Capacitation- and fertilization-related alterations in mouse sperm oxygen consumption

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Summary. On the basis of experiments performed some years ago, it has been generally accepted that mammalian sperm metabolic activity, as determined by manometric measurement of O_2 uptake, increases during capacitation. However, when mouse sperm suspensions were incubated for a total of 120 min in glucose-containing medium which promotes both capacitation and fertilization, O_2 consumption, measured with an O_2 electrode at 30 and 120 min, consistently declined with time. This same pattern was also observed when pyruvate was the sole exogenous energy substrate, despite the fact that these cells, although capacitated, cannot fertilize eggs until supplied with glucose. Since mouse spermatozoa require a suitable glycolysable substrate to express full functional ability, these results suggest that a shift in metabolic activity from oxidative phosphorylation to glycolysis normally accompanies the onset of fertilizing ability in this species. It was further demonstrated that this pattern, in the presence of glucose, can be markedly modified by alterations in the medium composition which have been shown to modulate fertilizing ability. All conditions chosen inhibit fertilization while retarding (Ca^{2+} -free), accelerating (hyperosmotic high Na^+) or not affecting (iso-osmotic high K^+) the rate of capacitation. When suspensions were assessed in these media, O_2 consumption increased rather than decreased over the 120 min, despite the continuous presence of glucose. When these same suspensions were assessed at 30 and 120 min in media altered to permit expression of fertilizing ability, the pattern observed was one of declining O_2 consumption. For the condition which accelerated capacitation, the decline relative to the inhibitory medium was seen at both 30 and 120 min, while with the other two, the shift was only observed at 120 min when suspensions would have obtained some degree of competence. The ionic composition of the extracellular environment therefore markedly affects sperm metabolic activity which in turn may modulate sperm functional ability both *in vitro* and *in vivo*.

Introduction

Mammalian spermatozoa are not immediately functional upon release from the male reproductive tract but must undergo further changes, collectively called capacitation, which confer on them potential fertilizing ability. Capacitation normally occurs in the female reproductive tract, although the development of culture systems now makes it possible to manipulate and capacitate spermatozoa from at least some mammalian species *in vitro*. Given that complete capacitation usually requires several hours (the length being species-dependent; Bedford, 1970), the possibility that changes in sperm metabolic activity might accompany capacitation was examined about 20 years ago in several studies (e.g. Hamner & Williams, 1963; Mounib & Chang, 1964; Murdoch & White, 1967). Comparisons were made between freshly ejaculated rabbit spermatozoa and those which had been incubated *in utero* for several hours as at least an approximation to capacitation. Since values for the latter group were the higher, it was concluded that metabolic activity, as measured by

manometric determinations of O_2 uptake, increased with capacitation. The development of the O_2 electrode has provided an easier, yet accurate, method to measure O_2 consumption by cells. Using both this approach and a Cartesian diver to study mouse spermatozoa incubated *in vitro*, Boell (1985) reported that, in contrast to the above, no time-dependent changes in metabolic activity occurred and that various modifications to the incubation medium failed to alter the rate of O_2 consumption. However, the cells were washed before incubation and similar treatment has been shown to accelerate mouse sperm capacitation by removing a surface-associated inhibitory factor (Fraser, 1984); therefore, the washed spermatozoa would not have been uncapacitated at the beginning of assessment.

It is important to know the functional state of spermatozoa at the time of assessment and we have a well-characterized in-vitro fertilization system which provides this information. We have demonstrated, for example, that fertilization but not capacitation in the mouse requires the presence of a glycolysable substrate (Fraser & Quinn, 1981), while other alterations in medium composition can significantly alter capacitation *per se* (e.g. Fraser, 1982, 1983). Using this system, O_2 consumption, as a measure of oxidative phosphorylation, has been determined in epididymal mouse sperm suspensions incubated under conditions previously shown to modify capacitation and fertilizing ability in specific ways. A preliminary report of this study has been published in abstract form (Fraser & Lane, 1986).

Materials and Methods

Media. A modified Tyrode's solution (Fraser, 1983) supplemented with albumin (crystalline BSA at 4 mg/ml; Sigma, Poole, Dorset, U.K.) was used throughout. The composition of complete medium is: 125 mM-NaCl, 25 mM- $NaHCO_3$, 2.68 mM-KCl, 1.80 mM- $CaCl_2$, 0.49 mM- $MgCl_2$, 0.36 mM- NaH_2PO_4 , 5.56 mM-glucose, 100 units penicillin/ml and phenol red. In Series I, modified media tested were: (a) glucose-free + 5.56 mM-fructose and (b) glucose-free + 4 mM-pyruvate. In all other series, glucose was present but other modifications were made; Series II, calcium-free ($CaCl_2$ omitted); Series III, iso-osmotic high potassium (27.68 mM-KCl, 100 mM-NaCl); Series IV, hyperosmotic high sodium (150 mM-NaCl). All media were gassed with 5% CO_2 (see below) before use.

Sperm suspension. These were prepared as for in-vitro fertilization using mature (>8 weeks) outbred TO male mice (Harlan Olac, Bicester, Oxon, U.K.). One cauda epididymidis from each of 2 males was released into 1 ml medium in a 30-mm culture dish (Sterilin, Teddington, Middx, U.K.) overlaid with autoclaved liquid paraffin (Boots, Nottingham, U.K.). Suspensions were incubated for a total of 120 min, with O_2 consumption measurements being made at 30 and 120 min. The gas phase used during incubations was either 5% CO_2 in air or 5% CO_2 -5% O_2 -90% N_2 . No differences were noted between the 2 mixtures and results have therefore been combined.

O_2 consumption measurements. Data were obtained using a Rank oxygen electrode (Rank Brothers, Bottisham, Cambs, U.K.) connected to a Gilson N_2 pen recorder (Anachem, Luton, Beds, U.K.). Water maintained at 37°C was circulated through the electrode's jacketed chamber and constant stirring by a magnetic flea ensured homogeneous distribution of O_2 throughout the contents of the chamber. The zero was set by adding a few grains of sodium dithionite to the chamber containing 700 μ l distilled water.

Except as noted, measurements were made by adding 350 μ l gassed medium to the chamber followed by 350 μ l sperm suspension. The plunger was inserted to expel air and O_2 consumption was monitored for approximately 5 min; there was no obvious alteration in pH, judging by the phenol red coloration. When 2 suspensions were being compared at both 30 and 120 min, these measurements had to be made sequentially since only 1 electrode was available. To avoid any discrepancies introduced by the inevitable delay in making the second measurement for a single time point, the order was reversed in alternate replicates. At the end of each measurement, an aliquant of the sperm sample in the chamber was removed, fixed in buffered formalin and counted in a haemocytometer; this figure was used in calculating O_2 consumption. The mean sperm concentration was 1.4×10^7 cells/ml (range 0.8 - 2.2×10^7 /ml). Data are presented as nmol O_2 consumed/min/ 10^8 cells.

In an earlier study it was shown that the O_2 consumption measured in mouse sperm suspensions by this method is associated with oxidative phosphorylation activity; the introduction of oligomycin caused a significant decrease in O_2 consumption, indicating that oxidation is tightly coupled to phosphorylation in these sperm cells (Fraser & Quinn, 1981). This was not repeated for the present study since the methods are essentially the same.

Statistical analysis. Data were analysed using the Friedman two-way analysis of variance (Siegel, 1956) to determine whether O_2 consumption altered with time in a consistent manner. This is a ranking test which uses matched samples.

Results

Series I: the effect of different exogenous substrates

Initial measurements were made at both 30 and 120 min on suspensions incubated in the standard Tyrode's medium containing glucose (+G) as substrate; this medium supports complete capacitation and fertilization (Fraser & Quinn, 1981). Sixteen replicates were carried out ($N = 16$). In the next experiments, 2 males were used for each replicate with 1 epididymis from each being released into 1 ml standard Tyrode's and the remaining epididymides being released into 1 ml modified glucose-free medium containing either 4 mM-pyruvate (+P; $N = 3$) or 5.56 mM-fructose (+F; $N = 3$). Under these conditions mouse spermatozoa can complete capacitation but cannot fertilize eggs due to inhibition of the acrosome reaction and hyperactivated motility (Fraser & Quinn, 1981).

In glucose-containing medium a consistent and significant ($P < 0.05$) reduction in the rate of O_2 consumption was observed at 120 min (Fig. 1a). When glucose and pyruvate were compared, a similar decline was noted in both groups (Fig. 1b), while O_2 consumption in the presence of fructose was very low and did not change appreciably over 120 min despite a significant decline in the glucose-incubated counterparts (Fig. 1c). Spermatozoa in +F medium are very sluggish and non-fertilizing until supplied with glucose. These results suggest that during capacitation there is a switch from oxidative to glycolytic metabolism which could explain the obligatory requirement for a suitable glycolytic substrate to support fertilization. As can be seen in Fig. 1 which is a composite of three different experiments, absolute values for O_2 consumption varied considerably among groups of replicates. However, all within-replicate observations were made on suspensions from the same mice and it is these within-replicate values that have been used to determine whether a consistent or random pattern was obtained. In all replicates using +G and +P, the O_2 consumption values at 120 min were consistently lower than the comparable readings obtained with the same suspensions at 30 min.

Series II: conditions which promote partial capacitation

Mouse spermatozoa incubated for 120 min in Ca^{2+} -free (–Ca) medium are non-fertilizing. Upon introduction of Ca^{2+} (1.80 mM) they are shown to be partially capacitated: a few spermatozoa are able to fertilize rapidly and the suspensions as a whole require only a maximum of 60 min rather than 120 min to acquire full functional competence (Fraser, 1987). In the first experiments of

Fig. 1. Oxygen consumption by epididymal mouse sperm suspensions after 30 and 120 min incubation in modified Tyrode's medium containing the following exogenous substrates: (a) glucose (+G); (b) glucose (+G) or pyruvate (+P); (c) glucose (+G) or fructose (+F). Values are mean $\pm$ s.e.m. * $P < 0.05$ compared with 30 min suspensions in the same medium.

this series, a single 2-ml sperm suspension was prepared using $-Ca$ medium and 2 males; 920 μ l of this were transferred to a separate dish and 80 μ l of a concentrated Ca^{2+} stock solution (22.5 mM- $CaCl_2$) were added to give a final concentration of 1.80 mM Ca^{2+} (+Ca). O_2 consumption was measured at 30 and 120 min in the $-Ca$ and +Ca suspensions ($N = 4$). In the second set of experiments, a 2-ml sperm suspension was prepared in $-Ca$ medium and incubated. At assessment, aliquants of the suspension were added to $-Ca$ medium or $\times 2 Ca$ medium, the latter to produce a final Ca^{2+} concentration of 1.80 mM ($N = 3$).

As observed in Series I, a consistent and significant decline in O_2 consumption occurred in suspensions incubated in +Ca medium; in contrast, a consistent increase was observed in the $-Ca$ suspensions (Fig. 2a). When suspensions incubated in $-Ca$ medium were assessed both in the absence and presence of Ca^{2+} , the patterns of O_2 consumption at 120 min were quite different. As in the first experiments of this series, a significant increase from 30 to 120 min was noted in $-Ca$ medium. However, while the introduction of Ca^{2+} at 30 min ($-Ca \rightarrow +Ca$) caused no obvious consistent alteration in O_2 consumption compared with the 30 min $-Ca$ suspensions, the same procedure at 120 min, which promotes the onset of fertilizing ability (Fraser, 1982, 1987), caused a consistent and significant decline from the 120 min $-Ca$ values and produced a pattern similar to that observed in +Ca medium (Fig. 2b). Thus alterations in ionic composition which modify fertilizing potential can markedly affect sperm metabolic activity.

Fig. 2. Oxygen consumption by epididymal mouse sperm suspensions: (a) after 30 and 120 min incubation in modified Tyrode's medium with (+Ca) or without ($-Ca$) calcium; (b) after 30 and 120 min incubation in $-Ca$ medium and assessed in both $-Ca$ and +Ca media. Values are mean $\pm$ s.e.m. * $P < 0.05$ compared with 30 min suspensions in the same medium; † $P < 0.05$ compared with 120 min $-Ca$ suspensions.

Series III: conditions which promote complete capacitation but inhibit fertilization

Mouse spermatozoa incubated for 120 min in iso-osmotic high K^+ (27.68 mM) medium are non-fertilizing but essentially completely capacitated since reduction in the K^+ concentration to < 10 mM promotes immediate fertilization (Fraser, 1983). In the first set of experiments, O_2 consumption was compared in suspensions incubated in standard Tyrode's (2.68 mM- K^+ ; control K) and high K (27.68 mM) media. In the second set, single sperm suspensions were prepared and incubated in high K medium, then assessed in high K or reduced K medium. Although ideally the suspensions should have been diluted 10-fold with $-K$ medium to reduce the K^+ to ~ 2.68 mM, this would have lowered the sperm concentration below that required to obtain a reading with the electrode. Consequently, one part of the high K sperm suspension was added to 2 parts of high K or $-K$ medium in the chamber, the latter reducing the K^+ concentration to ~ 9.2 mM which does promote fertilization (Fraser, 1983).

Again there was a consistent decline in O_2 consumption over 120 min in suspensions incubated and assessed in standard Tyrode's with low K. In high K the reverse was observed, with an increase from 30 to 120 min (Fig. 3a). When high K suspensions were assessed in high and reduced K media, however, the patterns of O_2 consumption differed markedly from each other. As in the first part of this series, a significant increase was observed over 120 min (Fig. 3b). In contrast, while reduction in K (high $\rightarrow$ reduced K) at 30 min caused no consistent alteration in O_2 consumption compared with the 30 min high K suspensions, reduction at 120 min, which promotes fertilizing ability, produced a consistent and significant decline from the 120 min high K values. Thus, as in Series II, conditions that modify fertilizing potential also modify metabolic activity.

Fig. 3. Oxygen consumption by epididymal mouse sperm suspensions: (a) after 30 and 120 min incubation in iso-osmotic Tyrode's medium containing potassium at control (2.68 mM-K^+) or high (27.68 mM-K^+) concentrations; (b) after 30 and 120 min incubation in high K medium and assessed in high K and reduced K. Values are mean $\pm$ s.e.m. * $P < 0.05$ compared with 30 min suspensions in the same medium; † $P < 0.05$ compared with 120 min high K suspensions.

Series IV: conditions which accelerate capacitation but inhibit fertilization

Mouse spermatozoa incubated in hyperosmotic high Na^+ (25 mM extra NaCl) medium are non-fertilizing but undergo accelerated capacitation; reduction in the Na^+ concentration after 30 min results in significantly higher rates of fertilization than counterparts incubated in standard Tyrode's medium (Fraser, 1983). Furthermore, reduction after 120 min still promotes high levels of fertilization. In Series IV experiments, single suspensions were prepared and incubated in hyperosmotic medium (high Na), then assessed in high Na or control Na (125 mM-NaCl). For the latter, medium with 100 mM-NaCl was prepared so that upon a 1:1 dilution of the high Na medium the final NaCl concentration would be 125 mM .

As seen in Series II and III, O_2 consumption by sperm suspensions incubated and assessed in conditions which inhibit fertilizing ability increased with time (Fig. 4). By reducing Na which permits expression of fertilizing ability, there was a consistent shift in the O_2 consumption pattern to one of decreasing rather than increasing values. This particular manipulation of environmental conditions permits maximal fertilization at both 30 and 120 min (Fraser, 1983) and, as shown in Fig. 4, there was a significant decrease in O_2 consumption at both times in response to reduction in Na^+ . This pattern was not seen in Series II and III, in which functional changes and metabolic changes occurred only in suspensions incubated for 120 min.

Fig. 4. Oxygen consumption by epididymal mouse sperm suspensions incubated for 30 and 120 min in hyperosmotic high Na (150 mM-NaCl) medium and assessed in high Na and control Na (125 mM-NaCl) media. Values are mean $\pm$ s.e.m. * P < 0.05 compared with 30 min high Na suspensions; † P < 0.05 compared with 120 min high Na suspensions.

Discussion

This study was designed to evaluate O_2 consumption by epididymal mouse spermatozoa incubated in conditions which have been shown to promote or inhibit capacitation and/or fertilization. The results indicate that O_2 consumption declines during capacitation *in vitro* in standard Tyrode's medium (glucose-containing). Given that expression of fertilizing ability by capacitated mouse spermatozoa requires a glycolysable substrate such as glucose (Fraser & Quinn, 1981), this result suggests that there is a shift from oxidative to glycolytic metabolism. A similar pattern of O_2 consumption was seen in suspensions incubated in the presence of pyruvate. Functionally, these cells are non-fertilizing, unable to undergo the acrosome reaction and to express hyperactivated motility until supplied with glucose (Fraser & Quinn, 1981). Despite being a glycolysable substrate, fructose fails to substitute for glucose and O_2 consumption is very low (Fig. 1c); the reasons for the inadequacy of fructose may include slower uptake and phosphorylation relative to glucose (Hoppe, 1976). Boell & Burkus (1984) also noted a low rate of O_2 consumption in the presence of fructose, although they reported these cells to be highly motile. We have never observed this (present study; Fraser & Quinn, 1981) and cannot explain the discrepancy unless it relates to the washing procedure. Certainly mouse spermatozoa cannot fertilize eggs in the presence of fructose alone (Hoppe, 1976; Fraser & Quinn, 1981). The present results are consistent with the obligatory requirement mouse spermatozoa show for an appropriate glycolysable substrate at the end of capacitation: the metabolic shift occurs in the presence of pyruvate and fructose but the glycolytic pathway cannot be utilized until a suitable substrate is available. The fact that this decline in O_2 consumption is observed both in the presence and absence of glucose provides evidence that this phenomenon is not an expression of the Crabtree effect, i.e. glucose-induced inhibition of respiration, as has been suggested for guinea-pig spermatozoa (Rogers *et al.*, 1979).

In experiments similar to the present ones, Boell (1985) reported that the rate of O_2 consumption by washed spermatozoa was constant during capacitation when cells were continuously monitored for 2–3 h. However, the washing procedure removes a surface-associated inhibitory factor and consequently washed spermatozoa are immediately fertile and retain full functional competence for at least 2 h (Fraser, 1984). Therefore, the observations of Boell (1985) are not directly

relevant to metabolic activity during capacitation. Once the alteration in metabolic activity which accompanies capacitation has been effected, it is quite probable that the rate remains constant for some time.

It is pertinent to address briefly the argument presented by Boell (1985) that the requirement for a glycolysable substrate is essentially artefactual, with the limited O_2 dissolved in the culture medium (due to the use of 5% O_2 –5% CO_2 –90% N_2) forcing the spermatozoa to turn to the anaerobic glycolytic pathway. During the preincubation phase the consumed O_2 can be at least in part replenished by diffusion of O_2 from the equilibrated liquid paraffin overlay into the medium. More importantly, however, the functional assessment demonstrating this specific requirement involves a 10-fold dilution of sperm suspension into fresh medium and thus O_2 depletion should not be a factor. Furthermore, although fertilization can only be assessed readily after about 1 h, the glucose-evoked responses in the spermatozoa themselves, i.e. induction of both the acrosome reaction and hyperactivated motility, occur within 5 min and in direct response to glucose; neither pyruvate nor lactate will substitute for glucose (Fraser & Quinn, 1981). In the present study some replicates were carried out in the presence of 5% CO_2 in air (21% O_2) and others in the O_2 – CO_2 – N_2 mixture. The pattern of O_2 consumption was the same in both gas phases.

All experimental assessments in Series II–IV were made in media which contained glucose but were modified in other ways so as to compromise fertilizing ability. Two of the modifications also affected the rate of capacitation, with $-Ca$ medium retarding it and hyperosmotic high Na medium accelerating it. All 3 modified media, including iso-osmotic high K medium which does not interfere with capacitation *per se*, inhibit fertilizing ability (Fraser, 1982, 1983, 1987). O_2 consumption measured under these same conditions consistently increased during 120 min incubation, despite the presence of glucose. Therefore alterations in medium composition which inhibit functional ability also prevent the downward shift in oxidative metabolism. However, changes in composition which allow expression of fertilizing potential promote the metabolic shift. Not only that, they do so at a time consistent with the onset of functional competence, e.g. at both 30 and 120 min for conditions which accelerate capacitation but only at 120 min for those which retard or have no effect on capacitation rate. While it is true that the sperm concentrations used for O_2 electrode monitoring were higher than those routinely used for fertilization studies, successful fertilization has been achieved with similarly high concentrations (Fraser & Drury, 1975). Therefore, there is no reason to believe that the samples might be intrinsically different.

Deutch *et al.* (1985) reported that O_2 consumption by human spermatozoa increased significantly when their concentration fell below 1.5 – 2.5×10^7 cells/ml. Thus, comparisons between suspensions of very different cell densities may prove to be invalid. In the present study all suspensions within a replicate were prepared from the same males, often from the same suspension, and the sperm concentration in all replicates was quite similar with a mean of 1.4×10^7 cells/ml. Therefore, it was felt that these comparisons were indeed valid.

Data obtained from the assessment of mammalian spermatozoa after incubation *in utero* (see 'Introduction') or *in vitro* in the presence of female reproductive tract fluids (Foley & Williams, 1967; Black *et al.*, 1968; Iritani *et al.*, 1969; Spilman *et al.*, 1970; Stone *et al.*, 1973) indicate an increase in oxidative metabolism with time rather than a decrease as found in the present study. However, it may be that these results are not as discordant as they initially appear. Analyses of female reproductive tract fluids have revealed that their ionic composition often differs considerably from that of blood, e.g. elevated K^+ at ~ 20 – 30 mM compared with ~ 5 mM for blood (Borland *et al.*, 1977, 1980; Johnson & Overstreet, 1982) and at least some of the earlier studies were carried out in the presence of such fluids. Despite the fact that capacitation *in vivo* occurs in these fluids, Fraser (1983) has demonstrated that elevated K^+ inhibits fertilization in the mouse. Since a reduction in K^+ leads to immediate egg penetration if spermatozoa have been incubated long enough for complete capacitation, she has suggested that localized alterations in K^+ concentration within the oviduct may represent a mechanism to modulate fertilizing potential *in vivo*. In the present study, these same conditions were shown to affect significantly the pattern of O_2

consumption and elevated K^+ concentrations, similar to those found in female tract fluids, promoted an increase in consumption with time, i.e. a result consistent with the other studies cited here. However, these mouse sperm suspensions proved to be non-fertilizing and the same may have been true for spermatozoa of those species examined in earlier studies. Certainly there is evidence that extracellular K^+ -related effects can be mediated via glycolysis (discussed by Fraser, 1983), an important consideration when glycolysis plays a central role in sperm function.

The above discussion is not intended to suggest that a decline in oxidative metabolism necessarily occurs in all mammalian species. Mouse spermatozoa are unusual in relying totally on a glycolysable substrate, but most spermatozoa require a glycolysable substrate, in addition to pyruvate and lactate, at some point in capacitation to achieve optimal function, e.g. rat (Tsunoda & Chang, 1975; Niwa & Iritani, 1978), hamster (Bavister & Yanagimachi, 1977; Dravland & Meizel, 1981), and human (Hoshi *et al.*, 1982). The exception is the guinea-pig for which inclusion of glucose retards the acrosome reaction, compared to medium containing only pyruvate and lactate (Rogers & Yanagimachi, 1975). A re-evaluation of metabolic activity, using defined culture media, in spermatozoa from species other than the mouse is required to resolve the question.

In conclusion, O_2 consumption declines with time in mouse suspensions incubated in a glucose-containing medium which promotes both capacitation and fertilization. If suspensions are incubated in conditions which, despite the presence of glucose, inhibit fertilization, the pattern observed is one of an increase with time but this can be reversed by altering the medium composition to one that permits expression of fertilizing ability. Mouse spermatozoa require glycolysable substrate to fertilize eggs and this pattern of O_2 consumption suggests that a shift from oxidative phosphorylation to glycolysis may accompany the onset of functional ability. The extracellular ionic composition, by affecting intracellular metabolic pathways, may play an important role in modulating sperm function.

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