Research

Induction of capacitation and the acrosome reaction of boar spermatozoa by L-arginine and nitric oxide synthesis associated with the anion transport system

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The aim of this study was to determine the effect of L-arginine on nitric oxide (NO) synthesis, capacitation and acrosome reaction of boar spermatozoa. Ejaculated boar spermatozoa were washed and then cultured in a bicarbonate:CO_2-buffered medium, modified NCSU-37, for 2 h. At the end of the culture, the status of spermatozoa was determined. The presence of (0.1–2.0 mmol l^{-1}) L-arginine in the culture medium induced an acrosome reaction as determined by fluorescein isothiocyanate–peanut agglutinin (FITC–PNA) and increased intracellular NO content, as quantified by a fluorescent indicator, diaminofluorescein-2 diacetate (DAF-2 DA). This stimulatory effect of L-arginine was neutralized by supplementation with an NO synthase inhibitor, N$^\omega$-nitro-L-arginine methyl ester (1 mmol l^{-1}). However, the inactive enantiomorph, N$^\omega$-nitro-D-arginine methyl ester, did not affect the stimulatory effect of L-arginine. These results indicate that L-arginine induces an acrosome reaction through the NO signal pathway in boar spermatozoa. Furthermore, the stimulatory effect of L-arginine was inhibited in the presence of an anion transport inhibitor, 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonic acid (SITS; 0.1 mmol l^{-1}), whereas any responses of spermatozoa to caffeine were not inhibited by SITS. A stimulatory effect of L-arginine on capacitation and acrosome reaction of spermatozoa was also observed in modified NCSU37 medium by using a chlortetracycline fluorescence assay, but not in supplemented bicarbonate-free Tris-buffered medium. These results indicate that the presence of L-arginine induces nitric oxide synthesis and stimulates capacitation and acrosome reaction of boar spermatozoa only when active sperm anion transport is present as a result of bicarbonate supplementation.

Introduction

Nitric oxide (NO) is a short-lived free radical synthesized by a class of NO synthases (NOS) that are responsible for the conversion of L-arginine to L-citrulline and NO (Palmer *et al.*, 1988; Palmer and Moncada, 1989). NO appears to be involved not only in the function of the male genitourinary system (Burnett *et al.*, 1992, 1995), but also in sperm physiology, such as hyperactivation, capacitation and acrosome reaction (de Lamirande *et al.*, 1997). The presence of NOS has been observed in the acrosome and tail of mouse (Herrero *et al.*, 1996, 1997a) and human spermatozoa (Herrero *et al.*, 1996; Lewis *et al.*, 1996). The NO-releasing compounds induce acrosome reaction in human (Herrero *et al.*, 1999) and rabbit spermatozoa (Guzman-Grenfell *et al.*, 1999). These data indicate that NO can be generated by spermatozoa and may regulate sperm function. As it is known that a NOS inhibitor N$^\omega$-nitro-L-arginine methyl ester (L-NAME), but not the inactive enantiomorph N$^\omega$-nitro-D-arginine methyl ester (D-NAME), produces a dose-dependent inhibition of spontaneous and progesterone-induced acrosome exocytosis of mouse spermatozoa *in vitro* (Viggiano *et al.*, 1996; Herrero *et al.*, 1997b), sperm

NOS appears to participate in the acrosome reaction. Relatively high concentrations (0.01–1.0 mmol l^{-1}) of sodium nitroprusside, a NO donor, inhibit motility and viability of human spermatozoa, but low concentrations (10–100 nmol l^{-1}) result in increased capacitation without an effect on motility (Sengoku *et al.*, 1998). Joo *et al.* (1999) demonstrated that sodium nitroprusside reduces both sperm motility and hyperactivation at 0.1–1.0 mmol l^{-1}, but increases the percentage of acrosome-reacted human spermatozoa at 0.01–1.0 mmol l^{-1}. NOS activity stimulated by follicular fluid proteins also increases the percentage of acrosome-reacted human spermatozoa (Revelli *et al.*, 1999). Although a similar role of NO may be expected in boar spermatozoa, present information is limited to a few species, such as mice and humans. Supplementation with L-arginine stimulates the motility of rabbit (Randany and Atherton, 1981) and human spermatozoa *in vitro* (Keller and Polakoski, 1975). Therefore, L-arginine as a resource of NO may induce capacitation and acrosome reaction through the NO signal pathway. However, little is known about the effect of L-arginine and NO on boar spermatozoa.

Furthermore, it has been shown that bicarbonate is a key inducer of capacitation and penetration of boar spermatozoa *in vitro* (Suzuki *et al.*, 1994; Harrison, 1996; Harrison *et al.*, 1996). Bicarbonate induces protein kinase A-dependent

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Table 1. Composition of mNCSU37 and mTBM media

Compound	mNCSU37 (mmol l^{-1})	mTBM (mmol l^{-1})
NaCl	108.73	113.1
NaHCO$_3$	25.07	–
KCl	4.78	3.0
KH$_2$PO$_4$	1.19	–
MgSO$_4$·7H$_2$O	1.19	–
CaCl$_2$·2H$_2$O	1.70	7.5
Tris	–	20.0
Glucose	5.55	11.0
Sodium pyruvate	0.91	5.0
Glutamine	1.00	–
Sorbitol	12.00	–
Insulin (mg l^{-1})	5	–
Gentamicin (mg l^{-1})	25	25
BSA (Sigma A7888) (w/v)	0.4%	0.4%

mNCSU37: modified North Carolina State University medium 37 supplemented with 0.91 mmol sodium pyruvate l^{-1}; mTBM: modified Tris-buffered medium.

changes in the plasma membrane of boar spermatozoa (Gadella and Harrison, 2000). An anion transport inhibitor, such as 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid (SITS), reversibly inhibits the bicarbonate-sensitive adenylyl cyclase in the plasma membrane of boar spermatozoa (Okamura *et al.*, 1991). Successful *in vitro* fertilization has been achieved not only in bicarbonate–CO_2-buffered media, such as Medium-199 (which originally contains L-arginine at 0.33 mmol l^{-1}), but also in the supplement of bicarbonate-free Tris-buffered medium (for reviews, see Funahashi and Day, 1997; Abeydeera, 2001). It would be interesting to clarify whether L-arginine or NO is associated with a modification of anion transporters, such as the HCO_3^-–Cl^- exchanger, in the induction of capacitation of boar spermatozoa.

The present study was undertaken to determine whether the addition of L-arginine affected NO synthesis and consequently induced the capacitation and acrosome reaction of boar spermatozoa, and whether the induction of capacitation and the acrosome reaction by L-arginine is associated with anion transporters.

Materials and Methods

Materials

KCl, KH$_2$PO$_4$, NaH$_2$PO$_4$, MgCl$_2$·6H$_2$O, MgSO$_4$·7H$_2$O, CaCl$_2$·2H$_2$O, sodium citrate·2H$_2$O and citric acid were purchased from Ishizu Pharmaceutical Co., Ltd (Osaka). NaCl and diaminofluorescein-2 diacetate (DAF-2 DA) were obtained from Nacalai Tesque Inc. (Kyoto) and Daiichi Pure Chemicals Co., Ltd (Kyoto), respectively. Other chemicals were purchased from Sigma Aldrich Japan K K (Tokyo).

Culture media

The basic medium for the incubation of spermatozoa was a modified North Carolina State University medium 37 supplemented with 0.91 mmol sodium pyruvate l^{-1} (mNCSU37; Table 1). Modified Tris-buffered medium (mTBM; Table 1) was also used for sperm culture. The culture media were equilibrated in an atmosphere of 5% CO_2 in air at 39°C for > 16 h before the start of incubation to obtain a pH of 7.2–7.4. The medium used for washing ejaculated spermatozoa was modified Tyrode's lactate–Hepes–polyvinyl alcohol (TL–Hepes–PVA) medium (Funahashi *et al.*, 1997).

L-Arginine, L-NAME, D-NAME, SITS and caffeine were prepared as stock solutions of 0.1 mol l^{-1} and stored at –30°C. For use, stock solutions were thawed and diluted with medium as required.

Preparation of boar spermatozoa

Sperm-rich fractions (30–45 ml) were collected from seven Berkshire boars by the gloved hand method at a local livestock centre and were diluted four times with a modified Modena solution (152.6 mmol trehalose l^{-1}, 23.5 mmol sodium citrate·2H$_2$O l^{-1}, 11.9 mmol NaHCO$_3$ l^{-1}, 6.3 mmol EDTA-2Na l^{-1}, 46.7 mmol Tris l^{-1}, 15.1 mmol citric acid l^{-1}, 5.0 mmol cysteine l^{-1} and 10.0 mg gentamycin ml^{-1}). The diluted semen samples were transported to the laboratory within 2 h of collection and used immediately. Seminal plasma was removed by washing 5 ml of diluted semen and mixing three times with TL–Hepes–PVA by centrifugation at 750 ***g*** for 3 min. After washing, the pellets containing spermatozoa were resuspended to a concentration of 1×10^6 cells ml^{-1} in mNCSU37 or mTBM (2 ml) with or without supplemented L-arginine, L-NAME, D-NAME, caffeine or SITS.

Acrosomal status of spermatozoa

Acrosomal status of live spermatozoa was monitored by means of fluorescein isothiocyanate-conjugated peanut agglutinin (FITC–PNA) under an epifluorescent microscope

(Fazeli *et al.*, 1997). Sperm suspension (1 ml) was exposed to FITC–PNA (10 µg ml^{-1}) and propidium iodide (PI, 12 µmol l^{-1}) for 5 min at 39°C and then immobilized by adding 1 µl of 12.5% (w/v) paraformaldehyde on 0.5 mol Tris l^{-1} (pH 7.4). Slides were prepared by placing 5 µl of the sperm suspension on to a glass slide and adding a cover-slip. Two hundred spermatozoa of each specimen were examined immediately under a phase-contrast microscope ($\times$ 1000) equipped with epifluorescent optics to assess the proportion of live and dead spermatozoa, and acrosomal status. Spermatozoa stained with PI were considered as dead cells; spermatozoa stained with FITC–PNA but without PI were classified as live acrosome-reacted cells and spermatozoa without any fluorescence were considered as acrosome-intact live cells.

Detection and quantification of NO synthesis

NO synthesis from spermatozoa was detected and quantified by using an NO detection reagent, DAF-2 DA. After washing, spermatozoa were resuspended to a concentration of 1×10^8 cells ml^{-1} in TL–Hepes–PVA. DAF-2 DA was added to the suspension at a final concentration of 10 µmol l^{-1} and incubated in air at 37°C for 1 h. After centrifugation at 750 *g* for 3 min, spermatozoa were resuspended to a concentration of 1×10^6 cells ml^{-1} in mNCSU-37 with or without the addition of L-arginine and then cultured in an atmosphere of 5% CO_2 in air at 39°C. For detection of NO, slides were prepared by placing 5 µl of the sperm suspension on to a poly-L-lysine-coated glass slide and adding a coverslip. Emission fluorescence at 522 nm from spermatozoa was observed by using a laser scanning confocal imaging system (MRC-1024: Nippon Bio-Rad Laboratories, Tokyo). The fluorescent image was stored as a PIC file, and NO in a sperm head was quantified according to the pixel density of fluorescence determined with a standard MRC-1024 program, LaserSharp (Nippon Bio-Rad Laboratories, Tokyo).

Chlortetracycline fluorescence assessment of spermatozoa

The methods used for chlortetracycline (CTC) analysis were performed according to Wang *et al.* (1995) with a few minor modifications as described previously (Funahashi *et al.*, 2000; Funahashi and Nagai, 2001). Treated spermatozoa were assessed immediately under a phase-contrast microscope, equipped with epifluorescent optics. Each cell was first observed under UV illumination to determine whether it was alive or dead; the sperm cells showing bright blue staining of the nucleus (bis-benzimide Hoechst 33258-positive cells) were considered as dead. One hundred live spermatozoa were examined under blue–violet illumination and classified according to CTC staining patterns. The three fluorescent staining patterns identified were: F: with uniform fluorescence over the whole sperm head; B: with a fluorescence-free band in the post-acrosome region; AR:

with almost no fluorescence over the sperm head except for a thin band of fluorescence in the equatorial segment (Wang *et al.*, 1995).

Experimental design

Primarily, the effect of L-arginine on acrosome reaction was determined by resuspending spermatozoa in 2 ml of mNCSU-37 containing various concentrations (0, 0.01, 0.05, 0.1, 0.5, 1.0 and 2.0 mmol l^{-1}) of L-arginine and culturing for 2 h in an atmosphere of 5% CO_2 in air at 39°C. The response of boar spermatozoa to L-arginine was assessed by means of FITC–PNA and PI staining.

Secondly, fluorescence from DAF-2 DA, a NO indicator, was quantified using a confocal microscope system at 0, 30 and 60 min after adding various concentrations (0, 1.0 and 2.0 mmol l^{-1}) of L-arginine to determine whether the effect of L-arginine was due to NO synthesis. The effect of L-arginine through the NO signal pathway was confirmed by culturing spermatozoa in mNCSU-37 without or with 1 mmol L-arginine l^{-1} alone or plus a constitutive NOS (cNOS) inhibitor L-NAME (0.5, 1.0 and 2.0 mmol l^{-1}) or the inactive enantiomorph D-NAME (1 mmol l^{-1}) for 2 h. After culture, the response to L-arginine and L-NAME (or D-NAME) on boar spermatozoa was determined by means of FITC–PNA and PI staining.

Finally, to clarify whether the stimulatory effect of L-arginine is associated with the anion exchanger activity, the effect of 1 mmol L-arginine l^{-1} in the absence or presence of 0.1 mmol SITS l^{-1} (an anion transport inhibitor) was compared with that of 1 mmol caffeine l^{-1} or no additives by chlortetracycline fluorescence assay. Furthermore, spermatozoa were resuspended to 2 ml mNCSU37 (which is a bicarbonate-buffered medium) or mTBM (which is a supplemented bicarbonate-free Tris-buffered medium) either without or with L-arginine alone (0.5 and 1.0 mmol l^{-1}) or in combination with 1 mmol L-NAME l^{-1}, cultured for 2 h in an atmosphere of 5% CO_2 in air at 39°C, and then assessed by FITC–PNA and PI staining and chlortetracycline fluorescence assay, to confirm the importance of exogenous bicarbonate ions for the effect of L-arginine

Statistical analyses

Statistical analyses of the results from three to seven replicated experiments were used for treatment comparisons and were carried out by one-way ANOVA using the STATVIEW (Abacus Concepts, Inc., Berkeley, CA) program. If significance was $P < 0.05$ in ANOVA, Fisher's protected least significant difference test was followed using the STATVIEW (Abacus Concepts, Inc., Berkeley, CA) program. All percentage data were subjected to arc–sine transformation before statistical analysis. For showing percentage data in figures, data were transformed back to the original percentages and are expressed as mean percentage with ranges. All other data are expressed as mean $\pm$ SEM. $P \leqslant 0.05$ was considered to be significant.

(a)

(b)
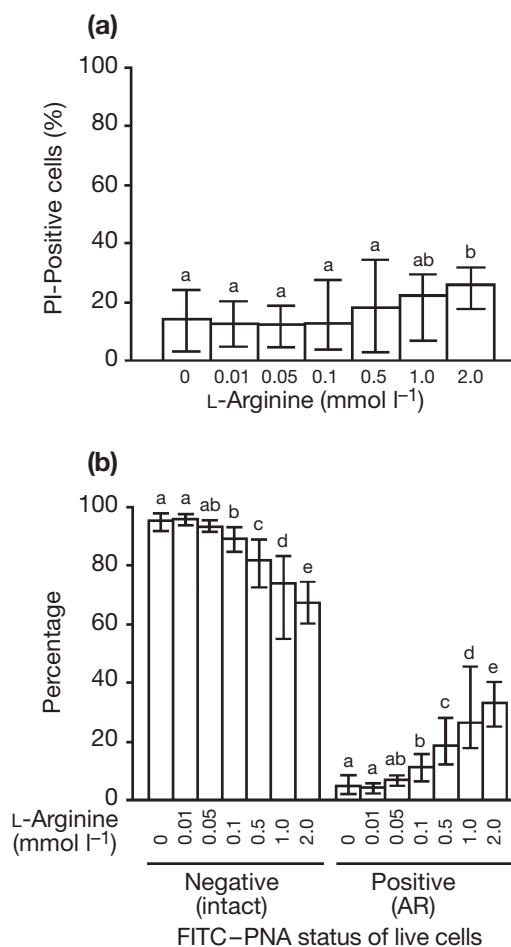
Fig. 1. The concentration-dependent effect of L-arginine on the (a) viability and (b) acrosome status of boar spermatozoa. Suspensions were cultured in the presence of 0–2 mmol L-arginine l^{-1} in modified North Carolina State University medium 37 supplemented with 0.91 mmol sodium pyruvate l^{-1} (mNCSU-37) for 2 h and assessed by propidium iodide (PI) and fluorescein isothiocyanate–peanut agglutinin (FITC–PNA) staining. Data are presented as mean percentage with ranges of cells expressing the different patterns in six replicate trials. Different letters above bars indicate significant differences within the same pattern ($P < 0.05$). AR: acrosome-reacted.

Results

Induction of acrosome reaction by L-arginine

The incidence of live spermatozoa was not different in suspensions treated with 0–0.5 mmol L-arginine l^{-1}, whereas there was a decrease in the percentage of live cells in suspension with 2 mmol L-arginine l^{-1} (Fig. 1a). The proportion of acrosome-intact live cells (no fluorescence) decreased in a dose-dependent manner even in the presence of only 0.1 mmol L-arginine l^{-1} (Fig. 1b). In contrast, the proportion of acrosome-reacted live cells (FITC–PNA positive cells) increased in medium containing a higher concentration of L-arginine, although there were no differences among media containing 0–0.05 mmol L-arginine l^{-1} (Fig. 1b). Therefore,
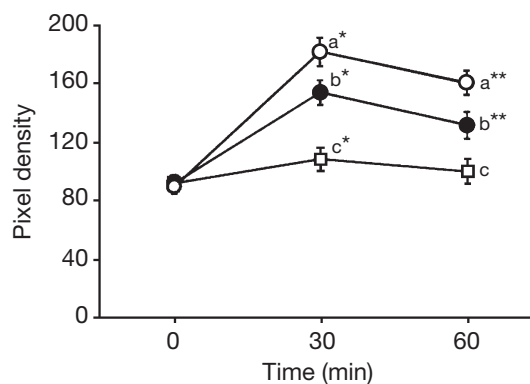
Fig. 2. Change of intracellular nitric oxide (NO) concentration of boar spermatozoa in the absence or presence of L-arginine. Fluorescence of boar spermatozoa loaded diaminofluorescein-2 diacetate (DAF-2 DA) was quantified at 0, 30 and 60 min after exposure to 0 (□), 1 (●) and 2 (○) mmol L-arginine l^{-1} in modified North Carolina State University medium 37 supplemented with 0.91 mmol sodium pyruvate l^{-1} (mNCSU-37). Data are presented as mean pixel density ± SEM of cells in three replicate trials. Different letters above bars indicate significant differences within the same observation time ($P < 0.05$). *Significant differences compared with 0 min ($P < 0.05$). **Significant differences compared with 0 and 30 min ($P < 0.05$).

these data indicate that L-arginine induces acrosome reaction of ejaculated boar spermatozoa in mNCSU-37.

Effect of L-arginine expression through the synthesis of NO

Culture with L-arginine triggered a dose-dependent increase in DAF-2 fluorescence of spermatozoa at 60 min after the addition of L-arginine (Fig. 2). At 30 min after the start of culture, DAF-2 fluorescence significantly increased even in the absence of L-arginine. Between 30 and 60 min after the addition of L-arginine, DAF-2 fluorescence decreased in the presence of 1 and 2 mmol L-arginine l^{-1}. In the absence of L-arginine, DAF-2 fluorescence at 60 min did not differ from that just before the addition of L-arginine (0 min). Therefore, these data indicate that L-arginine induces a dose-dependent increase in the NO content of spermatozoa.

The proportions of acrosome-reacted live cells decreased when 1 mmol L-arginine l^{-1} plus various concentrations (0.5, 1.0 and 2.0 mmol l^{-1}) of L-NAME were added, and the percentages of acrosome-reacted live cells in the presence of 1 or 2 mmol L-NAME l^{-1} were not different from that of control (no additives) (Fig. 3). However, supplementation with the inactive enantiomorph, D-NAME, did not affect the stimulatory effect of L-arginine. These data indicate that L-arginine induces acrosome reaction through the synthesis of NO.

Induction of capacitation and acrosome reaction by L-arginine when anion exchanger is active

Although the percentage of uncapacitated live (F-pattern) cells increased slightly in a medium containing caffeine
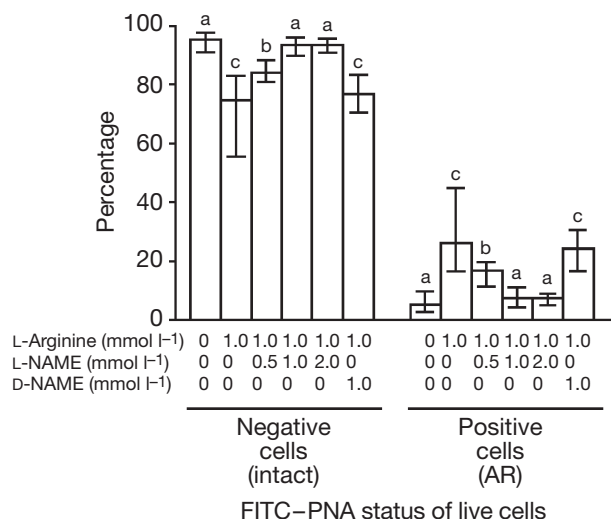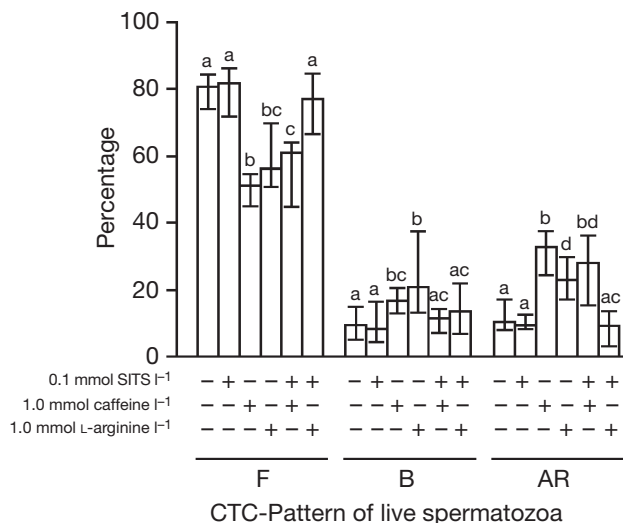
Fig. 3. Inhibitory effect of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) on sperm response to L-arginine. Sperm suspensions were cultured in the presence or absence of 1 mmol L-arginine l^{-1} plus 0–2 mmol L-NAME or 1 mmol D-NAME l^{-1} in modified North Carolina State University medium 37 supplemented with 0.91 mmol sodium pyruvate l^{-1} (mNCSU-37) for 2 h and assessed by fluorescein isothiocyanate–peanut agglutinin (FITC–PNA) and propidium iodide (PI) staining. Data are presented as mean percentage with ranges of cells expressing the different patterns in seven replicate trials. Different letters above bars indicate significant differences within the same pattern ($P < 0.05$). AR: acrosome-reacted.

Fig. 4. Inhibitory effect of 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) on response to L-arginine as determined by analysis of chlortetracycline fluorescence patterns of boar spermatozoa. Suspensions were cultured in the absence or presence of 0.1 mmol SITS l^{-1}, 1.0 mmol caffeine l^{-1} and/or 1.0 mmol L-arginine l^{-1} in modified North Carolina State University medium 37 supplemented with 0.91 mmol sodium pyruvate l^{-1} (mNCSU-37) for 2 h. Data are presented as mean percentage with ranges of cells expressing the different patterns in four to six replicate trials. Different letters above bars indicate significant differences within the same pattern ($P < 0.05$).
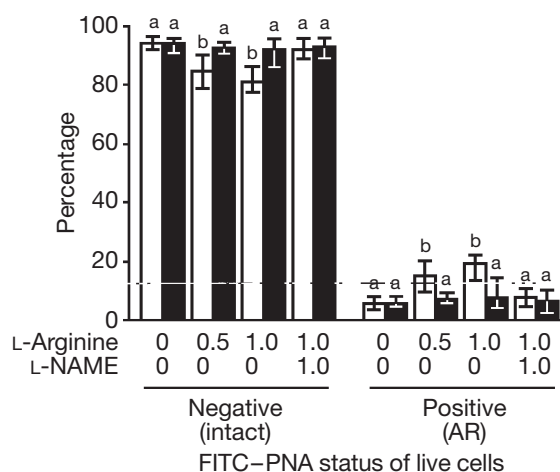
when SITS was added, the percentages of capacitated (B-pattern) and acrosome-reacted live cells were not affected by SITS, indicating that the response of spermatozoa to caffeine was not affected by SITS (Fig. 4). In contrast, the stimulatory effect of L-arginine on sperm capacitation was inhibited by SITS and the proportion of uncapacitated (F-pattern) live cells in media containing L-arginine plus SITS was not different from the control value. These results indicate that modification of anion transporter is associated with the induction of capacitation and acrosome reaction by L-arginine. When the location of anion transporter on the plasma membrane of boar spermatozoa was observed by detecting fluorescence from SITS, a strong fluorescence signal was detected in the post-acrosomal region of spermatozoa with a weak signal in the acrosomal region (data not shown).

Requirement for endogenous bicarbonate for the stimulatory effect of L-arginine on capacitation

When sperm samples were examined by means of FITC-PNA and PI assessment, culture with L-arginine reduced the proportion of live spermatozoa that were acrosome-intact and increased the proportion of acrosome-reacted spermatoza in mNCSU37, whereas culture with L-NAME neutralized the stimulatory effect of L-arginine (Fig. 5). In contrast,

Fig. 5. Different responses of spermatozoa to L-arginine in a bicarbonate-buffered medium (mNCSU-37, □) versus a Tris-buffered medium (mTBM, ■) as determined by fluorescein isothiocyanate–peanut agglutinin (FITC–PNA) staining of boar spermatozoa. Sperm suspensions were cultured in the absence or presence of L-arginine (0.5 and 1.0 mmol l^{-1}), or 1 mmol N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) l^{-1} in mNCSU-37 or mTBM for 2 h. Data are presented as mean percentage with ranges of cells expressing the different patterns in four to eight replicate trials. Different letters above the bars indicate significant differences within the same fluorescence pattern ($P < 0.05$). AR: acrosome-reacted.
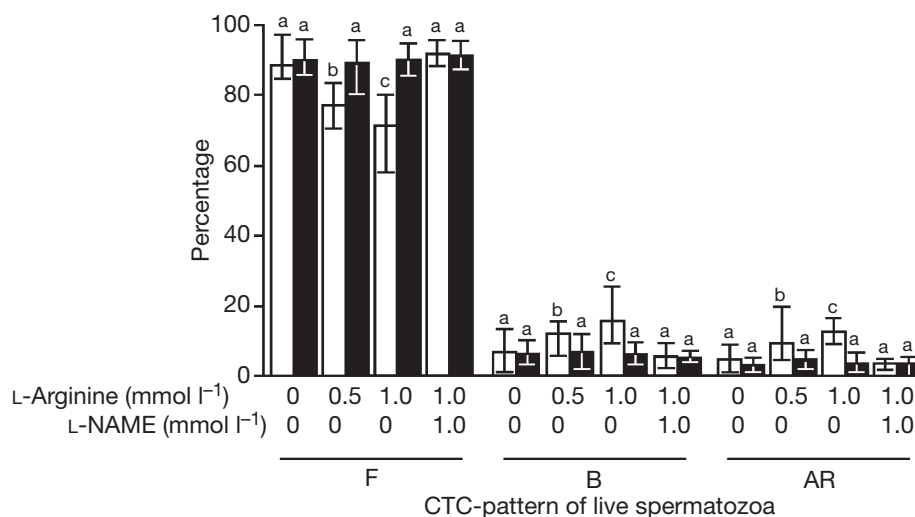
Fig. 6. Different responses of spermatozoa to L-arginine in a bicarbonate-buffered medium (mNCSU-37, □) versus a Tris-buffered medium (mTBM, ■) as determined by analysis of chlortetracycline fluorescence patterns of boar spermatozoa. Sperm suspensions were cultured in the absence or presence of L-arginine (0.5 and 1.0 mmol l^{-1}), or 1 mmol N^{ω}-nitro-L-arginine methyl ester (L-NAME) l^{-1} in mNCSU-37 or mTBM for 2 h. Data are presented as mean percentage with ranges of cells expressing the different patterns in nine replicate trials. Different letters above the bars indicate significant differences within the same pattern ($P < 0.05$).

any stimulatory effects of L-arginine were not observed in mTBM regardless of the presence of L-NAME. When spermatozoa were assessed by CTC fluorescence assay, culture with L-arginine again reduced the percentage of uncapacitated (F-pattern) cells and increased the percentage of capacitated (B-pattern) and acrosome-reacted (AR-pattern) cells, whereas the effect of L-arginine was inhibited in the presence of L-NAME (Fig. 6). However, the presence of L-arginine did not affect the proportions of cells expressing any of the patterns of fluorescence when in mTBM.

Discussion

The present study demonstrated that L-arginine induces both NO synthesis and acrosome loss of boar spermatozoa in a bicarbonate–CO_2-buffered medium, mNCSU-37. The stimulatory effect of L-arginine on acrosome reaction was inhibited in a dose-dependent manner by an inhibitor of cNOS, L-NAME, but not by the inactive enantiomorph, D-NAME. These results are the first to indicate that ejaculated boar spermatozoa contain a cNOS activity and that the acrosome reaction induced by L-arginine in a bicarbonate-buffered medium occurs through the NO signal pathway. It has been reported that concentrations of L-arginine in the oviductal fluid of oestrous sows and rabbits were 3.72 ± 1.25 mg $(100 \text{ ml})^{-1}$ (0.214 ± 0.072 mmol l^{-1}) and 2.62 ± 0.60 mg $(100 \text{ ml})^{-1}$ (0.150 ± 0.034 mmol l^{-1}), respectively (Engle *et al.*, 1968). Similar concentrations of L-arginine have also been detected in the oviductal fluid of oestrous ewes (2.14 mg $(100 \text{ ml})^{-1}$; 0.123 mmol l^{-1}) (Perkins and Goode, 1967) and humans (0.19 mmol l^{-1}) (Tay *et al.*,

1997). The results of the present study indicate that L-arginine promoted acrosome reaction of boar spermatozoa even at 0.1 mmol l^{-1}, indicating that L-arginine in the oviductal fluid has a minimal physiological role on spermatozoa in the oviduct.

The results of the present study indicate that the viability of boar spermatozoa was not affected even in 0.01–1.0 mmol L-arginine l^{-1} and that acrosome exocytosis of the spermatozoa was stimulated at 0.1–2.0 mmol l^{-1}. The results from the CTC analysis in the present study also demonstrated that L-arginine at concentrations examined (0.5 and 1.0 mmol l^{-1}) stimulated both capacitation and acrosome reaction of boar spermatozoa. Therefore, an increased intracellular NO content of boar spermatozoa stimulated by L-arginine (up to 1 mmol l^{-1}) appears to induce capacitation and acrosome loss of the cells without any reduction in viability. This result could be supported by evidence that sperm penetration *in vitro* has been achieved in pigs in modified Medium-199, which originally contained 0.33 mmol L-arginine l^{-1} (for reviews, see Funahashi and Day, 1997; Abeydeera, 2001). Interestingly, even supplementation of modified Medium-199 containing 0.4% BSA with 1 mmol L-arginine l^{-1} results in an increased incidence of acrosome reaction of boar spermatozoa, without any reduction in viability (H. Funahashi, unpublished).

Furthermore, the present study assessed by means of FITC–PNA staining and CTC fluorescence assay showed that L-arginine induced both capacitation and acrosome reaction in a bicarbonate–CO_2-buffered medium, but not in a supplemented bicarbonate-free Tris-buffered medium. Bicarbonate is known to induce capacitation and penetra-

tion of boar spermatozoa *in vitro* (Suzuki *et al.*, 1994; Harrison 1996; Harrison *et al.*, 1996), via protein kinase A-dependent changes in the plasma membrane (Gadella and Harrison, 2000). Under a CO_2-containing atmosphere, CO_2 dissolved in medium will be converted to carbonic acid and then to dissociated bicarbonate ion and H^+ (Umbreit, 1957). The precise concentration of bicarbonate ion will depend on the buffer composition and its pH. In bicarbonate-free Hepes-buffered modified Tyrodes' medium, which was not equilibrated in an atmosphere of 5% CO_2 in air, no sperm penetration occurred in pig oocytes even in the presence of caffeine (Suzuki *et al.*, 1994). Bicarbonate ion derived from only CO_2 dissolved in caffeine-free media may not be sufficient to induce efficient capacitation and the acrosome reaction of boar spermatozoa via the L-arginine–NO pathway. Additional supplementation with bicarbonate increases the incidence of sperm penetration into pig oocytes in the presence of caffeine in an atmosphere of 5% CO_2 in air (Wang *et al.*, 1995; Abeydeera *et al.*, 1997). Therefore, the results of the present study indicate that supplementation with bicarbonate appears to be required for the induction of capacitation and acrosome reaction of boar spermatozoa by the L-arginine–NO pathway.

The present study demonstrates that the response of boar spermatozoa to L-arginine was markedly inhibited in the presence of SITS in a bicarbonate–CO_2-buffered medium. An anion transport inhibitor, SITS, reversibly inhibited the bicarbonate-sensitive adenylyl cyclase in the plasma membrane of boar spermatozoa (Okamura *et al.*, 1991) and prevented the acrosome reaction of bull (Spira and Breitbart, 1992) and hamster spermatozoa (Visconti *et al.*, 1999) in the bicarbonate-containing medium. However, in general, bicarbonate can pass rather freely across membranes via interconversion with CO_2, which is highly permeable, and the bicarbonate–chloride exchanger acts to externalize the bicarbonate ion (Madshus, 1988). Anion channel blockers, phosphotungstate and 4,4'-diisothio-cyanatostilbene-2,2'-disulphonate, enhanced bicarbonate-induced activation in the motility, respiration rate and the cAMP content of boar epididymal spermatozoa because its efflux of endogenous bicarbonate ion derived from metabolic CO_2 was inhibited and thence because bicarbonate ion was accumulated intracellularly (Tajima and Okamura, 1990). SITS, which is impermeable and acts by binding to the membrane band-3 polypeptides, is known as an inhibitor of anion transporters, not specific to the bicarbonate–chloride transporter (Cabantchik *et al.*, 1978). It is known that NO donors stimulate ion transport in human colonic muscle cells *in vitro* (Stack *et al.*, 1996). In the present study, the anion exchanger, detected by the fluorescence of SITS, on the plasma membrane of boar spermatozoa was detected mainly in the post-acrosomal region. Endothelial NOS activity was also evident in the post-acrosomal and equatorial regions of morphologically normal human spermatozoa (O'Bryan *et al.*, 1998). Therefore, these reports and results of the present study

indicate that active anion transport, at least without bicarbonate–chloride exchanger, appears to be highly associated with the L-arginine–NO pathway to induce capacitation–acrosome reaction of boar spermatozoa. However, it is still unclear how the entrance of a cationic amino acid, such as L-arginine, is affected by the inhibition of anion transport by SITS in the present study.

The addition of dibutyryl cAMP has been known to induce the acrosome reaction in bicarbonate-free medium (Visconti *et al.*, 1999) and partially to reverse the inhibition of the acrosome reaction induced by SITS (Spira and Breitbart, 1992; Visconti *et al.*, 1999). These data and the results from the present study indicate that NO may be contained in the bicarbonate-sensitive cAMP–adenylyl cyclase signal pathway via active anion transport to induce capacitation and acrosome reaction of boar spermatozoa. Herrero *et al.* (2000) suggested that NO acts as a cellular messenger by modulating the cAMP pathway involved in capacitation and protein tyrosine phosphorylation. However, an NO donor, sodium nitroprusside was demonstrated to increase the percentage of acrosome reaction in capacitated human spermatozoa via stimulation of an NO-sensitive sCG, cGMP synthesis and protein kinase G activation (Revelli *et al.*, 2001). This effect appears to involve the activation of protein kinase C in the presence of extracellular calcium ion (Revelli *et al.*, 2001). An increase in intracellular cGMP has also been observed during NO-dependent stimulation of acrosome reaction in capacitated bull spermatozoa. Since a growing number of experimental results indicate that NO can induce its biological effect via pathways that are not cGMP-dependent, the target of NO on capacitation and acrosome reaction of boar spermatozoa may not be only cGMP-dependent pathways. Further studies are required to clarify the mechanism of the L-arginine–NO signal pathway.

In conclusion, the results of the present study demonstrate that L-arginine induces the capacitation and acrosome reaction of ejaculated boar spermatozoa through the NO signal pathway. Experiments using an anion transport inhibitor in different culture media showed that an active anion exchanger and supplementation with bicarbonate are required for the L-arginine–NO pathway.

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