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Capacitation status of activated bovine sperm cultured in media containing methyl-β-cyclodextrin affects the acrosome reaction and fertility

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Summary

Mammalian sperm undergo a series of biochemical transformations in the female reproductive tract that are collectively known as capacitation. One of the key processes involved in capacitation is the activation of sperm motility. Here, we investigated the capacitation and fertility status of activated sperm which had been cultured in media containing methyl-β-cyclodextrin (MBCD). In order to do this, single activated sperm were caught using a micropipette and stained with chlortetracycline (CTC). Firstly, we investigated the effects of preincubation upon motility, capacitation of activated sperm and fertility. Culture in preincubation media supplemented with MBCD increased the rates of activation and fertilization compared with sperm cultured by control methods ($p < 0.05$). Following capture, individual activated sperm mostly exhibited a pattern characteristic of capacitation.

Secondly we examined the effects of culturing sperm in media with or without glucose (G) and pyruvate acid (P) upon activated motility, the capacitation of activated sperm and fertility. Supplementation of culture media with G and P resulted in higher proportions of activated sperm and increased fertilization rates compared to culture without G and P ($p < 0.05$). Most of the sperm activated by culture in G and P exhibited patterns characteristic of capacitation. Without G and P, individual activated sperm mostly exhibited patterns characteristic of the acrosome reaction ($p < 0.05$). In conclusion, activated sperm exhibited patterns characteristic of capacitation. In addition, sperm activated in media containing an energy source (glucose and pyruvate acid) appeared to exhibit acrosome reactiveness and fertility.

Keywords: Acrosome reaction, Activated motility, Capacitation, Sperm

Introduction

Mammalian sperm undergo a series of biochemical transformations in the female reproductive tract that are collectively known as capacitation (Austin, 1951; Chang, 1951). Capacitation has previously been correlated with changes in sperm plasma membrane fluidity, intracellular ion concentrations, metabolism and motility (Yanagimachi, 1994). Following capacitation, the sperm binds to the egg zona pellucida and undergoes the acrosome reaction, which allows the sperm to penetrate the egg. The signalling pathways involved in sperm capacitation have been characterised and current evidence indicates that cholesterol efflux (Davis *et al*., 1981), pH (Breitbart *et al*., 2002), Ca^{2+} (Parrish *et al*., 1999), actin polymerization (Etkovitz *et al*., 2008), cAMP (Lamirande *et al*., 2008) and tyrosine phosphorylation (Moseley *et al*., 2005; Hung *et al*., 2008) are important regulatory components.

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The process of capacitation includes the activation of sperm motility and hyperactivation, which usually occur at some point during the fertilization process *in vitro* (Ho *et al*., 2001). Following the activation of motility, the sperm flagella beat in almost symmetrical fashion, propelling the sperm in nearly linear trajectories (Ho *et al*., 2002; Kumar *et al*., 2006; Suarez, 2008). In a hyperactivated state, the spermatozoa flagella beat vigorously, producing high amplitude, asymmetrical beat pattern. These motility changes assist the sperm in penetrating the oocyte zona pellucida and are essential for fertilization. Numerous earlier studies have reported the mechanism of hyperactivation, which is generally considered part of the capacitation process since sperm have been observed to hyperactivate while undergoing capacitation *in vitro* (Yanagimachi, 1994). Hyperactivation and capacitation have also been recognized as multifaceted phenomena involving associated increases in intracellular levels of calcium, ATP and pH (Suarez, 2008; Etkovitz, *et al*., 2008). The increased level of tyrosine phosphorylation in sperm proteins is mainly distributed within the flagellum (Naz *et al*., 2004). The activity of pyruvate dehydrogenase A2, which induces tyrosine phosphorylation, has been positively correlated with hyperactivation (Kumar *et al*., 2006). Capacitation phenomena may therefore be related to hyperactivation. However, there have been cautionary reports (Ho *et al*., 2001) advising that although activated motility has been related to capacitation, the precise capacitation status of activated sperm is still poorly understood.

In earlier reports, sperm were evaluated collectively as discrete groups, thus the capacitation state of each individual sperm could not be evaluated in detail (Marquez *et al*., 2004; Kumar *et al*., 2006; Etkovitz *et al*., 2008). Therefore, in the present study, we caught individual sperm by micropipette and investigated whether the capacitation state of activated sperm are related to the acrosome and fertility. Furthermore, when sperm activity was reduced by culturing sperm in media without energy (glucose and pyruvate), we demonstrated a defined change of capacitation pattern in activated sperm, in turn causing effect upon fertility, the acrosome reaction and the expression level of progesterone receptor (PRs).

Materials and methods

Oocyte collection and *in vitro* maturation

Bovine ovaries were obtained from an abattoir, placed in saline containing a 0.1% solution of antibiotic/antimycotic (Gibco Laboratories, AB) and transported to the laboratory within 1–3 h. Oocyte collection and *in vitro* maturation of oocytes were carried out as previously described (Saeki *et al*., 1990), with some modifications. Bovine oocytes were obtained from abattoir-derived ovaries. Oocytes with intact cumulus cells and evenly granulated cytoplasm were selected, washed and cultured in modified TCM199 (m-TCM199) for 22–25 h at 39°C under 5% CO_2 in air with high humidity. m-TCM199 consisted of HEPES-buffered medium 199 (Gibco Laboratories) supplemented with 0.1% (w/v) polyvinyl alcohol (PVA; Sigma Chemical Co.), 0.5 mM sodium pyruvate (Nacalai Tesque Inc.), 1% AB, 0.02 AU/ml FSH (Antrin, Denka) and 1 μg/ml estradiol-17β (Sigma Chemical Co.).

Washing and pre-incubation of sperm

Sperm washing and pre-incubation (PI) was carried out described below. Frozen–thawed sperm were washed with a discontinuous percoll solution. The washed sperm (2.0×10^8 cells/ml) were cultured in Brackett and Oliphant medium (BO) (Brackett *et al*., 1975) supplemented with 0.05% (w/v) PVA, 1% AB (BO · PVA) and methyl-β-cyclodextrin (MBCD) for 0, 2 and 4 h at 39°C under 5% CO_2 in air with a high humidity. Sperm were pre-incubated and then used for insemination.

In vitro fertilization of bovine oocytes

Matured oocytes were inseminated with cultured sperm (2×10^6 cells/ml) in BO · PVA. Oocytes were incubated with sperm for 18–20 h after insemination. All incubations were performed at 39°C under 5% CO_2 in air at high humidity.

Assessment of fertilization

In order to determine the pronuclear stage during fertilization, subsets of oocytes after IVF were fixed on a slide with 3:1 acetic acid:ethanol and stained with 1% orcein. Oocytes considered to be fertilized were classified into three groups: '2PN' (female pronucleus, male pronucleus and sperm tail; normal fertilization); 'polyspermy' (two or more sperm tails in the cytoplasm with condensed heads, or two or more decondensed heads in the cytoplasm); and 'asynchrony' with a condensed sperm head (female pronucleus and a condensed sperm head). The percentage of each category of fertilization was calculated from the total number of oocytes inseminated.

Analysis of sperm motility

Sperm motility was categorized according to motility patterns as previously reported (Ho *et al*., 2002; Marquez, *et al*., 2004). Treated sperm were examined in PVP solution in order to reduce sperm motility

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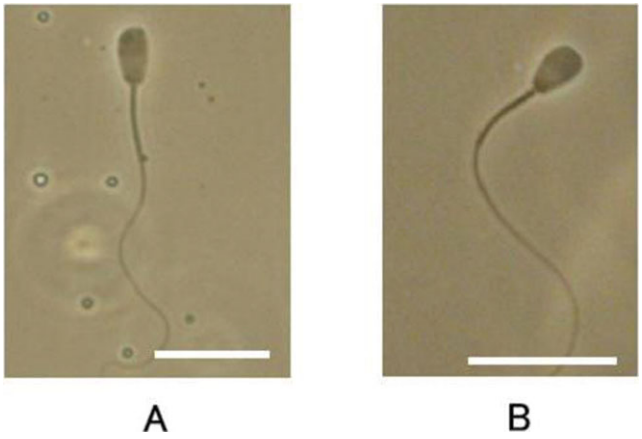

Figure 1 Sperm motility patterns of bovine spermatozoa cultured in BO · PVA supplemented with MBCD for 4 h. A) normal motility B) activated motility. Bars = 20 μm.

on an interference contrast microscope (Olympus, BH-2). Normal motility and activated motility were characterized by symmetrical beating of the flagellum (norma; Fig. 1*A*) resulting in a linear swimming trajectory (activated; Fig. 1*B*).

Activated motility sperm: chlortetracycline fluorescence assay

Normal and activated sperm were collected and examined at 0, 2 and 4 h after PI. Single sperm were collected by injection pipette immediately prior to CTC staining. Isolated sperm were injected in BO · PVA on a glass slide (Matsunami). The chlortetracycline (CTC) stain was used to evaluate capacitation status as reported by Fraser *et al.* (1995). Precipitated sperm were mixed thoroughly with 750 μM CTC solution (Sigma Chemical Co.), 5 mM cysteine (Sigma Chemical Co.), 130 mM NaCl (Kanto Chemical), 20 mM Tris (Sigma Chemical Co., pH 7.8), fixed with 12.5% (w/v) paraformaldehyde in 0.5 M Tris–HCl (pH 7.4) and finally placed on a glass slide and covered with a coverslip. Those sperm were observed for CTC staining using a 400 to 440 nm filter and a DM455 dichroic mirror (U-MWBV2, Olympus) under a fluorescence microscope (BX-51, Olympus). CTC staining patterns were classified into three types according to the method described by Fraser *et al.* (1995): F (uncapacitated)-pattern, bright fluorescence detected over the whole region of the sperm head; B (capacitated)-pattern, fluorescence detected on the sperm head but not over the post-acrosomal region; and AR (acrosome-reacted) pattern, weak fluorescence observed over the sperm head with a bright band sometimes present in the equatorial region (Fig. 2).

CTC staining of sperm in contact with cumulus cells

Sperm in contact with cumulus cells were stained by the CTC method described above. At 3–5 h after insemination, embryos were removed from BO · PVA, washed five times in BO · PVA and stripped of cumulus cells by vortexing. After vortexing, embryos were gathered and sperm in direct contact with the cumulus cells were collected by centrifugation of the BO · PVA and vortexing. Sperm collected were also stained by CTC staining.

Immunofluorescent studies of the progesterone receptor

Localization studies of the progesterone receptor in bovine sperm was carried out by indirect immunofluorescence using C-19 (sc-538: Santa Cruz Biotechnology Inc.) and rabbit anti-mouse IgG conjugated with FITC (RAM-FITC; Zymed). PR (C-19) is an affinity purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of the PR of human origin.

Following PI, 500 ml of sperm suspension was mixed with 500 ml 4% paraformaldehyde in PBS for 10 min. The sperm suspension was then centrifuged at 600 *g* for 6 min. After supernatant removal, fixed sperm cells were washed with 1 ml PBS and centrifuged at 600 *g* for 3 min to remove remaining aldehyde residues. The washing procedure was repeated twice. Following removal of the supernatant, the sperm pellet was incubated with primary antibody C-19 (diluted by 1:10) in PBS supplemented with 3 mg/ml BSA for 1 h at room temperature. The sperm suspension was then centrifuged at 600 *g* for 3 min. After removal of the supernatant, the sperm were washed twice as described above to remove unbound primary antibody. The sperm pellet was then incubated with RAM-FITC (diluted by 1:100) in PBS supplemented with 3 mg/ml BSA for 1 h at room temperature. The sperm suspension was then centrifuged at 300 *g* for 3 min. After removal of the supernatant, the sperm were washed twice as described above to remove unbound secondary antibody. Samples were then mounted and kept for up to one day at 4°C in the dark and examined by fluorescence microscopy (Olympus). Sperm were examined by fluorescence microscopy to identify positive progesterone receptor localization (Fig. 3).

Experimental design

Experiment 1

To investigate the effects of culturing sperm in BO · PVA supplemented with MBCD upon activated motility, fertility and the capitation/acrosome reaction (AR) of individual sperm with normal and activated motility, we incubated sperm in BO · PVA (control)

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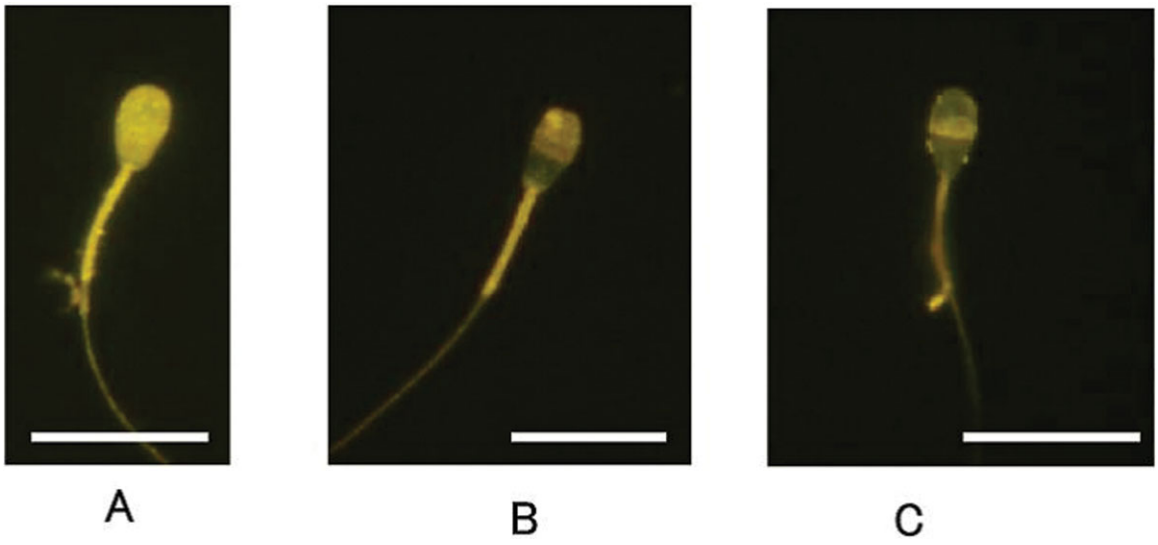

Figure 2 F, B and AR pattern. A) F (uncapacitated)-pattern, bright fluorescence detected over the whole region of the sperm head, B) B (capacitated)-pattern, fluorescence detected on the sperm head but not over the post-acrosomal region, C) AR (acrosome-reacted)-pattern, weak fluorescence observed over the sperm head with a bright band sometimes present in the equatorial region. Bars = 20 μm.

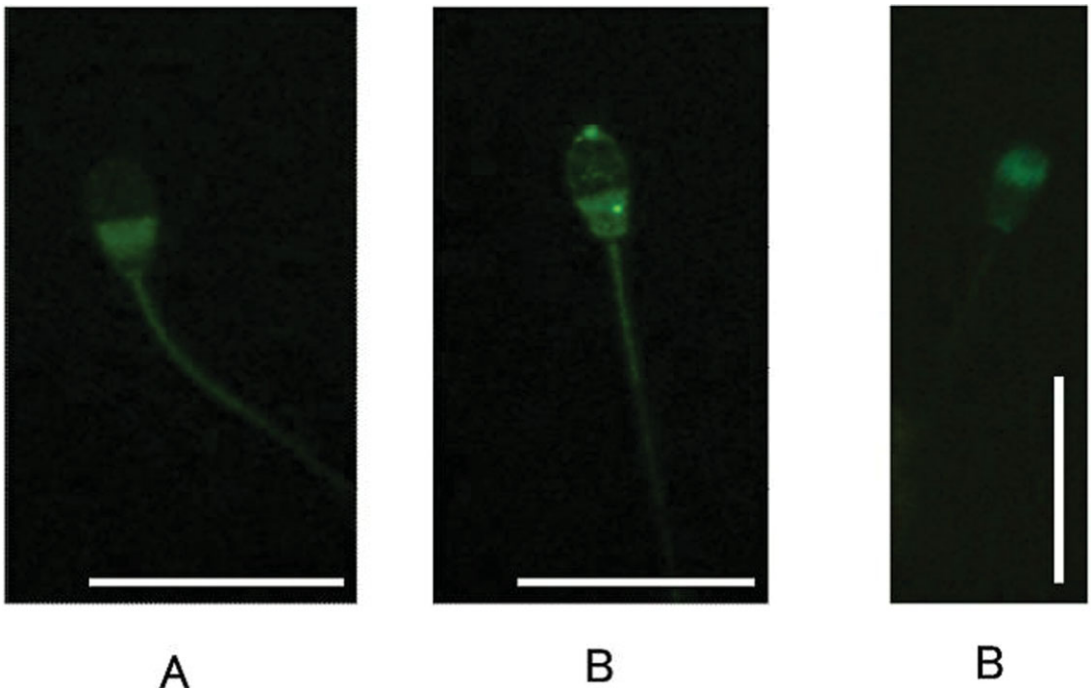

Figure 3 Fluorescence pattern of sperm stained for progesterone receptor localization. A) Negative control (isotype control). B) Positive progesterone receptor localization. Bars = 20 μm.

and BO·PVA supplemented with 1.5 mM MBCD (BO·MBCD). Following sperm culture, individual sperm exhibiting normal and activated motility from the control and BO·MBCD groups were caught by micropipette and stained by CTC in order to determine the rate of activation and fertilization.

Experiment 2

To investigate how the presence of glucose and pyruvate acid in BO·MBCD caused effect upon activated motility, fertility, capitation and the acrosome reaction of activated sperm, we incubated sperm in BO·MBCD with glucose (G) and pyruvate acid (P)

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(PI^+), without G and P(PI^-), or BO · MBCD without G and P initially but supplemented with glucose and pyruvate acid after 2h PI (supplementation group). Following sperm culture, individual activated sperm from all groups were caught by micropipette and stained by CTC to determine the rate of activation and fertilization.

Experiment 3

In order to investigate the AR in sperm from the PI^+ and supplementation groups at 4 h that were in contact with cumulus cells and PR localization of the sperm cultured in those media, we cultured sperm and inseminated in BO · PVA, respectively.

Statistical analyses

All experiments were replicated three to eight times. In Experiment 1 and 2, the rates of fertilization were evaluated for significance using the chi-squared test. In Experiment 1 and 2, the proportion of sperm exhibiting B and AR patterns and group and sperm motility (normal and activated motility) among each treatment were evaluated for significance using one-way factorial ANOVA with Fisher's PLSD post-hoc test. In Experiment 3, the proportion of immunopositive staining for the PR in sperm cells and the rate of sperm exhibiting hyperactivation, were evaluated for significance using the chi-squared test. In Experiment 3, the proportion of sperm exhibiting B and AR patterns between each treatment were evaluated for significance using the chi-squared test. In all analyses, $p < 0.05$ was considered significant.

Results

Experiment 1

After 2 h and 4 h of PI, the proportions of individual activated sperm exhibiting patterns characteristic of capacitation were higher than that of sperm with normal motility in BO · MBCD and the control group (Fig. 4, $p < 0.05$, ANOVA). Conversely, the proportion of individual sperm with normal motility exhibiting capacitation patterns did not significantly change in BO · MBCD and control ($p > 0.05$, ANOVA).

A time-dependent increase in the proportion of sperm displaying activated motility was observed in the BO · MBCD group (Fig. 5, $p < 0.05$, ANOVA). However, the proportion of activated sperm in the control group did not increase significantly with the length of time cultured ($p > 0.05$, ANOVA).

Normal fertilization rates in the BO + MBCD group (70.0% and 55.0%) were higher than that in the control group (26.2% and 18.1%) after 2 h and 4 h PI respectively (Table 1, $p < 0.05$, chi-squared test).

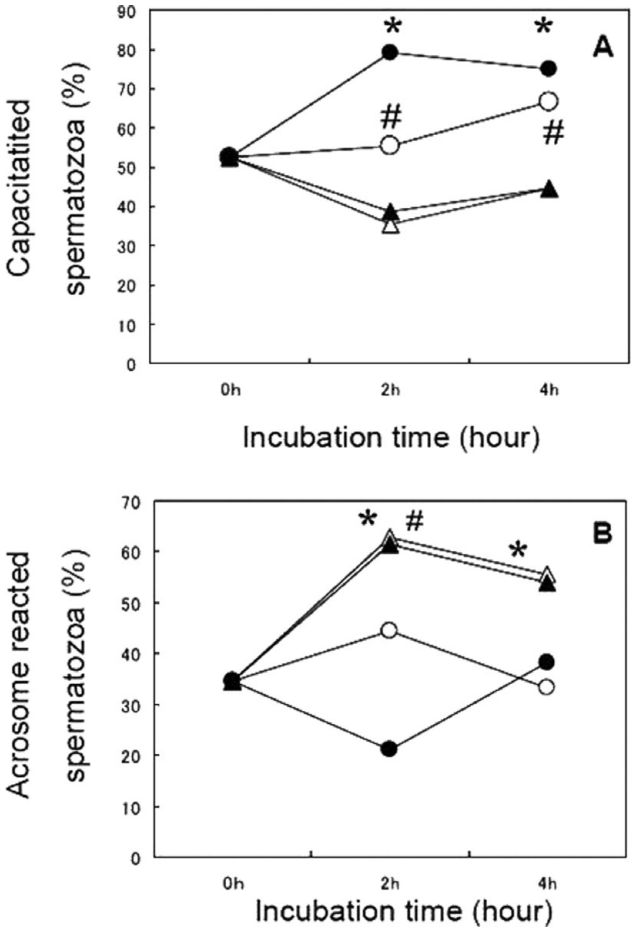

Figure 4 A and B. Capacitation and acrosome reaction of activated sperm in pre-incubation media supplemented with MBCD (●, activated sperm in BO · PVA supplemented with MBCD; ▲, normal motility sperm in BO · PVA supplemented with MBCD; ○, activated sperm in BO · PVA; △, normal motility sperm in BO · PVA) and the proportion of capacitated and acrosome reacted bovine sperm in vitro. * indicates significant differences between activated and normal motility sperm in BO · MBCD ($p < 0.05$), # indicates significant differences between activated and normal motility sperm in controls ($p < 0.05$) as determined by one-way ANOVA. Experiments were repeated 4–9 times.

Experiment 2

After 2 h and 4 h PI, the incidence of capacitation in activated sperm were 71.4% and 60.9% in PI^+, 42.9% and 36.0% in PI^- and 42.9% and 58.8% in the supplementation group (Fig. 6). After 2h PI, the incidence of capacitation in activated sperm in PI^+ was higher than that of the other treatment groups ($p < 0.05$, ANOVA). After 4 h PI, the incidence of capacitation in activated sperm

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Table 1 Effect of preincubation of sperm in media containing MBCD on fertility[1]

Media for sperm incubation	Time (h) of sperm incubation					
	0		2		4	
	Normal (%)[2]	Total (%)[3]	Normal (%)[2]	Total (%)[3]	Normal (%)[2]	Total (%)[3]
BO (control)	41/127 (32.3)	42/127 (33.1)	27/103 (26.2)[a]	28/103 (927.2)[a]	20/111 (18.1)[a]	21/111 (18.9)[a]
MBCD	41/127 (32.3)	42/127 (33.1)	100/145 (70.0)[b]	110/145 (86.6)[b]	87/146 (55.0)[b]	92/146 (63.0)[b]

Statistical significance compared to control: *a* vs. *b*, $p < 0.001$ (chi-squared test).
[1]Repeat no. 5.
[2]No. of 2PN formation in oocytes /total oocytes.
[3]No. of 2PN, more than 3PN or sperm head in embryos/ total embryos.

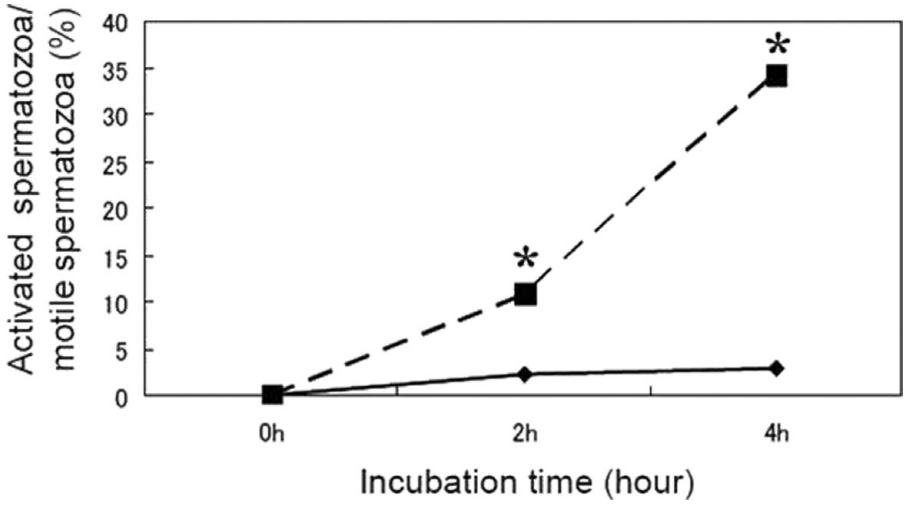

Figure 5 Effect of sperm cultured in BO · PVA supplemented with or without MBCD (■: BO · MBCD, ◆: control for the motility of activation. * indicates significant differences between BO · MBCD and controls by one-way ANOVA. Experiments were repeated 6 times.

in PI^+ and the supplementation group were higher than that of the PI^- groups ($p < 0.05$, ANOVA).

The incidence of normal motility in all treated groups was 31.0–41.6% and 21.0–32.1% at 2 h and 4 h, respectively ($p > 0.05$, ANOVA). The rate of activated motility in the PI^+ group was higher than that in the other treatment groups after 2 h incubation (Fig. 7, $p < 0.05$, ANOVA). After 4 h PI, the rate of activated motility in the PI^+ and supplementation groups were significantly higher than that in the PI^- group ($p < 0.05$, ANOVA).

After 2 h and 4 h PI, the normal fertilization rates were 70.2% and 56.5% in PI^+, 55.4% and 16.4% in PI^- and 55.4% and 32.4% in the supplementation group, respectively (Table 2). The rate of fertilization in PI^+ was significantly higher than that of the other treatment groups ($p < 0.05$, chi-squared test) at 2h PI. After 4 h PI, the fertilization rate of PI^+ was highest of all the groups ($p < 0.05$, chi-squared test). Fertilization rate in the supplementation group was lower than that in PI^- ($p < 0.05$, chi-squared test).

Experiment 3

After 3–5 h of insemination, the proportion of sperm displaying the AR pattern of CTC staining in the PI^+ groups (81.4%) was significantly higher than that in the supplementation group (65.2%) ($p < 0.05$: chi-squared test). The proportion of sperm in the PI^+ group which were immunopositive for PR (42.7%: 70/164) was higher than the supplementation group (31.3%: 47/150) ($p < 0.05$, chi-squared test).

Discussion

In Experiment 1, activated sperm exhibited a pattern characteristic of capacitation in BO · MBCD (Fig. 4) and sperm cultured in BO · MBCD exhibited activated motility rates *in vitro* comparable to controls (Fig. 5). Activated sperm acquired hyperactivated motility (Ho *et al.*, 2001). In a previous study, ram sperm exhibiting capacitation, as indicated by CTC staining, was

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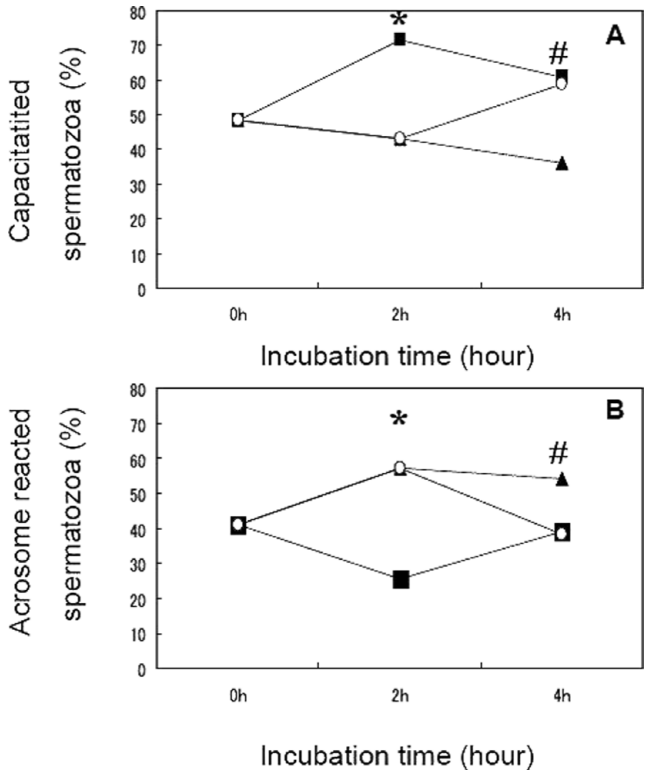

Figure 6 Capacitation of activated sperm during PI in BO · MBCD with, without or supplemented (■, PI^+; ▲, PI^-; ○, supplementation) with glucose and pyruvate. *indicates significant differences among PI^+, PI^- and supplementation groups ($p < 0.05$), and # indicates differences among PI^+, supplementation groups and PI^- groups ($p < 0.05$) as determined by one-way ANOVA. Experiments were repeated nine times.

correlated with tyrosine phosphorylation, a characteristic fundamental to sperm capacitation (Grasa *et al.*, 2006). In mouse and bull sperm, exposure to β-cyclodextrins increased the proportion of fertilization rates and the incidence of capacitation (Choi *et al.*, 1998; Dinkins *et al.*, 2000). Tyrosine phosphorylation was induced during capacitation and hyperactivation (Hung *et al.*, 2008). We thus infer that in the present study, activated sperm induced capacitation in BO · MBCD.

In Experiment 1, sperm cultured in BO · MBCD exhibited higher fertility and activated motility rates *in vitro* compared with sperm cultured in the control group (Table 1 and Fig. 4). In the process of fertilization, cholesterol loss from the surface membrane of sperm is one of the molecular events of capacitation *in vitro* (Davis, 1981; Moseley *et al.*, 2005). When cholesterol is removed from the sperm cell membrane, the cholesterol:phospholipid ratio of the sperm plasma membrane decreases, the lipid concentration changes and the cAMP-signalling cascade is the first process of capacitation to activate, thereby allowing the sperm to fertilize (Witte *et al.*, 2007). Activated motility and hyperactivation are known to be regulated by the cAMP-signalling cascade which is stimulated by adenylyl cyclase, HCO_3^- and Ca^{2+} (Ho *et al.*, 2001; Marquez *et al.*, 2008). These changes in motility may help sperm swim faster and generate enough force to penetrate cumulus cells and the zone pellucida during fertilization (WHO Health Organization, 1999). Increasing the proportion of activated sperm and the number of activated sperm exhibiting patterns characteristic of capacitation resulted in enhanced rates of fertilization. In the present study, we infer

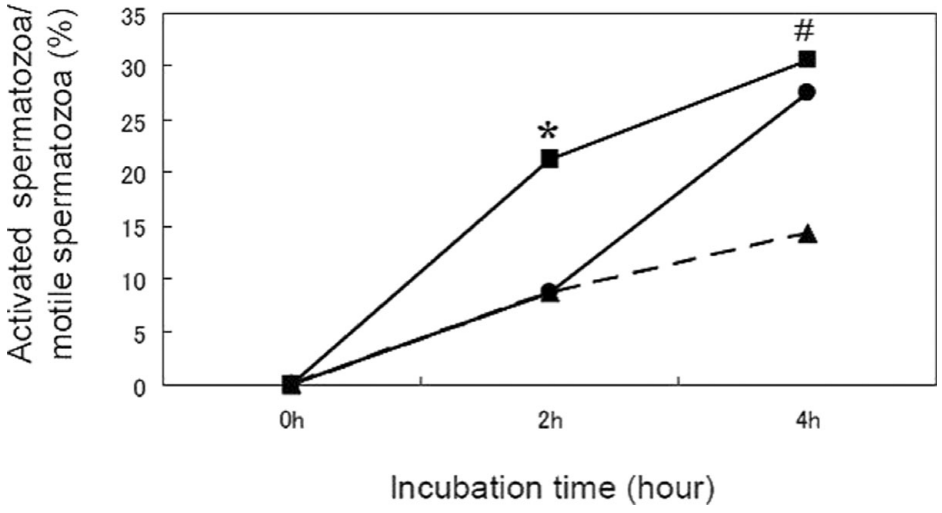

Figure 7 The proportion of activated sperm in BO · MBCD on the proportion of *in vitro* (■, PI^+; ▲, PI^-; ●, supplementation) of glucose and pyruvate. * indicates significant differences among PI^+, PI^- and supplementation groups ($p < 0.05$), # indicates differences among PI^+, supplementation groups and PI^- groups ($p < 0.05$) as determined by one-way ANOVA. Experiments were repeated nine times.

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Table 2 Effect of glucose and pyruvate acid in BO • PVA supplemented with MBCD on fertility[1]

Media for sperm incubation	Time (h) of sperm incubation					
	0		2		4	
	Normal (%)[2]	Total (%)[3]	Normal (%)[2]	Total (%)[3]	Normal (%)[2]	Total (%)[3]
PI^+ (control)	57/163 (35.0)	58/163 (35.6)	106/151 (70.2)^a	124/151(82.1)^c	108/191 (56.5)^e	116/191 (60.7)^h
PI^-	57/163 (35.0)	58/163 (35.6)	82/148 (55.4)^b	83/148 (56.1)^d	32/195 (16.4)^f	33/195 (16.9)^i
Supplementation	57/163 (35.0)	58/163 (35.6)	82/148 (55.4)^b	83/148 (56.1)^d	73/225 (32.4)^g	73/225 (32.4)^j

Statistical significance compared with 0 h: *a* vs. *b*, $p < 0.01$; *c* vs *d* $p < 0.001$; *e* vs *f*, *e* vs *g*, *f* vs *g*, $p < 0.001$; *h* vs *i*, *h* vs *j*, *i* vs *j* $p < 0.003$ (chi-squared test).
[1]Repeat no. 9.
[2]No. of 2PN formation in oocytes/total oocytes.
[3]No. of 2PN, more than 3PN or sperm head in embryos/total embryos.

that activated sperm has undergone capacitation and therefore possess the ability to fertilize.

In Experiment 2, activated sperm in the PI^+ groups at 2 and 4 h and the supplementation groups at 4 h, exhibited patterns characteristic of capacitation. This result could be explained by mitochondrial function. Gur & Breitbart reported that mitochondrial activity was related with the capacity of fertilization (Gur & Breitbart, 2007). Mitochondria produce certain proteins that are essential for sperm function at fertilization, such as catsper, actin filaments and the progesterone receptor (Gur & Breitbart, 2006). Mitochondrial activity is thus important in capacitation and the acquisition of fertilizing potential (Gur *et al.*, 2007). In Experiment 2, the rate of activated sperm exhibiting the capacitation pattern in the PI^+ group was higher than the PI^- group (Fig. 6). Activated sperm in the PI^- group induced the acrosome reaction (Fig. 6). The acrosome reaction is known to be induced by an increase of Ca^{2+} in sperm cells (Parrish *et al.*, 1999). Actin polymerization occurs during mammalian sperm capacitation as a result of mitochondrial activity (Etkovitz *et al.*, 2008) and the F-actin polymers are dispersed prior to the acrosome reaction (Brener *et al.*, 2003). Actin filaments may create a barrier in Ca^{2+} channels and thus prevent the acrosome reaction in capacitated sperm. Thus, we infer that activated sperm in the PI^- group did not undergo actin polymerization and thus influx into sperm cells resulted in the induction of the acrosome reaction.

In Experiment 2, sperm cultured in the PI^+ group exhibited higher fertility rates *in vitro* compared with sperm cultured in either the PI^- or supplementation groups (Table 2). Mitochondrial activity may be a factor related to increased rates of fertilization in the PI^+ group. Sperm cultured in the PI^+ group exhibited activated motility rates *in vitro* compared with sperm cultured in the PI^- group (Fig. 6). Mitochondria produce ATP for tail motion (Hung *et al.*, 2008). It is well established that sperm cells become motile and travel to oocytes via tail motion. Activation and hyperactivation allow sperm to penetrate the cumulus and zona pellucida surrounding the egg (Ho *et al.*, 2001; Suarez *et al.*, 2007). Progressive motility is one of the most important criteria for the fertilization potential of sperm (WHO Health Organization, 1999). Increased mitochondrial activity may have resulted in the increased rates of fertilization in the PI^+ group than the PI^- group. Sperm cultured in BO • MBCD supplemented with only pyruvate (without glucose) increased the rates of fertilization and activated motility (data not shown). Thus, we infer that capacitation may require an energy source, such as glucose and pyruvate acid.

In Experiment 2, there were similar proportions of activated motility and sperm exhibiting capacitation patterns in PI^+ and supplementation groups after 4 h PI. Motility and capacitation have been correlated with fertilization rate (Austin, 1951; Chang, 1951; Perl *et al.*, 2006). However, sperm cultured in our supplementation group exhibited lower fertility rates *in vitro* compared with sperm cultured in the PI^+ group after 4 h PI (Table 2). These results suggest that the factors associated with low fertilization rates in the supplementation group were not motility and the capacitation state of activated sperm. In Experiment 3, we examined the identity of the factor responsible for the low fertilization rates in the supplementation group.

In Experiment 3, sperm cultured in the supplementation group exhibited lower rates of acrosome reaction and reduced immunopositivity to PRs (Table 2) *in vitro* compared with the PI^+ group. During fertilization, progesterone is present at high levels in the cumulus oophorus cells (Osman *et al.*, 1989). Progesterone is also known to induce the acrosome reaction (Witte *et al.*, 2007). Some earlier studies succeeded in identifying and further characterizing the sperm proteins that bind to progesterone, termed progesterone receptors (Tesarik, *et al.*, 1992; Ambhaikar *et al.*, 1998; Gadkar *et al.*, 2002). We infer that expressing

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progesterone receptors in the sperm membrane is essential for increased rates of acrosome reactivity and the acquisition of fertilizing ability.

In conclusion, individual activated sperm were caught and stained by the CTC stain. Captured sperm exhibited patterns characteristic of capacitation. Sperm cultured in a medium containing an additional energy source were able to induce the acrosome reaction and prepare progesterone receptors. During fertilization, activated motility closely correlates with capacitation and activated sperm were shown to be able to maintain capacitation, induce acrosome reactiveness and enhance fertility.

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