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Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa

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Summary. Carboxyfluorescein diacetate and propidium iodide were used as fluorescent stains to assess membrane integrity in sperm populations from ram and boar. The living spermatozoa were immobilized with low concentrations of formaldehyde so that individual stained cells could be observed in a suspension with the aid of a fluorescence microscope. Intracellular esterases liberated impermeant-free carboxyfluorescein from the permeant carboxyfluorescein diacetate and caused the product to accumulate and fluoresce green within the acrosome and the mitochondria as well as within the cytoplasm. Most of the spermatozoa (the intact ones) accumulated carboxyfluorescein in all compartments; however, a few cells (those with damaged plasma membranes) accumulated the stain only in the acrosome and/or the mitochondria, while others (all of whose membranes were damaged) remained entirely unstained. The impermeant propidium iodide did not stain any of the (intact) spermatozoa that accumulated carboxyfluorescein throughout their length, but stained all the others (the heads fluoresced red).

The technique appeared to provide more reliable estimations of the percentage of functional cells than did motility estimations or assessments of acrosomal integrity (presence of normal apical ridge). The technique also demonstrated the sensitivity of the sperm plasma membrane to cold shock: virtually all cells rapidly became permeable to the stains after such stress. Assessments of boar sperm samples during preparative incubation for in-vitro fertilization indicated a considerable increase in the percentage of cells with damaged plasma membranes as incubation proceeded, in advance of the increase in the percentage of cells with discharged acrosomes.

Keywords: spermatozoa; fluorescent probes; membranes

Introduction

Laboratory assessment of sperm fitness is a fundamental problem in semen research and technology. The integrity of the sperm plasma membrane is an obvious parameter for assessment, because it is an essential requirement for general cell function. Garner *et al.* (1986) have described the use of two fluorescent probes, carboxyfluorescein diacetate and propidium iodide, to assess the membrane integrity of spermatozoa of cattle and other species. Propidium iodide, which binds to and stains cellular DNA, is impermeant to the plasma membrane and therefore can only enter and stain damaged (permeable) cells. Carboxyfluorescein diacetate, on the other hand, is permeant but is de-esterified inside the cell by non-specific esterases; the resultant free carboxyfluorescein is fluorescent and impermeant, and builds up in the cytoplasm of intact cells, causing them to fluoresce green throughout.

Unfortunately the procedure as published implied a need for flow cytometry as a means of quantitation, a resource which is not available to the large majority of laboratories; quantitation

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could not readily be carried out by direct observation with a fluorescence microscope because the live spermatozoa were still motile. This paper describes the use of very low concentrations of formaldehyde to immobilize the living spermatozoa so that assessment of membrane integrity can be made using a standard fluorescence microsope. Results are presented to demonstrate the sensitivity of the technique and its ability to detect lesions in organelles as well as in the plasma membrane of individual spermatozoa.

Materials and Methods

Spermatozoa. Semen was obtained from Clun Forest rams and Large White boars in the Institute's animal colonies, using an artificial vagina or the gloved-hand technique respectively; only the sperm-rich fractions of boar ejaculates were used. Ram semen was used immediately. Boar semen was diluted with 4 volumes of a milk-containing medium and stored overnight; the milk medium was composed of 3 parts by volume of a protein-free boar semen diluent (developed by Cheng (1985) from the 'Zorlesco' medium of Gottardi *et al.* (1980)) and 1 part by volume of skimmed milk containing 0·05 (v/v) 2-mercaptoethanol (details to be published elsewhere). (The purpose of this storage in a milk medium was to disperse the clumps of cytoplasmic droplets in the boar semen and render the spermatozoa less 'sticky' so that the cells could be resuspended easily after washing; see Harrison & White, 1972.)

The saline medium in which spermatozoa were routinely diluted and incubated contained 140 mM-NaCl, 10 mM-glucose, 2·5 mM-K^+, 0·5 mg polyvinyl alcohol/ml, 0·5 mg polyvinyl pyrrolidone/ml, and 20 mM-Hepes; it had been adjusted to pH 7·55 at 20°C with NaOH, and its osmolality (measured by freezing point depression) was 300 mosmol/kg. Spermatozoa were washed by sedimentation through cushions of a similar medium containing sucrose in place of NaCl (Harrison *et al.*, 1982).

6-Carboxyfluorescein diacetate was obtained from Calbiochem (via Novabiochem UK Ltd, Highfields Science Park, Nottingham, UK) and propidium iodide from Sigma Chemical Company Ltd (Poole, Dorset, UK).

Staining with fluorescent probes. A staining medium was prepared by adding to each ml of saline medium: 20 µl of a stock solution of formaldehyde (2·5 mg/ml in water, stored at 2°C for not more than 4 days), 20 µl of a stock solution of carboxyfluorescein diacetate (0·46 mg/ml in dimethyl sulphoxide, kept dark and stored at −20°C), and 10 µl of a stock solution of propidium iodide (0·5 mg/ml in isotonic saline, kept dark and stored at −20°C); final concentrations were 1·7 mM-formaldehyde, 7·3 µM-propidium iodide and 20 µM-carboxyfluorescein diacetate, and the staining medium was made up not more than 1 h before use. Samples of spermatozoa were diluted in aliquants of this medium to a final concentration of about 10^7 cells/ml and were then incubated for at least 8 min at 30°C.

Sub-samples (5 µl) of the stained suspension were placed on clean microscope slides and overlaid carefully with coverslips. Random fields were observed under × 400 magnification with epifluorescence illumination. Staining with carboxyfluorescein diacetate was assessed using the standard fluorescein filter set, while staining with propidium iodide was assessed using the standard rhodamine filter set. On occasions, fields were also observed with phase-contrast illumination.

For quantitative assessment of plasma membrane integrity, at least 100 cells were counted in each of two 5-µl aliquants from a stained sample. Spermatozoa that fluoresced green throughout their length after staining with carboxyfluorescein diacetate were classified as being intact while all others were classified as damaged. Spermatozoa with heads that fluoresced red after staining with propidium iodide were classified as damaged, unstained cells as intact.

Assessment of motility. Sperm suspensions were observed under × 50 magnification in uncovered drops on glass slides on a warm stage maintained at 37°C. They were scored using a discontinuous qualitative scale of 0–5 in which 0 signified complete absence of motile cells and 5 signified a sample in which all cells exhibited vigorous motility (Harrison *et al.*, 1982). The minimum scoring above 0 was +/−, which signified that most cells were immotile but that some exhibited twitching movements; score categories for increasing degrees of motility were +, 1, 1+, 2 etc. (This type of scale was used to emphasize the qualitative nature of the scoring.)

Assessment of acrosomal status. The presence or absence of a normal apical ridge was assessed by inspection of glutaraldehyde-fixed cells under phase-contrast illumination (Shams-Borhan & Harrison, 1981).

Results

General

The appearance of ram spermatozoa stained with both carboxyfluorescein diacetate and propidium iodide is shown in Fig. 1. In every semen sample, four categories were observed. The majority of the cells had accumulated carboxyfluorescein throughout both head and flagellum, and

had not taken up propidium iodide; they clearly possessed an intact plasma membrane. A second category had accumulated carboxyfluorescein only within the midpiece, which presented a more or less speckled appearance depending on the individual cell. The heads (nuclei) of these cells had taken up propidium iodide, demonstrating that their plasma membranes were damaged both in the head region and along the flagellum (no accumulaton of carboxyfluorescein in the principal piece); the speckled appearance of the midpieces was interpreted as accumulation of carboxyfluorescein within intact mitochondria as a result of esterase action within these organelles. A third category of cells showed accumulation of carboxyfluorescein not only within the midpiece but also over the acrosomal region, although there was no accumulation of the dye along the length of the flagellum and the head was stained with propidium iodide. These cells were interpreted as having damaged plasma membranes but intact acrosomes as well as intact mitochondria. In the final category of cells (not illustrated in Fig. 1), there was no accumulation of carboxyfluorescein in any part and the head had taken up propidium iodide. This category was interpreted as having a damaged plasma membrane, a damaged acrosome and no intact mitochondria.

Inclusion of low concentrations of formaldehyde immobilized the intact cells, allowing easy counting of the categories, but did not affect the accumulation of free carboxyfluorescein within the sperm cytoplasm or organelles. Use of glutaraldehyde or higher levels of formaldehyde (16 mM or more) was, however, detrimental.

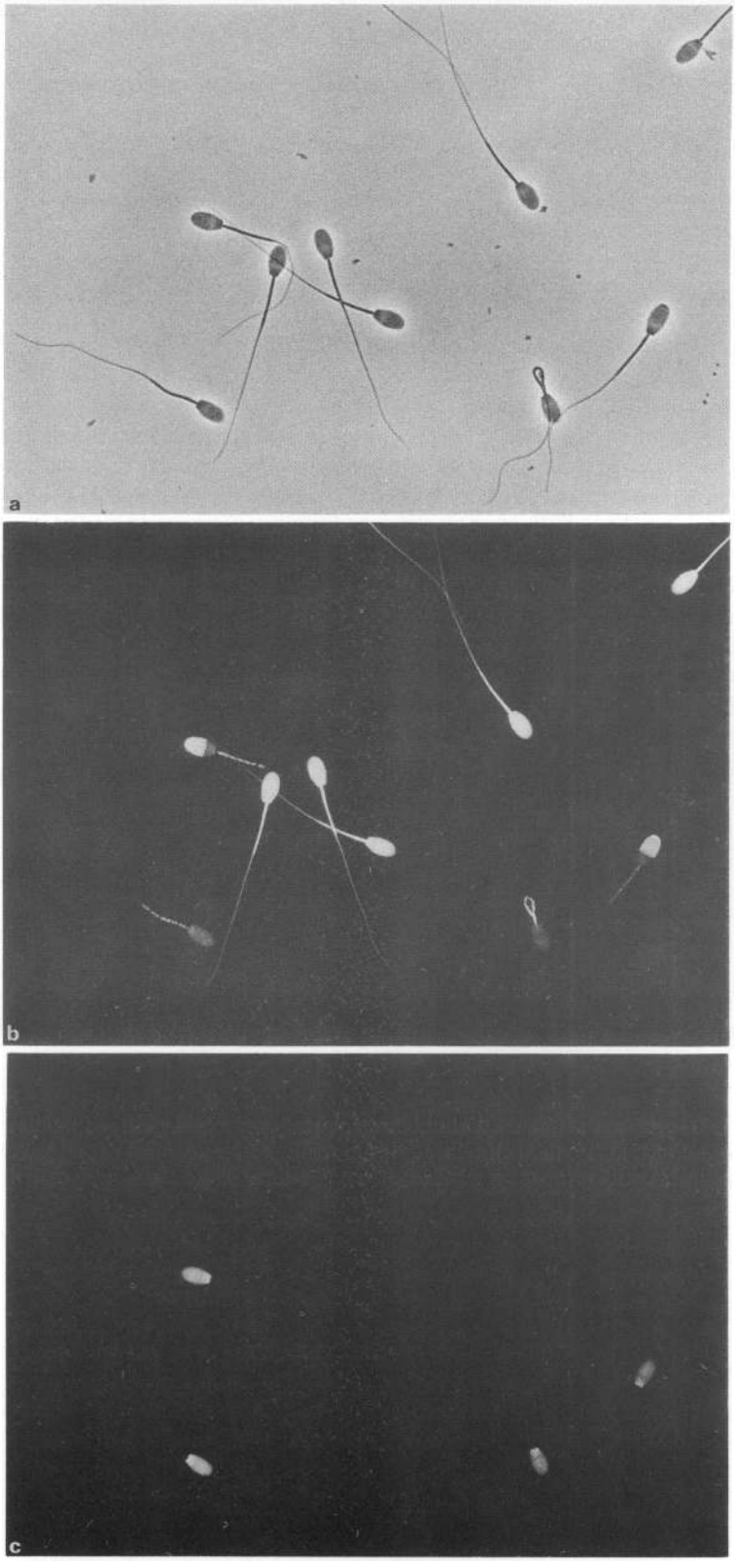
For the purposes of the work described in the rest of this paper, cell populations were classified simply as being intact (stained throughout their length with carboxyfluorescein diacetate, unstained with propidium iodide) or damaged (only partly or not stained with carboxyfluorescein diacetate, stained with propidium iodide).

Use of carboxyfluorescein diacetate and propidium iodide separately and in combination

Sub-samples of a suspension of washed boar spermatozoa were subjected to staining, with carboxyfluorescein diacetate alone, with propidium iodide alone or with carboxyfluorescein diacetate and propidium iodide together. When the dyes were used alone, the total number of spermatozoa observed in each field was counted directly using phase-contrast illumination; when the dyes were used in combination, the total number of cells observed comprised the number seen stained with propidium iodide plus the number seen to retain carboxyfluorescein throughout their length (both observed under epifluorescent illumination). Five replicates of each procedure were performed on the same sperm suspension, with the three procedures being tested in sequence within each replicate (to avoid any bias due to ageing of the sperm suspension). The results were as follows: for each 100 spermatozoa counted, 76·5 $\pm$ 5·5 (s.d.) were judged to be intact by using carboxyfluorescein diacetate alone, while 80·3 $\pm$ 5·7 were judged intact by using propidium iodide alone, and 78·3 $\pm$ 4·6 by using the two dyes in combination. The agreement between the different methods showed that propidium iodide stained all the cells not fully retaining carboxyfluorescein, and therefore that the three procedures were equivalent; the repeatability of the procedure was also established. Subsequent experience with washed and unwashed samples from both ram and boar semen has confirmed these observations, although it has generally proved more convenient or informative for research purposes to use the combination of dyes.

Stability of stained cells

The stability of the stained preparations was investigated. A sample of ram semen was diluted in saline medium at room temperature ($\sim$20°C). A sample was then taken from this stock suspension and stained by the standard procedure at 30°C for 8 min, after which the stained suspension was kept at room temperature. At about 10, 55 and 100 min after the spermatozoa had first come into contact with the staining solution, sub-samples were taken from the stained suspension and examined for plasma membrane integrity. Further samples from the original stock suspension were placed in



staining solution about 55 and 100 min after the initial dilution, and sub-samples from these were examined immediately after staining (i.e. 10 min later). Four replicate experiments were performed.

An analysis of variance of the results (not presented in detail) revealed that no significant change in the staining pattern could be seen when the spermatozoa remained in the staining solution for up to 130 min. A slight decline in the mean proportion intact seen after 100 min of staining (59·7% intact as compared with 64·2% originally) was mirrored in a similar decline (58·5% intact) seen in the diluted suspension that had been held for 100 min before staining. We ascribed this decline to inherent senescence changes rather than to any detrimental effect of the staining solution.

Application of the method of semen assessment

Samples from 7 ram ejaculates (each from a different male) and 5 different boar ejaculates (from 2 different males) were subjected to: (a) visual assessment of motility, (b) staining with carboxyfluorescein and propidium iodide, and (c) fixation with glutaraldehyde for assessment of acrosomal status. Ram semen was examined within 1 h of collection while boar semen was examined after storage overnight in a milk-containing diluent (see 'Methods'). The results are presented in Table 1. For both species, the acrosomal status was a poor reflection of the state of the plasma membrane, many fewer cells showing an intact plasma membrane than showed a normal apical ridge. Moreover, whereas for ram semen visual motility assessment was approximately correlated with the proportion of cells with intact plasma membranes, for boar samples there was little relationship between observed motility and proportion of intact cells. Many of the boar spermatozoa were immotile even though they were apparently intact. That the integrity estimations were essentially reliable could be demonstrated by incubating such poorly motile samples briefly with 3 mM-dithiothreitol. This treatment stimulated motility very markedly (Table 2), showing that the immotile cells were indeed undamaged and capable of expressing motility under the right environmental conditions.

The sensitivity of the method for assessment of plasma membrane integrity could be demonstrated via the effects of 'cold shock'. Rapid cooling (to 0°C) of suspensions of ram and boar spermatozoa resulted in every case in more than 97% of the cells becoming permeable to the fluorescent dyes; acrosomes were also permeabilized, although mitochondria mostly remained intact (see Watson, 1981, for a review of the phenomenon of cold shock). In the case of cold-shocked ram spermatozoa, damage as judged by phase-contrast assessment of acrosomal status was almost as complete as that assessed by the fluorescent probes, whereas a larger percentage of cold-shocked boar spermatozoa presented a normal apical ridge than had intact plasma membranes (Table 3).

Finally, in a preliminary trial, the method was used in combination with others to assess sperm status during preparative preincubation for in-vitro fertilization. The results from two experiments are shown in Table 4. The proportion of cells with permeable plasma membranes increased with incubation, and at each stage this proportion was equal to or greater than the proportion of cells judged to have discharged their acrosomes.

Fig. 1. Simultaneous staining of ram spermatozoa with carboxyfluorescein diacetate and propidium iodide. Staining was carried out as described in the 'Methods'. (**a**) Phase contrast. (**b**) The same field as (a), seen by epifluorescence using FITC filter set; 4 spermatozoa (plus that in the top right-hand corner) are intact and are brightly stained throughout, 2 (on the left and right extremes of the group) possess bright acrosomes and brightly spotted midpieces but their tails and posterior heads are unstained, while only midpieces are brightly spotted in the 2 spermatozoa furthest towards the bottom; because of the relatively broad band width of the FITC filter system, heads stained with rhodamine (see (c)) are also seen as weakly fluorescent, but they contrast clearly with the carboxyfluorescein staining of the acrosome (compare the 2 cells at bottom right). (**c**) The same field as (a), seen by epifluorescence using rhodamine filter set; note that only those cells seen as partly stained in (b) fluoresce. × 575.

Table 1. Assessment of ram and boar semen samples taken from freshly collected ram ejaculates (7 different males) and from boar ejaculates stored overnight in a milk-containing diluent (2 different males)

Species	Ejaculate no.	Motility (qualitative scale: 0–5)	% with intact plasma membrane	% with normal apical ridge
Ram	1	3	51·4	88·5
	2	2	48·8	86·0
	3	2+	51·6	82·5
	4	3+	70·1	90·0
	5	4+	85·1	94·6
	6	3+	60·4	91·0
	7	2+	38·8	68·0
Boar	1	2+	64·2	95·5
	2	2+	76·9	86·0
	3	2	77·1	80·0
	4	2	81·0	96·0
	5	1	77·0	95·0

Table 2. Comparison of motility and plasma membrane integrity in boar sperm suspensions

Exp.	Treatment	Motility (qualitative scale: 0–5)	% with intact plasma membrane
1	Untreated	1	59·6
	3 mM-DTT	3+	66·7
2	Untreated	1	82·1
	3 mM-DTT	4	76·3

Spermatozoa from boar ejaculates stored overnight in a milk-containing diluent were sedimented by centrifugation for 15 min at $600\,g_{max}$ and gently resuspended in saline medium; 3 mM-dithiothreitol (final conc.) was added to one half of this suspension, and the two halves were then incubated at 37°C for 15 min. Treated and control cells were then washed, resuspended in saline medium, and examined for motility and plasma membrane integrity.

Discussion

The use of impermeant fluorescent probes for monitoring the integrity of membranes is well known in other cell systems. The methodology has also been applied before to spermatozoa, but its potential appears to have been overlooked by many sperm biologists. The most comprehensive paper using fluorescent probes for sperm assessment (Garner *et al.*, 1986) largely involved the use of flow cytometry; to workers lacking such sophisticated instrumentation, visual microscopic analysis of samples containing motile cells may have appeared a serious difficulty. We have developed a means of circumventing this problem, and have been able to apply it, using a simple fluorescence microscope, to mouse, human and guinea-pig spermatozoa (unpublished observations) as well as to ram and boar. Our technique therefore seems generally applicable.

Table 3. Effect of cold shock on boar sperm membrane integrity

Exp.	Treatment	Motility (qualitative scale: 0–5)	% with intact plasma membrane	% with normal apical ridge
1	Untreated	2+	77	86
	Cold-shocked	+	3	32
2	Untreated	2	71	80
	Cold-shocked	+/−	0	16
3	Untreated	2	66	51
	Cold-shocked	0	1	32

Spermatozoa from boar ejaculates stored overnight in a milk-containing diluent were washed through sucrose medium and resuspended in saline medium. Then 1-ml aliquants were placed in medium-walled glass tubes, incubated for 5 min at 37°C, and plunged into an ice-water mix. After gentle agitation for 2 min, they were left in the mix for a further 8 min. Finally, they were incubated for 10 min at 37°C. The assessment of plasma membrane integrity was carried out by staining with carboxyfluorescein diacetate alone.

Table 4. Boar sperm membrane integrity during incubation for in-vitro fertilization

Treatment	Motility (qualitative scale: 0–5)	% with discharged acrosomes	% with permeable plasma membranes
Exp. 1			
(a) Overnight storage and washing	1+	28	27
(b) +2·5 h at 37°C and pH 7·8	+/−	29	41
(c) +4 h at 37°C and pH 7·4 (10^6/ml)	NA	63	74
Exp. 2			
(a) Overnight storage and washing	2	8	21
(b) +2·5 h at 37°C and pH 7·8	2+	12	31
(c) +2 h at 39°C and pH 7·4 (10^6/ml)	+/−	54	44
(d) +further 2·5 h at 39°C and pH 7·4	NA	61	72

Boar spermatozoa were subjected to the sequence of treatments developed by Cheng (1985) to obtain successful fertilization *in vitro*: briefly, part of the sperm-rich fraction from an ejaculate was incubated overnight at about 22°C, after which it was diluted in a saline medium and centrifuged to obtain a "partially capacitated" washed sperm suspension (a); this was then diluted to 2×10^8 cells/ml in Medium 199 at pH 7·8, and incubated for 2·5 h at 37°C, to yield a "fully capacitated" suspension (b). Further dilution to 10^6 cells/ml in lactate- and pyruvate-supplemented Medium 199 at pH 7·4 and incubation for up to 4·5 h at 39°C simulated "insemination" conditions (c and d). For clarity, the data for acrosomal status and plasma membrane integrity have been expressed differently from those in Tables 1–3, and are given respectively as % of cells with discharged acrosomes (i.e. absence of normal apical ridge, possibly due to an acrosome reaction) and as % of cells with permeable (i.e. damaged) plasma membranes. NA, not assessed.

Noland *et al.* (1986) and Dey & Majumder (1988) have used spectrofluorometry of sperm suspensions to assess plasma membrane integrity. Apart from the fact that only the average response of the sample can be monitored, this approach has a major imperfection: for practical purposes it is impossible to obtain samples free of damaged individuals, so as to set the lower end of the scale. Moreover, cell suspensions tend to scatter both incident and emitted light, making responses complicated and dependent both on cell density and on dye concentration.

In contrast, microscopic observation of live sperm populations interacting with impermeant fluorescent probes enables direct assessment of individual cells. The use of low concentrations of

formaldehyde to immobilize the motile cells for observation does not harm them: motility is restored when the formaldehyde is removed, and treated spermatozoa remain potentially fertile (Dott *et al.*, 1976). Our observations show that the presence of such low concentrations of formaldehyde does not alter the impermeability of the cell membranes nor does it prevent the intracellular de-esterification of carboxyfluorescein diacetate; the spermatozoa are stable in the staining solution for at least 2 h. However, it should be emphasized that the cells are not 'fixed' under these conditions and will almost certainly continue to undergo changes to which they are susceptible. The immobilizing effect of formaldehyde is presumably mediated through reversible interaction with amino groups on the surface of the spermatozoa. (Such formaldehyde treatment cannot be performed in media containing significant concentrations of free primary amine groups, e.g. Tris or glycine buffers.)

We, and also Garner *et al.* (1986), have compared two fluorescent probes, an impermeant nuclear stain and a member of the so-called 'vital' probes; the latter enter the cell as permeant esters but are then broken down by intracellular esterase action to impermeant fluorescent products. The basis of the use of both types of probe is well established biochemically and biophysically (see Shapiro, 1988). Of the nuclear stains available, propidium iodide is better than the often used ethidium bromide (e.g. Jeulin *et al.*, 1982) because, being more highly charged, propidium iodide is less lipophilic and therefore less membrane-permeant; bisbenzimidazole nuclear stains like DAPI and Hoechst 33258 are much too permeant and are used to stain living cells (see, e.g., Keeler *et al.*, 1983). Of the common 'vital' probes, carboxyfluorescein diacetate would appear to be better than fluorescein diacetate because the charge on the carboxyfluorescein product renders it much less permeant than fluorescein (Weinstein *et al.*, 1977). Babcock (1983), when comparing the two, was unable to monitor sperm mitochondrial pH with carboxyfluorescein and deduced that this derivative was only produced in the cytoplasm (in contrast to fluorescein); a similar localization had been deduced by Thomas *et al.* (1979) with respect to ascites tumour cells. However, both deductions were made on indirect evidence, whereas direct observation by ourselves and by Garner *et al.* (1986) has revealed that carboxyfluorescein is produced and retained within the mitochondria and the acrosome, as well as within the cytoplasm, of all the sperm species examined. Carboxyfluorescein diacetate is therefore a valuable probe because it can be used to assess the integrity of the organelle membranes as well as the plasma membrane; it is, moreover, particularly sensitive, because the carboxyfluorescein product has a molecular weight of only 376 and is therefore rapidly lost after membrane damage.

Use of fluorescent probes compared well with some other methods of semen assessment. The results with boar semen shown in Table 2, as well as studies of the reversible effects of serum albumin on motility estimation in ram and rabbit (Harrison *et al.*, 1982), demonstrate that even under physiological conditions expression of motility is not an entirely reliable parameter of sperm integrity: poor motility does not always indicate cellular damage. In this respect, therefore, we disagree with Garner *et al.* (1986) who classified as moribund those immotile spermatozoa that retained carboxyfluorescein throughout their cytoplasm. On the other hand, there is evidence that maintenance of plasma membrane integrity in media of normal Na^+/K^+ ratio needs a supply of intracellular ATP (Bredderman & Foote, 1971), and so membrane damage as measured by fluorescent probes may indicate prior metabolic failure as well as concomitant loss of vital intracellular metabolites such as adenine nucleotides.

In our hands, the fluorescent probes were also clearly more sensitive indicators of sperm damage than phase-contrast assessment of acrosomal status (see Tables 1 and 3). In corroboration of this, we always noted some cells in each sample whose acrosomes were accumulating carboxyfluorescein (and were therefore intact) but whose plasma membranes were damaged. It seems, therefore, that the plasma membrane is intrinsically more labile than the outer acrosomal membrane.

The plasma membrane also appears considerably more labile than the mitochondrial membranes. We found that sperm plasma membrane integrity was very readily disrupted during cold shock (see Table 3), whereas most of the mitochondria in the midpieces of such cold-shocked cells continued to accumulate carboxyfluorescein (results not illustrated). Holt *et al.* (1988) investigated sperm

mitochondrial integrity after cold shock, using the dye rhodamine 123 as probe; they concluded that the mitochondria remained intact, and they inferred that metabolic activity persisted after cold shock. However, Holt & North (1988) deduced that some permeabilization of plasma membranes occurred after cold shock: vanadate, an impermeant inhibitor of dynein ATPase, caused a reduction in motility in mildly cold-shocked spermatozoa. Our observations are consistent with the findings of both Holt *et al.* (1988) and Holt & North (1988), and indicate that the responses of the plasma membrane and mitochondrial membrane to cold shock are very different. Loss of plasma membrane impermeability to carboxyfluorescein infers similar loss of impermeability to intracellular metabolites. Hence it is highly probable that after cold shock rapid leakage of such intracellular components occurs, with the immediate result that insufficient adenine nucleotides are available to couple oxidative phosphorylation and motility, regardless of the potential capability of the mitochondria for supplying energy.

A further point of interest from our studies concerns the decrease in intact plasma membranes concomitant with or even preceding the increase in discharged acrosomes in sperm populations being prepared for use in in-vitro fertilization (see Table 4). Mortimer *et al.* (1988) showed that, amongst human spermatozoa incubated in in-vitro-fertilization media, very few live acrosome-reacted spermatozoa could be seen at any given stage, although the number of dead acrosome-reacted cells increased steadily with time; the authors deduced that human spermatozoa that had undergone the acrosome reaction were short-lived. However, in their preparations about 50% of the population remained viable, even after some 25 h. Our findings with boar spermatozoa, although too sparse to allow firm conclusions to be drawn, suggest that acrosome-reacted cells in this species may have a very short survival time indeed; certainly, they indicate a surprisingly high and rapid sperm mortality in in-vitro-fertilization media. Methods so far published for simultaneously detecting acrosome reactions and sperm viability have been indirect, involving air-dried smears with inherent possibilities of artefactual damage (e.g. Talbot & Chacon, 1981; Aalseth & Saacke, 1986; Mortimer *et al.*, 1988). Carboxyfluorescein diacetate should permit acrosome-reacted live cells to be distinguished from unreacted live cells, as well as dead reacted from dead unreacted cells, and thus allow direct examination of such changes in living cell suspensions.

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