The distribution of VAP and LHD for both preparations are shown (Fig. 1). Results from spermatozoa prepared on the Percoll gradient were skewed towards a high proportion of fast-moving spermatozoa with a large LHD. The Percoll technique significantly enhanced ($P < 0.0001$) all velocity parameters measured (Table 1). By contrast, the distribution of

the washed spermatozoa was markedly skewed towards slower speeds with a mode of 20–40 $\mu\text{m s}^{-1}$ and a correspondingly smaller lateral head displacement.

Characteristics of sperm velocity (i.e. VAP, VSL and VCL) were strongly correlated with each other and with LHD in both preparations. There was a negative correlation between BCF and all other measurements.

Incubation time had a minimal effect with a small but significant ($P < 0.05$) decrease in percentage of motile spermatozoa in both preparations and in percentage of rapidly motile Percoll-prepared spermatozoa after 1 h. Velocity parameters increased and reached maximum values after 1 and 3 h for the washed spermatozoa and Percoll-gradient-prepared spermatozoa, respectively.

Acrosomal status of spermatozoa

The number of spermatozoa without acrosomes increased significantly during incubation of each preparation (Table 2).

Table 1. Mean ($\pm$ SEM) of boar spermatozoa motility and movement characteristics assessed by the Hamilton Thorne Motility Analyser for Percoll-prepared and washed spermatozoa

Sperm parameter	Sperm preparation	Incubation time (h)			
		0	1	3	5
Motile spermatozoa (%)	Percoll	80.0 $\pm$ 3.7 ^e	65.1 $\pm$ 4.4 ^g	70.2 $\pm$ 3.6	67.4 $\pm$ 6.3
	Wash	77.5 $\pm$ 3.2	61.9 $\pm$ 8.4	75.0 $\pm$ 5.7	75.5 $\pm$ 3.6
Rapid motility (%)	Percoll	77.7 $\pm$ 3.5 ^{ae}	61.2 $\pm$ 4.8 ^{ag}	63.9 $\pm$ 4.3 ^a	64.6 $\pm$ 6.4
	Wash	39.5 $\pm$ 4.5 ^c	40.4 $\pm$ 8.0 ^d	46.6 $\pm$ 4.8 ^d	47.4 $\pm$ 7.2
Mean path velocity (VAP, $\mu\text{m s}^{-1}$)	Percoll	95.8 $\pm$ 4.6 ^{ae}	98.8 $\pm$ 4.7 ^a	113.4 $\pm$ 8.8 ^{ag}	111.7 $\pm$ 4.9 ^a
	Wash	38.5 $\pm$ 2.0 ^b	44.0 $\pm$ 2.1 ^b	40.5 $\pm$ 1.9 ^b	35.3 $\pm$ 3.5 ^b
Progressive velocity (VSL, $\mu\text{m s}^{-1}$)	Percoll	83.3 $\pm$ 5.7 ^a	89.5 $\pm$ 4.5 ^a	105.3 $\pm$ 9.2 ^a	104.3 $\pm$ 4.8 ^a
	Wash	27.5 $\pm$ 0.6 ^b	37.4 $\pm$ 1.2 ^b	33.8 $\pm$ 1.7 ^b	27.6 $\pm$ 2.0 ^b
Track velocity (VCL, $\mu\text{m s}^{-1}$)	Percoll	109.3 $\pm$ 4.5 ^a	107.0 $\pm$ 4.4 ^{ae}	122.7 $\pm$ 7.7 ^{ag}	121.6 $\pm$ 5.0 ^{ag}
	Wash	43.6 $\pm$ 2.4 ^b	47.6 $\pm$ 3.3 ^b	43.8 $\pm$ 2.3 ^b	38.4 $\pm$ 3.7 ^b
Beat cross frequency (BCF, Hz)	Percoll	7.7 $\pm$ 0.9 ^e	10.7 $\pm$ 0.7 ^f	10.5 $\pm$ 0.6 ^f	11.0 $\pm$ 0.8 ^f
	Wash	9.5 $\pm$ 0.9	9.9 $\pm$ 0.8	9.7 $\pm$ 0.3	9.3 $\pm$ 0.5

^{a,b,c,d}Within columns, values with different superscripts are significantly different (a versus b, $P < 0.0001$; a versus c, $P < 0.001$; a versus d, $P < 0.05$).

^{e,f,g}Within rows, values with different superscripts are significantly different (e versus f, $P < 0.01$; e versus g, $P < 0.05$).

Table 2. Percentage of spermatozoa without acrosomes after 0, 1, 3 and 5 h incubation for Percoll and washed sperm preparations

Sperm preparation	Incubation time (h)			
	0	1	3	5
Percoll	3.8 ^c	21.2 ^{ad}	24.1 ^d	24.7 ^d
Wash	3.4 ^{ce}	8.7 ^{bcd}	22.5 ^d	23.5 ^d

^{a,b}Within columns, values with different superscripts are significantly different (a versus b, $P < 0.0001$).

^{c,d,e,f}Within rows, values with different superscripts are significantly different (c versus d, $P < 0.0001$; e versus f, $P < 0.001$).

After 1 h, a significantly greater ($P < 0.0001$) percentage of Percoll gradient prepared spermatozoa had no acrosomes, but after 5 h (i.e. usual time of insemination), there was no significant difference between the two preparations.

Polyspermic fertilization rate

A significantly greater ($P < 0.001$) proportion of oocytes inseminated with Percoll-gradient-prepared spermatozoa (37.5%) were polyspermic compared with those inseminated with washed spermatozoa (4.5%).

Embryo cleavage rates

Table 3 shows the comparative cleavage rates. The chi-squared test demonstrated a significantly ($P < 0.0001$) improved cleavage rate by the oocytes inseminated with

Percoll-gradient-prepared spermatozoa compared with the washed spermatozoa preparation.

Electron microscopy

An example of the transmission electron micrographs developed from the sperm preparations after 5 h incubation is shown (Fig. 2). Although no unique ultrastructural differences could be detected between the two preparations, the washed spermatozoa contained a notable amount of dead or damaged spermatozoa and amorphous material.

Discussion

The study reported here clearly demonstrates that, compared with conventional washing techniques, boar spermatozoa exhibit an enhanced function after a combination of washing and Percoll gradient centrifugation. These results support the extensive data from human studies (Akerlof *et al.*, 1987; Gellert-Mortimer *et al.*, 1988; Guerin *et al.*, 1989; McClure *et al.*, 1989; Englert *et al.*, 1992) and confirm the observations that the penetration of hamster oocytes is significantly greater by boar spermatozoa washed on a Percoll gradient compared with those prepared by dilution (Berger and Horton, 1988) or swim-up (Berger and Parker, 1989).

Although it was not possible to distinguish the precise aspect of sperm function that was enhanced by the Percoll technique, the computer-generated data clearly demonstrated a significant increase in motility and head movement characteristics after Percoll centrifugation compared with after simple washing. It has been suggested that increased sperm motility plays an important role in zona penetration, by thrusting the acrosome through the zona using released acrosomal enzymes (Fraser, 1984). Hence, the faster propulsion of Percoll-prepared

Table 3. Cleavage rate and stage of embryonic development achieved by oocytes inseminated with Percoll-prepared or washed spermatozoa

Sperm preparation	Cleavage rate	Embryo stage				
		2–3-cell	4–5-cell	6–7-cell	8–10-cell	11–12-cell
Percoll	117/194 ^a (60.3%)	4 (2.0%)	34 ^a (17.5%)	29 ^a (14.9%)	32 ^a (16.5%)	18 ^a (9.3%)
Wash	25/215 ^b (11.6%)	3 (1.3%)	10 ^b (4.7%)	6 ^b (2.8%)	4 ^b (1.7%)	2 ^b (0.9%)

^{a,b}Within columns, values with different superscripts are significantly different (a versus b, $P < 0.0001$).

Fig. 2. Electron micrographs of (a) washed and (b) Percoll-prepared sperm preparations after incubation for 5 h at 37°C. Note the large amount of debris in the washed sample. Arrows indicate degenerating dead spermatozoa and amorphous material. Scale bar represents 1.7 µm.

spermatozoa, in association with vigorous head movement, may contribute to improved fertilizing capacity. The tendency for beat cross frequency to decrease linearly with increasing velocity suggests that this parameter is not crucial for optimum fertilization.

Although the rate of spontaneous acrosome reactions was similar with both techniques after 5 h incubation (i.e. at an equivalent time to insemination), the initial rate was much higher for the Percoll-prepared spermatozoa than for the washed spermatozoa. However, it seems unlikely that this

difference directly influenced the observed fertilization and cleavage rate since evidence from human studies indicated that an accelerated rate of spontaneous acrosome reactions does not result in a concomitant increase in oocyte penetration (Fenichel *et al.*, 1991).

In contrast to studies in humans (Tanphaichitr *et al.*, 1988), no unique ultrastructural differences were detected between the spermatozoa in each preparation. However, the electron micrographs confirmed that the Percoll procedure yielded pure fractions of spermatozoa, whereas the washing technique produced cell pellets that contained a heterogeneous population of spermatozoa in addition to amorphous material and seminal plasma. Seminal plasma components have previously been shown to compromise the motility (Jeng *et al.*, 1993) and capacitation process (Chang, 1957) of spermatozoa. Furthermore, evidence from human studies suggests that the presence of non-functional gametes within these unfractionated sperm suspensions impairs the fertilizing ability of normal spermatozoa, by the generation of an excessive production of reactive oxygen species that irreversibly damage membrane function (Aitken, 1988; Aitken and Clarkson, 1988) and influence sperm movement characteristics (Aitken *et al.*, 1993). The significantly reduced motility and fertilizing ability of the washed spermatozoa certainly lends support to these observations.

Although many porcine IVF experiments have reported high oocyte penetration rates (Nagai *et al.*, 1983; Cheng *et al.*, 1986; Yoshida, 1987; Mattioli *et al.*, 1989; Wang *et al.*, 1991), few studies have cultured oocytes matured and fertilized *in vitro* beyond 44 h (Rath, 1992; Wu *et al.*, 1992; Zhu *et al.*, 1992). The embryo cleavage rate achieved with the Percoll spermatozoa in this experiment was notably better than that achieved in these studies and supports the proposal derived from human studies (Le Lannou and Blanchard, 1988; Van der Zwahlen *et al.*, 1991) that the selection of highly motile, morphologically normal spermatozoa from the gradient leads to an improvement of the quality of the embryos fertilized *in vitro* and ultimately embryonic development. Human clinical pregnancy and birth rates were almost doubled when spermatozoa were centrifuged on a Percoll gradient compared with standard migration techniques (Guerin *et al.*, 1989). Additional embryo transfer experiments are required to extend these observations to pigs.

The differing rate of polyspermic fertilization recorded for each sperm preparation further emphasized the enhanced penetrating ability of the Percoll-gradient-prepared spermatozoa, and confirmed that the poor development of the oocytes inseminated with washed spermatozoa was not due to excessive polyspermy. Although the incidence of polyspermy was comparable with previous studies (Nagai *et al.*, 1983; Cheng, 1985; Yoshida, 1987; Mattioli *et al.*, 1989; Kikuchi *et al.*, 1993), this level is still unacceptably high. Since one of the major factors influencing polyspermy is the number of capacitated spermatozoa at the site of fertilization (Hunter, 1973, 1976), further research is required to optimise the 'Percoll' *in vitro* fertilization system without suppressing the present high rate of fertilization.

The computer-assisted semen analysis enabled a rapid assessment of each sample and an accurate evaluation of the sperm motility parameters. This technique alleviated the problem of head to head agglutination that routinely occurs during incubation (Berger and Horton, 1988) and which rapidly form

on a haemocytometer during manual analysis. Evidence from porcine (Tuli *et al.*, 1992), bovine (Anzar *et al.*, 1991; Tuli *et al.*, 1992) and ovine (Suttiyotin and Thwaites, 1992) studies support the efficacy of the HTM analyser for assessing animal spermatozoa but emphasize the importance of determining the correct computer program options so that accurate cell concentrations and motility measurements are obtained.

The results of this study confirm the findings of many previous reports (Forster *et al.*, 1983; Berger *et al.*, 1985; McClure *et al.*, 1989; Chan and Tucker, 1992) that separation of spermatozoa from seminal plasma by centrifugal washing in culture medium on a two-step Percoll gradient is a convenient and efficient way to prepare a population of fertile spermatozoa, but the reasons for its superiority over other separation methods remain unknown. Since the majority of porcine IVF studies have routinely used simple spermatozoa washing and incubation procedures, we propose that fertilization and cleavage rates could be markedly improved by a simple modification of spermatozoa preparation techniques.

The authors thank A. Skews for the excellent preparation of the electron micrographs, JSR Healthbred for the generous donation of boar semen and the Department of Obstetrics and Gynaecology, University of Bristol, for allowing us to use their Hamilton Thorne Motility Analyser. This research was supported by a grant from the Ministry of Agriculture, Fisheries and Food.

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