A highly efficient method for washing mammalian spermatozoa

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Summary. A simple method has been developed for washing spermatozoa: the cell suspension is layered over a solution containing Ficoll and, after centrifugation, the original suspending medium remains as an undiluted layer above the Ficoll solution while the spermatozoa form a loose pellet at the bottom. Removal of the supernatant layers is easily and completely accomplished by aspiration.

When ram spermatozoa were washed by this method, as much as 99.6% of the original medium could be removed during a single washing cycle, while up to 98% of the cells were recovered. Mechanical damage to the cells was minimal and repeatability was high.

Introduction

Studies of spermatozoa usually require that these cells are in the form of a viable population which has been washed free of seminal plasma or other body fluids. Such fluids contain a wide variety of components, some of which tend to interfere and interact with the spermatozoa. Most methods of washing spermatozoa have been of the 'classical' type: the suspension is diluted with an artificial medium and centrifuged to concentrate the cells, after which the cells are resuspended in further washing medium; the process is repeated until contamination by the original milieu is sufficiently low. The problems inherent in such a method were discussed by Harrison & White (1972), who proposed a variation designed to obviate some of these problems. However, a major drawback to this variation was that it involved cooling, a process known to cause irreversible damage to spermatozoa (see Mann, 1964).

Another type of washing method has been described (Flipse, 1968; Wales & Humphries, 1969; Prakash, 1974): the spermatozoa are layered in suspension on top of a dense medium and sedimented through this dense medium by centrifugation. The original milieu remains undiluted above, and the cells are separated from it as they pass through the interface. Any remnants of the milieu are removed from the cells as they pass down the dense layer. This type of washing method is likely to be efficient in terms of milieu removal, and fewer washing cycles should be required to achieve a given level of removal than with the classical type of method.

However, neither Flipse (1968) nor Wales & Humphries (1969) attempted to recover motile cells, since they immobilized their cell pellets in formalin. Although Prakash (1974) obtained motile cells, the quantitative information given did not allow true assessment of the efficacy of his method.

I have developed a simple washing method, based upon the 'dense layer' procedure, which is effective at normal temperatures and has been characterized with respect to various parameters.

Washing Procedure

Any sperm suspension can be washed by this method. Routinely, bull and ram semen is diluted in 0.9% (w/v) NaCl or in sperm-Ringer solution (Mann, 1964) to give a maximum of 8×10^8 cells/ml. Rabbit and stallion semen is filtered to remove gel but is not diluted.

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Boar semen is filtered to remove gel, concentrated by centrifugation (Harrison & White, 1972) (unless sperm-rich fractions are being used), and then diluted with 4 vols of a detoxified skim-milk preparation, after which it is incubated at 30°C for 30–45 min. (The milk preparation is made as follows: fresh milk is centrifuged at 6000 $g_{\max}$ for 45 min at 2°C, and the infranatant layer is recentrifuged at 100,000 g for 60 min at 2°C; the supernatant from this high-speed spin is detoxified by addition of 50 μ l 2-mercaptoethanol/100 ml (modified from Johnson, Flipse & Almquist, 1955). The preparation is stored at –20°C.) This treatment disaggregates cytoplasmic droplet clumps and dissolves seminal mucous material; without it, the washed boar spermatozoa can neither be re-suspended easily, because the pellet ‘sticks’, nor be freed from cytoplasmic droplets (Harrison & White, 1972). The milk treatment does not appear to have any effect upon the subsequent livability of the washed spermatozoa.

Washing is carried out in graduated 10 ml conical plastic (‘TPX’) tubes (Xlon Products). The Ficoll-containing washing medium (5 ml) is placed in each tube, and the sperm suspension is layered carefully on top. Up to 8×10^8 cells are normally washed in each tube, i.e. 1 ml aliquots of diluted bull or ram semen, 2 ml aliquots of diluted boar semen, or 2.5 ml aliquots of rabbit or stallion semen. Routinely, the concentration of Ficoll in the washing medium is 7.5% (w/v) as some samples of semen have been observed to fall through 5% Ficoll solutions. Apart from the Ficoll, the composition of the washing medium can be varied at will within the normal limits required for cell survival; at present, I am using a sucrose-based medium or a saline-based medium, made up as follows: in 1 litre, 4.77 g *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES, Hopkin & Williams) (20 mM final concentration), 1.80 g glucose (10 mM), 0.716 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (2 mM), 0.813 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (4 mM), 2.5 ml *N*-KOH (2.5 mM) and 7.5 ml *N*-NaOH (to give pH 7.5–7.55 at 20°C), 100 mg kanamycin sulphate (Sigma), 3 g bovine serum albumin (crystallized, Sigma), and sucrose or NaCl to iso-osmolality (300–310 mosmol/kg, as measured by freezing-point depression). Since Ficoll contains appreciable amounts of NaCl, sucrose or NaCl additions must be varied to obtain iso-osmolality according to the amount of Ficoll in the medium: e.g. if no Ficoll is present, 77.0 g sucrose or 8.01 g NaCl are included per litre; if 7.5% Ficoll is present, 69.8 g sucrose or 7.25 g NaCl are included per litre.

After loading, the tubes are submitted to a two-stage centrifugation procedure using a swing-out rotor: after a gentle initial acceleration, 500 $g_{\max}$ for 5 min, then 1200 g for 10 min (dial settings 3 and 7½ respectively on an M.S.E. Minor Centrifuge). If the concentration of Ficoll in the washing medium is varied, the centrifugation details are also varied, e.g. for 5% Ficoll solutions, the second stage is 950 g for 10 min. The rationale behind a two-stage procedure is that during the initial slow-speed stage the spermatozoa are sedimented slowly through the interface, and ‘matting’ of the cells at the interface is avoided; a ‘mat’, formed at higher speeds, will drag down cytoplasmic droplets and original milieu and thereby reduce the washing efficiency considerably.

After centrifugation the supernatant layers are removed by aspiration, running the pipette tip gently round the walls of the tube at the surface of the liquid. In this way complete removal of the ‘original milieu’ layer is readily achieved and the spermatozoa, which form a loose pellet, can be collected in the Ficoll washing medium at a suitable concentration by leaving a measured volume of cells plus liquid in the bottom of the tube; provided the spermatozoa are motile, they can be readily resuspended merely by agitation of the tube.

Characterization of the Method

To characterize it, the new method was used to wash ram spermatozoa: washing efficiency, cell recoveries and damage caused by the procedure were assessed, and the effect of procedural variations on these parameters was studied. Ram semen was diluted with a suitable volume of 0.9% (w/v) NaCl, and 1.0 ml aliquots of the suspension were washed as described above; the sperm pellets were resuspended in a volume of 1.0 ml.

Washing efficiency was assessed by measuring the removal of macromolecular markers which were added to the sperm suspension before washing. Tritiated inulin (500 μ Ci/mg; Radiochemical

Centre, Amersham, Bucks) was added to the diluted semen at the rate of 0.3 $\mu\text{Ci/ml}$ suspension, and yeast glucose-6-phosphate dehydrogenase (135 units/mg; Boehringer Corporation (London) Ltd) was added at the rate of 3.5 units/ml. Aliquots of the sperm suspensions before and after washing were cooled and centrifuged, and the clear supernatants were assayed in duplicate for tritium and for glucose-6-phosphate dehydrogenase using standard methods. Inulin, a carbohydrate polymer of molecular weight about 5000, does not enter the sperm cell and would appear to resemble a typical non-interacting plasma macromolecule (it is, of course, frequently used in medical physiology to measure the total extracellular fluid space). However, it has been claimed that the carbohydrate, dextran, can be adsorbed to erythrocytes in salt solutions (Brooks & Seaman, 1973); therefore, since glucose-6-phosphate dehydrogenase activity is virtually absent from ram seminal plasma and very low in ram spermatozoa (Harrison, 1972), this enzyme was used as a second protein marker, in case inulin interacted with the sperm cells.

Sperm counts were performed on the initial and final cell suspensions using a method evolved by H. M. Dott and G. M. Foster (unpublished). Suitable dilutions (to give about 10^6 cells/ml) were made in duplicate into 0.9% (w/v) NaCl containing 0.1% (w/v) formaldehyde. Three 5 μl drops of each dilution were air-dried onto clean microscope slides, then covered with 10% (v/v) ethanol under a coverslip and counted using phase-contrast microscopy and a Quantimet 720 image analysing computer (see Dott, 1975) connected to a Wang 600 desk-top calculator. Approximately 1200 spermatozoa were counted in each drop, and a count was rejected if it did not agree with a duplicate to within 20% of their means.

Cell damage was assessed either by measuring the proportion of glucose phosphate isomerase extracellular in the washed sperm suspension (Harrison & White, 1972), or by examining at intervals over several hours the motility of aliquots of the cell suspensions which had been diluted to about 2×10^7 cells/ml in suitable Ficoll-free media and incubated at 30°C.

Experimental results

Two experiments were performed. In the first, different media were compared, and the effect of rinsing the walls of the tube during removal of the supernatant after centrifugation (expected to ensure maximum removal of the original milieu) was studied; washing efficiency was assessed only by use of tritiated inulin. The first experiment was carried out in triplicate using approximately constant cell numbers and the results are presented in Table 1. In the second experiment, using two media, the removal of inulin was compared with that of glucose-6-phosphate dehydrogenase from sperm suspensions over a range of cell numbers; the results are presented in Table 2. Standard statistical methods were used to analyse the data.

Table 1. Comparison of media with respect to washing efficiency and sperm recoveries, using two variations of the dense-layer method to wash ram spermatozoa ($7.54 \pm 0.14 \times 10^8$ cells/tube)

		Saline-based medium containing:				Sucrose-based medium containing:	
		7.5% Ficoll	7.5% Ficoll (no albumin)	5% Ficoll	5% Ficoll (no albumin)	7.5% Ficoll	5% Ficoll
(a) Washing efficiency (as mean % added tritiated inulin remaining after washing, $n = 3$)	Walls of tube rinsed	2.38	2.53	4.15	4.72	0.95	1.28
	Not rinsed	2.55	2.60	4.45	4.49	0.86	1.31
(b) Sperm recovery (as mean % of population remaining after washing, $n = 3$)	Walls of tube rinsed	88.5	88.9	84.1	91.3	78.9	86.9
	Not rinsed	81.5	100.1	94.4	90.1	81.7	88.9

Table 2. Removal of inulin and glucose-6-phosphate dehydrogenase (G6PDH) from ram sperm suspensions, and sperm recoveries: relationship to sperm numbers washed

Sperm no. washed/tube ($\times 10^8$)	Washed in saline-based medium containing 7.5% Ficoll			Washed in sucrose-based medium containing 7.5% Ficoll		
	Recovery (%)	Residual inulin (%)	Residual G6PDH (%)	Recovery (%)	Residual inulin (%)	Residual G6PDH (%)
1.67	96.2	1.05	0.61	72.7†	0.36	0.16
1.79	90.9	1.14	0.71	66.0†	0.47	0.24
2.69	104.9	1.49	0.93	87.4	0.48	0.28
2.99	98.7	1.50	0.94	81.1	0.59	0.31
5.42	108.7	2.19	1.62	86.5	0.83	0.59
5.71	90.6	2.12	1.65	85.9	0.80	0.64
8.59	103.0	2.66	2.49	93.2	1.06	1.00
11.1	94.9	3.31	3.12	84.8	1.44	1.33
12.9	89.3	3.57	3.12	96.7	1.44	1.15
16.9	101.5	4.73	4.16	88.2	2.08	1.93
Correlation with sperm no.	0.0485	0.997*	0.995*	0.485	0.992*	0.984*
Slope	—	0.228	0.233	—	0.105	0.109
Standard error of estimates from sperm numbers	—	0.107	0.135	—	0.075	0.108

* $P < 0.001$, d.f. = 9.

† These values were inaccurate due to methodological difficulties and were not included in the analyses.

Washing efficiency. No difference could be detected between rinsing tube walls during removal of the supernatant after centrifugation and not rinsing (Table 1a, upper rows versus lower rows: $F = 0.193$, d.f. = 1,24) and so the simpler procedure was adopted.

No effect of omission of albumin from the medium could be detected (Table 1a: $t = 1.57$, d.f. = 10 for media with 5% Ficoll; $t = 0.55$, d.f. = 10 for media with 7.5% Ficoll).

Using 7.5% rather than 5% Ficoll or using a sucrose-based rather than a saline-based medium increased the efficiency of washing (Table 1a: $F = 181$, $P < 0.001$, d.f. = 1,16; $F = 767$, $P < 0.001$, d.f. = 1,16 respectively). The efficiencies observed ($= 100(1 - [\text{residual inulin}/\text{initial inulin}])\% \pm \text{S.E.M.}$) when 7.5×10^8 cells were washed per tube ($n = 6$ for each) were: 5% Ficoll, saline-based medium, $95.77\% \pm 0.11\%$; 7.5% Ficoll, saline-based medium, $97.54\% \pm 0.10\%$; 5% Ficoll, sucrose-based medium, $98.71\% \pm 0.07\%$; 7.5% Ficoll, sucrose-based medium, $99.10\% \pm 0.04\%$.

Over a range between 1.67×10^8 and 16.9×10^8 cells washed per tube, there was little difference between removal of inulin and removal of glucose-6-phosphate dehydrogenase (compare slopes in Table 2); each appeared to be a reasonably accurate measure of washing efficiency, and both were removed more efficiently in the sucrose-based medium (compare slopes in Table 2). Thus it could be seen that higher viscosity (sucrose versus saline, 7.5% Ficoll versus 5% Ficoll) coincided with a true higher washing efficiency. It seems reasonable to suppose that the latter is a resultant of the former and that the viscosity is a controlling factor in the washing efficiency obtained using any particular dense-layer system.

However, the number of spermatozoa washed per tube is also an important factor affecting washing efficiency, since the two were linearly related in the saline-based and the sucrose-based media (see correlations in Table 2). As far as I have observed, the number of cells is more important in this respect than the volume in which they are contained. Presumably the more cells that pass down through the dense layer, the more of the original milieu they drag down with them.

Sperm recoveries. Although they varied more between replicates than did washing efficiencies (see Table 1), sperm recoveries were high under most conditions and were independent of the method of removal of the supernatant (Table 1b, upper rows versus lower rows: $F = 0.950$, d.f. = 1,24), of the presence of albumin (Table 1b: $t = 0.22$, d.f. = 10 for 5% Ficoll; $t = 1.51$, d.f. = 10 for 7.5%

Ficoll) and of sperm numbers (see correlations in Table 2). However, they were higher in a saline-based medium than in a sucrose-based medium, especially when 7.5% Ficoll was used (from Table 2: $97.87\% \pm 2.09\%$ in 7.5% Ficoll, saline-based medium ($n = 10$); $87.98\% \pm 1.73\%$ in 7.5% Ficoll, sucrose-based medium ($n = 8$)). The washing media which yielded lower recoveries of spermatozoa also yielded higher efficiencies of washing and were more viscous. It is axiomatic that viscosity affects sedimentation; the spermatozoa are less readily sedimented in more viscous media.

Damage to spermatozoa. In 3 ram sperm suspensions which had been washed twice, the mean proportion of glucose phosphate isomerase which was extracellular was found to be 4.7% of a total content of $17.1 \text{ units}/10^9 \text{ cells}$. This compared favourably with figures given by Harrison & White (1972) for other washing methods. However, the response of sperm suspensions to washing by the dense-layer method varied considerably with species (unpublished observations); in particular, bull spermatozoa lost motility very rapidly.

Further observations

The dense-layer procedure is a convenient means of obtaining a sample of the extracellular environment in a cell suspension, since if the washing process has been carried out carefully, the supernatant layer over the Ficoll washing medium will not have been diluted: aliquots of this layer were found to be representative of the original milieu (see Foulkes & Watson, 1975). The method has two advantages: firstly, the cells are certainly not damaged mechanically during their passage from the supernatant layer, and therefore leakage of intracellular constituents is unlikely to have occurred during the preparation of the cell-free sample (see Pace & Graham, 1970). Secondly, because the spermatozoa can be recovered undamaged after the separation procedure (although see 'Discussion'), their subsequent characteristics can be monitored.

In preliminary experiments, it was found that two washing cycles were required to remove, cytoplasmic droplets from spermatozoa and that sucrose-based media were more effective than saline-based media. Originally the spermatozoa were washed first through 5% Ficoll, then through 10% Ficoll; later, two washes through 7.5% Ficoll in the sucrose-based medium were found to be adequate, the spermatozoa being diluted to two vols in Ficoll-free medium before being relayered. The cytoplasmic droplets may be removed as efficiently after two cycles by this method of washing as by the procedure of Harrison & White (1972). They may be further separated from spermatozoa by differential centrifugation of the combined supernatants, and may be washed by carefully layering the final supernatant over a medium containing a suitable concentration of Ficoll and spinning the droplets through the interface. Such a method appears more suitable than that of Garbers, Wakabayashi & Reed (1970), because the droplets are not subjected to hypertonic environments and do not have to be washed by resuspending a pellet (when damage can occur to these fragile particles).

The dense-layer washing method did not separate ejaculated rabbit spermatozoa from the contaminating particles usually found in rabbit semen (Metz, Hinsch & Anika, 1968): large numbers of these particles were always found in the final sperm suspensions.

Discussion

Characterization of a washing method has rarely been carried out in detail. Dott & Walton (1960) investigated the effects of washing on spermatozoa and published data on the removal of seminal plasma constituents from sperm suspensions using a classical washing procedure. Harrison & White (1972) published results which indirectly indicated the efficiency of their washing procedure. Multamäki & Niemi (1972) and Prakash (1974) measured the citric acid content of sperm suspensions before and after washing to assess the washing efficiency; since, however, citrate can enter the spermatozoa and may be metabolized by them (Flipse, 1967; Keyhani & Storey, 1973), its levels may decrease in sperm suspensions, particularly when present in low concentration, and its measurement cannot be assumed a valid estimate of seminal plasma removal. On theoretical grounds, measurement of removal of high molecular weight components which do not interact with the spermatozoa must

be the best assessment of general seminal plasma removal. Some components of seminal plasma interact with the spermatozoa (e.g. the so-called 'sperm-coating antigen' (Weil, 1967)): general measurement of removal of seminal plasma proteins (Dott & Walton, 1960) may not therefore yield a true assessment of removal of non-interacting material. Measurement of removal of labelled inulin appears a simple and suitable method, with the caveat that it might interact with certain cell types (Brooks & Seaman, 1973); measurement of removal of an added enzyme that is not normally found in the semen should therefore be used as a check.

Using these latter methods, the dense-layer washing procedure was shown to be very efficient in removing seminal plasma (between 95.3% and 99.6% in a single cycle depending on conditions) and replicates were in close agreement. If 1 ml sperm suspension were diluted with 5 ml washing fluid and centrifuged to yield 1 ml washed suspension by the classical washing method, in theory 83% could be removed at best. The dense-layer method is therefore potentially more efficient.

I cannot find any published information on sperm losses during washing. However, losses can be as low as 5% using the dense-layer method; replicates were in close agreement. Because of the high recovery, the likelihood of biased loss within the sperm population is low and the resultant suspension is then representative of the starting population.

Damage caused to cells during washing has been the subject of many investigations (see, for example, Jones & Holt, 1974, and references quoted therein). As far as can be seen, minimal mechanical damage is imposed upon the cells by the use of the dense-layer procedure. However, after washing, cell motility is considerably impaired in some species. The only unusual component which was present in high concentrations was Ficoll; but it seems unlikely that Ficoll exerts a toxic effect upon the cells, for Beatty (1964) reported that rabbit spermatozoa exposed to Ficoll solutions of up to 30% (w/w) were both motile and fertile, and Prakash (1974) reported that bull spermatozoa exposed to 14% (w/v) Ficoll were very motile. It seems most likely that the loss of motility observed after washing by the dense-layer method is an expression of the 'dilution' effect (Mann, 1964) brought about by the very high degree of seminal plasma removal resulting from this washing method. Such an effect has not been observed previously after a single washing cycle, because other types of washing methods are much less efficient; mortality of spermatozoa following thorough washing has usually been due as much to repeated resuspension as to removal of seminal plasma.

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