THE EFFECTS OF WASHING
ON THE ULTRASTRUCTURE AND CYTOCHEMISTRY
OF RAM SPERMATOZOA

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Summary. Washing ram spermatozoa two or four times with Krebs-Henseleit-Ringer solution, to achieve dilution rates of 100- or 10,000-fold, damaged the plasma membranes, acrosomes and mitochondria of some spermatozoa. The proportion of spermatozoa with broken plasma membranes over the acrosome increased with the number of washes.

Washing once to achieve a dilution rate of 10-fold was sufficient to remove from spermatozoa all the substrate available for NADPH diaphorase and probably glucose-6-phosphate dehydrogenase, but not quite all the substrate for succinic dehydrogenase. A 10-fold dilution was also sufficient to remove glucose-6-phosphate dehydrogenase from a considerable proportion of spermatozoa, but even three washes to achieve a dilution rate of 1000-fold had little effect on succinic dehydrogenase and NADPH diaphorase activity in spermatozoa.

The effects of washing could not be attributed to repeated centrifugation of spermatozoa during the process.

INTRODUCTION

The detrimental effects of washing semen or diluting to high rates have been described by a number of authors. For example, Emmens & Swyer (1948) and Blackshaw (1953) showed that sperm motility was depressed, while Mann & Lutwak-Mann (1948), Dott & Walton (1960) and Wales & Wallace (1965) observed a reduction of metabolic activity. Workers from several laboratories have measured the loss of a number of intracellular substances, including inorganic ions (Dott & White, 1964), hexokinase and glucose phosphate isomerase (Harrison & White, 1972), glyceraldehyde-3-phosphate dehydrogenase (Smith, Mayer & Merilan, 1957), cytochrome (Mann, 1951), plasmalogens, protein, hyaluronidase and lipoprotein, from the acrosome (Mann, 1964). In addition to the reduction in metabolic activity, Dott & Walton (1960) noticed that washing caused an increase in the proportion of eosinophilic spermatozoa and an increased tendency towards agglutination. These observations suggest that washing causes a change in the permeability of the plasma membrane associated with the removal of antiagglutinins. Most of these

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studies measured the overall effects of diluting semen and it is not possible to conclude whether the effects were due to changes occurring to all or only to some of the spermatozoa in the samples examined. Since the structure of the cells was not examined, it is not possible to decide whether the losses of substances were due to increased permeability of the cell membranes or to their destruction.

The experiments reported in this paper were carried out to determine the effects of washing on the ultrastructure and cytochemical activity of individual spermatozoa in the samples that were affected by the washing process. The oxidative enzymes, succinic dehydrogenase and glucose-6-phosphate dehydrogenase, were chosen for the cytochemical studies as representatives of membrane-bound and soluble enzymes. Since the cytochemical demonstration of glucose-6-phosphate dehydrogenase is dependent upon the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase, this enzyme was also studied.

**MATERIALS AND METHODS**

Semen was collected from Southdown rams following electrical stimulation (Nichols & Edgar, 1964) and from Border Leicester rams using an artificial vagina; only those samples showing wave motion with a score of 3-5 to 4-0 (Emmens, 1947) were used. Samples of undiluted semen containing 200 x 10⁶ spermatozoa, approximately 0·1 ml, were fixed for electron microscopy or diluted into the media for incubation within 15 min of collection. Washing was carried out by diluting the semen samples 10-fold with Krebs–Henseleit–Ringer solution (Jones, 1971a: fructose was omitted for the cytochemical studies), reconcentrating by centrifugation at 700 g for 10 min, removing the supernatant (the volume of diluent used in the initial dilution) and resuspending the spermatozoa using a Pasteur pipette. The process was repeated from one to four times to achieve dilution rates of 10-, 100-, 1000- or 10,000-fold. In each experiment, samples which were to receive less than the maximum number of washes were stored after the initial dilution. Subsequent handling was arranged so that the terminal washes of all the different treatments were performed simultaneously.

All diluted samples were finally reconcentrated into 0·1 ml and fixed for electron microscopy or diluted for incubations.

The procedures used for fixing, preparing and examining spermatozoa with the transmission electron microscope have been described by Jones (1971a, b, 1973b). The method included coding and randomization of treatments, and preparation of low power electron micrographs (initial magnification X500 followed by a photographic enlargement X2) of different areas of an araldite-embedded section from each treatment. These were subsequently studied and the different modifications of the acrosome and plasma membrane around the head (the nine classes shown in Table 1) or the mid-piece (the five types shown in Table 2) were scored into classes corresponding to those described in detail by Jones (1973a) and Jones & Martin (1973).

All the media used for the cytochemical studies contained 50 mM-phosphate
buffer (pH 7.5) and 1 mg nitroblue tetrazolium/ml. Disodium succinate (50 mm) was used as the substrate for succinic dehydrogenase. The medium for the glucose-6-phosphate dehydrogenase reaction contained 5 mm-magnesium chloride, 0.25 mg NADP/ml as co-factor and 1.25 mg glucose-6-phosphate/ml as substrate, and NADPH (2 mg/ml) was used as substrate for NADPH diaphorase. Spermatozoa in 0.1 ml were added to 0.9 ml of the reaction media and incubated for 30 min (succinic dehydrogenase and NADPH diaphorase) or 1 hr (glucose-6-phosphate dehydrogenase) at 37°C. They were then concentrated by centrifugation and removal of the supernatant, fixed in 10% formalin in phosphate buffer (pH 7.0), smeared on microscope slides precoated with gelatin, and mounted in glycerin-jelly. Before microscopical examination, the slides corresponding to each experimental treatment were coded and randomized so that the observer was unaware of the treatment being examined. Depending on the density of the cytochemical reaction product, spermatozoa were scored into the four classes shown in Pl. 1, Figs 3 to 6.

One hundred spermatozoa were scored per slide and the number in each of the classes was counted. Analyses of $\chi^2$ (Claringbold, 1961) were used to examine the data obtained from the electron microscope studies; details of these methods were described by Jones & Martin (1973). The standard errors shown in Tables 1 and 2 were computed from error mean squares in analyses of variance carried out on each of the classes (columns) of spermatozoa shown in the Tables. In the cytochemical studies, counts of percentages were transformed to angles for the analyses of variance and orthogonal polynomial coefficients were used to test the validity of the hypotheses proposed in the experimental design. In determining the activities of succinic dehydrogenase and glucose-6-phosphate dehydrogenase, the coefficients were used to examine separately the effects of washing and incubating spermatozoa in the absence or presence of substrate. The standard errors shown in the Text-figures were computed from the residual mean square in the analysis of variance.

RESULTS

Electron microscopy

Undiluted semen and samples washed twice or four times were examined in the first experiment which was replicated with ejaculates from four rams. The effects of washing on the structures in the head of the spermatozoa are shown in Pl. 1, Figs 1 and 2, and Table 1. Most spermatozoa from undiluted samples (Pl. 1, Fig. 1) had intact plasma membranes around the head and undamaged acrosomes (first column in Table 1). Washing (Pl. 1, Fig. 2) reduced the incidence of these ‘normal’ spermatozoa and increased the incidence of spermatozoa with broken plasma membranes ($P<0.001$) and the proportion showing acrosomal changes ($P<0.01$). Increasing the number of washes from two to four increased the incidence of spermatozoa with intact acrosomes but broken plasma membranes ($P<0.01$). Different ejaculates varied in their susceptibility to treatment. For example, the mean percentages (mean effects) of normal heads for Ejaculates 1 to 4 respectively were 80.7, 68.7, 58.0 and 80.0.
Table 1. The effects of washing semen two and four times, to achieve dilution rates of 100- and 10,000-fold, respectively, on the structure of the head of ram spermatozoa

<table>
<thead>
<tr>
<th>Classification of sperm heads†</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane: intact</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>broken or lost</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acrosome: swollen</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>vesciculated</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>vacuolated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>loss of outer membrane</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>loss of contents</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Undiluted  | 0† | 0† | 0† | * | 11† | * | 1† | * |
Washed: 2-times | 70 | * | 0 | 0 | 3 | 14 | 1 | 9 | 4 |
Washed: 4-times | 59 | 0 | 0 | 0 | 2 | 23 | 1 | 7 | 9 |
S.E. of mean | 4-4 | 0-0 | 0-0 | 1-1 | 2-4 | 0-6 | 2-3 | 1-6 |

† Examples of the spermatozoa in Classes 1 to 9 are shown in Jones & Martin (1973: Pl. 1, Figs 1 to 9).
‡ Mean percentage values for four ejaculates have been rounded up to the nearest whole number; asterisks in body of Table denote that mean percentage values were equal to 0·25.

The mid-pieces of most spermatozoa in the samples from undiluted semen had intact plasma membranes and mitochondria of homogeneous electron density (Pl. 1, Fig. 1). Overall, washing reduced the incidence of these 'normal' types (first column, Table 2) and increased the incidence of spermatozoa with damaged mid-pieces ($P<0·001$). The susceptibility of spermatozoa to damage varied from one ejaculate to another. The mean proportion (mean effects) of mid-pieces scored as normal for Ejaculates 1 to 4 were, respectively, 90·0, 46·7, 49·0 and 62·7. Dilution also reduced the percentage of motile spermatozoa ($P<0·05$, Table 2). Increasing the number of washes from two to four had no effect on the structure of the mid-piece or the mean scores of % motile spermatozoa.

In order to determine whether the deleterious effects of washing that were observed in the first experiment should be attributed to dilution or the physical effects of centrifugation, two semen samples from each of two rams (replicates)

EXPLANATION OF PLATE 1

Fig. 1. Electron micrograph of undiluted ram semen fixed immediately after collection, showing intact plasma membranes and well-preserved acrosomes and mitochondria. × 19,000.

Fig. 2. Electron micrograph of ram semen washed four times to achieve a dilution rate of 10,000-fold, showing that considerable damage has occurred to the plasma membrane, acrosome and mitochondria. × 20,000.

Figs 3 to 6. Light micrographs of ram spermatozoa allowed to react with tetrazolium salt showing the four degrees of staining which were observed in the demonstrations of dehydrogenase activities. Spermatozoa scored as reacting at least as intensely as those in Fig. 3 were counted as reactive spermatozoa; the percentage of these spermatozoa was used as unit observation for the analyses of variance and this measure is the ordinate in Text-figs 1 to 3. × 1000.

Fig. 7. Light micrograph of ram spermatozoa smeared on a microscope slide after the cytochemical reaction to demonstrate dehydrogenases. Note the variation in intensity of staining with tetrazolium. × 800.
Washing ram spermatozoa

were diluted 10-fold; one was stored and the other centrifuged and resuspended three times without removing the supernatant. Both samples were then fixed after centrifugation and removal of the supernatant. The mean percentages of undamaged heads were 91·0 and 87·0, respectively, for the samples centrifuged once and four times. The corresponding mean percentages of undamaged mid-pieces were 93·0 and 86·0. Neither of these effects was statistically significant.

Cytochemistry

In all the experiments, cytochemical reaction products were only observed in the mid-pieces of spermatozoa. Each spermatozoon examined was scored according to the intensity of reaction into the four classes shown in Pl. 1, Figs 3 to 6. On completion of the experiment, it was considered that only those spermatozoa which showed the strongest cytochemical reaction could be consistently distinguished from the remainder and only the effect of treatment on this parameter was considered in the analyses.

Table 2. The effects of washing semen two and four times, to achieve dilution rates of 100- and 10,000-fold respectively, on the structure of the mid-piece of ram spermatozoa and scores of % motile spermatozoa

<table>
<thead>
<tr>
<th>Treatment of spermatozoa</th>
<th>Mid-piece</th>
<th></th>
<th></th>
<th></th>
<th>Motile sperm. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact plasma membrane</td>
<td>Broken plasma membrane</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal mitochondria</td>
<td>Condensed mitochondria</td>
<td>Pale mitochondria</td>
<td>Normal mitochondria</td>
<td>Pale mitochondria</td>
</tr>
<tr>
<td>Undiluted</td>
<td>86*</td>
<td>7*</td>
<td>2*</td>
<td>1*</td>
<td>5*</td>
</tr>
<tr>
<td>Washed: 2-times</td>
<td>48</td>
<td>15</td>
<td>10</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>4-times</td>
<td>52</td>
<td>18</td>
<td>4</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td>S.E. of mean</td>
<td>7·7</td>
<td>4·5</td>
<td>4·6</td>
<td>1·5</td>
<td>3·9</td>
</tr>
</tbody>
</table>

The five columns describing changes to the mid-piece correspond to the structures shown in Jones & Martin (1973: Pl. 1, Figs 10 to 14).

* Mean percentage values for four ejaculates have been rounded up to the nearest whole number.

Succinic dehydrogenase activity was examined in a $4 \times 2$ factorial experiment, replicated with ejaculates from five rams, in which undiluted semen and samples washed one, two or three times were incubated in the absence or presence of substrate (Text-fig. 1) when determining the cytochemical activity. The analysis of variance showed that omission of substrate from the reaction medium markedly reduced the percentage of spermatozoa showing the intense dehydrogenase activity ($P<0·001$); the effect was greater in washed than unwashed samples ($P<0·05$). Increasing the number of washes from one to three did not significantly affect the proportion of reacting spermatozoa when substrate was included in the reaction medium.

Glucose-6-phosphate dehydrogenase activity was examined in a $5 \times 2$ factorial experiment, replicated with ejaculates from seven rams, which examined the same treatments that were used in the previous experiment. In addition, the effects of repeated centrifugation without further dilution were examined; semen was diluted once (10-fold) then centrifuged and resuspended twice
without removing the supernatant, before the final reconcentration for the cytochemical incubation (Text-fig. 2). The analysis of variance showed that, overall, the exclusion of substrate reduced the proportion of spermatozoa showing the strong cytochemical reactions ($P<0.01$). When substrate was excluded, washing reduced the incidence of reacting spermatozoa to about 20\% ($P<0.001$), but increasing the number of washes had little further effect. When substrate was included in the cytochemical reaction media, the incidence of reacting spermatozoa was reduced by washing (for undiluted versus diluted, $P<0.001$) and there was a linear relationship between incidence of reacting spermatozoa and number of washes ($P<0.001$). The response of spermatozoa diluted 10-fold was much the same whether they were centrifuged once or three
times. The mean responses also varied between ejaculates ($P<0.001$). An interaction between ejaculates and semen treatment showed that some ejaculates were more sensitive than others to the washing procedures ($P<0.01$).

The activity of NADPH diaphorase was studied in a $2 \times 2$ factorial experiment, replicated with ejaculates from four rams, in which semen was washed once or three times and allowed to react with tetrazolium in the absence or presence of substrate. The mean percentages of spermatozoa showing an intense cytochemical reaction after one wash were 58.25 and 0.25 after incubation in the presence and absence of substrate respectively. After three washes, the mean percentages were 61.5 and zero respectively. Clearly, there was a statistically significant difference due to the inclusion of substrate ($P<0.001$), but no significant change was caused by washing. The mean responses of samples
incubated in the presence of substrate were high and much the same in three of the ejaculates, but lower in the fourth. This was reflected in the analysis of variance by a difference between ejaculates ($P < 0.05$).

**TEXT-FIG. 2.** The effects of washing ram semen on the percentage of spermatozoa showing intense formazan deposition in the mid-piece following cytochemical reactions for glucose-6-phosphate dehydrogenase in the absence (■) and presence (●) of 1.25 mg/ml glucose-6-phosphate. U, undiluted semen; R, semen diluted 10-fold and concentrated by centrifugation and resuspended twice in this medium before a final concentration and removal of the supernatant for the cytochemical reaction (see text). Vertical lines indicate the S.E.M. and the solid connecting lines are calculated regression lines.

**DISCUSSION**

The studies with the electron microscope provide a structural basis to account for some of the loss of substances and viability of ram spermatozoa that has been associated with dilution and washing of semen (see ‘Introduction’ for references). In these studies, however, only a proportion of spermatozoa were adversely affected whilst about 50% of cells remained structurally intact even when washed to achieve a dilution rate of 10,000-fold. This suggests that some biochemical determinations of semen may be measuring the response of a specific proportion of spermatozoa and not a change in the composition of all cells.

The localization of succinic dehydrogenase activity in the mid-piece of ram spermatozoa is in agreement with findings of other workers who studied spermatozoa from the bull, cat, dog, guinea-pig, mouse, rabbit and man (Edwards & Valentine, 1963; Balogh & Cohen, 1964; Hrudka, 1965; Mathur, 1971). The absence of an effect of washing on succinic dehydrogenase activity is also in agreement with the general opinion that the enzyme is bound to the mitochondrial membrane (Lehninger, 1965). Our findings do not, however, support those of Nelson (1959) who observed succinic dehydrogenase activity in the main-piece as well as the mid-piece. There is insufficient information
to determine why some dehydrogenase activity was found in a proportion of washed spermatozoa which were allowed to react with tetrazolium in the absence of succinate (Text-fig. 1). Possibly, the activity was due to the presence of endogenous substrate which is only slowly leached from the cells (Hrudka, 1965).

The demonstration of glucose-6-phosphate dehydrogenase in ram spermatozoa is in agreement with findings for spermatozoa from the boar, mouse, rat, guinea-pig, cat, dog and man (Balogh & Cohen, 1964; Bolton & Linford, 1970; Mathur, 1971). The ease with which it was removed from spermatozoa is in agreement with other histochemical studies (Kalina & Gahan, 1965) and the opinion that the enzyme is not membrane-bound. The presence of about 20% of spermatozoa that were cytochemically active even when incubated in the absence of glucose-6-phosphate may indicate that some of the NADP used in the cytochemical reaction medium was reduced by alternative routes and served as a substrate for the enzyme NADPH diaphorase. The demonstration that NADPH diaphorase was not washed from spermatozoa supports this proposal. The absence of an effect of washing on NADPH diaphorase activity also supports the suggestion that, in the second cytochemical experiment (Text-fig. 2), it was glucose-6-phosphate dehydrogenase that reacted in the undiluted samples and that washing removed this enzyme and its substrate from the spermatozoa at different rates.

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Washing ram spermatozoa


