Reprod Med Biol (2010) 9:23–31
DOI 10.1007/s12522-009-0036-7

ORIGINAL ARTICLE

# Metabolism of exogenous fatty acids, fatty acid-mediated cholesterol efflux, PKA and PKC pathways in boar sperm acrosome reaction

**Md. Sharoare Hossain · Sadia Afrose · Tomio Sawada · Koh-ichi Hamano · Hirotada Tsujii**

Received: 3 April 2009 / Accepted: 16 September 2009 / Published online: 27 October 2009


**Abstract**
*Purpose* For understanding the roles of fatty acids on the induction of acrosome reaction which occurs under association of cholesterol efflux and PKA or PKC pathways in boar spermatozoa, metabolic fate of alone and combined radiolabeled $^{14}$C-oleic acid and $^{3}$H-linoleic acid incorporated in the sperm was compared, and behavior of cholesterol and effects of PKA and PKC inhibitors upon fatty acid-induced acrosome reaction were examined.
*Methods* Semen was collected from a Duroc boar, and the metabolic activities of fatty acids in the spermatozoa were measured using radioactive compounds and thin layer chromatography. Cholesterol efflux was measured with a cholesterol determination assay kit. Participation of fatty acids on the AR through PKA and PKC pathways was evaluated using a specific inhibitor of these enzymes.
*Results* Incorporation rate of $^{14}$C-oleic acid into the sperm lipids was significantly higher than that of $^{3}$H-linoleic acid ($P < 0.05$). The oxidation of $^{14}$C-oleic acid was higher in combined radiolabeling rather than in one. The highest amounts of $^{3}$H-linoleic acid and $^{14}$C-oleic acid were recovered mainly in the triglycerides and phospholipids fraction, and $^{14}$C-oleic acid distribution was higher than the $^{3}$H-linoleic acid in both labeled ($P < 0.05$) sperm lipids. In the $^{3}$H-linoleic and $^{14}$C-oleic acid combined radiolabeling, the incorporation rate of the radioactive fatty acids in all the lipid fractions increased 15 times more than the alone radiolabeling. Boar sperm utilize oleic acid to generate energy for hyperactivation ($P < 0.05$). Supplementation of arachidonic acid significantly increased ($P < 0.05$) cholesterol efflux in sperm. When spermatozoa were incubated with PKA or PKC inhibitors, there was a significant reduction of arachidonic acid-induced acrosome reaction (AR) ($P < 0.05$), and inhibition by PKA inhibitor is stronger than that by PKC inhibitor.
*Conclusions* Incorporation of unsaturated fatty acids, especially oleic acid, into triglycerides and phospholipids provides prerequisite energy for AR. Cholesterol efflux by arachidonic acid triggers AR. Arachidonic acid activated PKA and PKC pathway participate in induction of the AR.

**Keywords** Acrosome reaction · Boar sperm · Cholesterol efflux · Incorporation and oxidation of fatty acid · PKA and PKC

M. S. Hossain · S. Afrose · K. Hamano · H. Tsujii (✉)
Laboratory of Animal Biotechnology,
Faculty of Agriculture, Shinshu University,
Minamiminowa-mura, Nagano 399-4598, Japan
e-mail: htsujii@shinshu-u.ac.jp

T. Sawada
The Sawada Women's Clinic, Nagoya Reproduction Center,
Chikusaku, Nagoya, Aichi, Japan

## Introduction

Bovine serum albumin (BSA) containing about 2–3 mol of fatty acids/mol of protein [1] is well recognized as an important inducer of the sperm capacitation and acrosome reaction (AR) [2]. Thus, as much as 15 μg of fatty acids are introduced in the culture medium for induction of AR [3]. We previously reported that fatty acids which bind to the BSA (BSA-V) enhanced much more boar sperm AR than fatty acid-free BSA (BSA-FAF) [4], and the rate of AR in BSA-FAF was restored to the level in the presence of BSA-V when a fatty acids mixture was added. We further reported that unsaturated fatty acids (esp. oleic, linoleic and arachidonic acids) that have double bonds and are more

potent for cell function than saturated fatty acid are the major AR-inducing fatty acids for boar sperm [5]. A similar observation was reported by Meizel and Turner [6] in hamster sperm, showing that AR was enhanced by oleic acid.

Many investigators have established that the process of AR represents a series of elegant intracellular communication and mechanism, and these intracellular events most frequently depend on the supply of energy to the sperm. Adenosine triphosphate (ATP) converted in the form of cyclic adenosine monophosphate (cAMP) is necessary for AR through protein tyrosine phosphorylation (PTP) process [7]. The potentiality of sperm AR strongly depends on its energy production by the ATP, and in general, sperm produce energy from triglycerides (TG) and phospholipids (PL) [8]. The PL is noticed as an integral component of the intracellular signal transduction process, and fatty acids and TG are important sources of energy for cellular metabolism [9]. Furthermore, PL, cholesterol, and cholesterol ester (CE) are known to be involved in maintaining the sperm plasma membrane integrity. In addition, it was proposed that unsaturated fatty acids increase membrane fluidity and change the hamster sperm head membrane architecture [10, 11], which is associated with cholesterol efflux, suggesting that unsaturated fatty acids induce boar sperm AR via cholesterol efflux. The combined isotope concept for the measurement of lipid turnover was used to estimate the differences in the rate of turnover of fatty acids in subcellular fractions prepared from boar sperm. Combined radiolabeling technique will also help to our understanding how sperm handle metabolism of two different fatty acids synergistically. However, the roles of fatty acid mediated energy balance, cholesterol efflux and PKA or PKC pathways in the boar sperm AR have not yet been studied elsewhere.

Here, we selected oleic and linoleic acid as representatives of unsaturated fatty acid for the metabolism study, and compared the metabolic activity of alone and combination of radiolabeled oleic and linoleic acid in boar spermatozoa. We also examine the effects of fatty acids on cholesterol efflux from the sperm and the roles of PKA and PKC on the induction of AR.

## Materials and methods

### Chemicals

The cholesterol determination kit (439-17501) was obtained from Wako Pure Chemicals (Tokyo, Japan). Chelerythrine chloride, KT 5720, oleic acid, linoleic acid and arachidonic acid (all are approximately 99% pure) were from Sigma Chemical Company (St Louis, MO, USA). H-89 and Calphostin C were obtained from Alexis Biochemicals (CH-4415 Lausen, Switzerland). Other chemicals were analytical grade.

### Semen collection and preparation

Semen was collected by the gloved-hand technique from Duroc boars aged between 2 and 3 years at Nagano Animal Industry Experiment Station, Nagano Prefecture, Japan. The semen was diluted with the Modena extender giving a sperm concentration of $1 \times 10^8$/ml at room temperature according to the method of Johnson et al. [12]. A detailed method for preparation of sperm from the semen is described in our previous report [4]. Spermatozoa were suspended in a basic TALP medium [13] containing BSA-FAF (Sigma Chemical Company, St Louis, MO, USA) instead of BSA-V (Sigma) for swimming up the spermatozoa. Fatty acids were then added to the culture media according to the previously described method [5]. For measuring total incorporation of fatty acids and conducting thin layer chromatography, 18.5 kBq of $^3$H-linoleic (specific activity 370 MBq/mmol) and $^{14}$C-oleic acid (specific activity 385 MBq/mmol) were added to 1 ml of the swim-up spermatozoa and then incubated at 37°C for 0.5–3 h in the same condition. The final sperm concentration in the medium was $1 \times 10^8$/ml.

### Sperm motility and hyperactivity

Sperm motility and hyperactivity were assessed by the subjective observation as described previously [14]. Briefly, motility and hyperactivation of sperm motility were observed in a 2 µl drop of sperm suspension on a heated stage at 37°C under a light-microscope. Sperm showing high flagellar bend amplitude and asymmetrical beating were estimated as sperm with hyperactivated motility. Spermatozoa showing motility and hyperactivity were estimated as percentage of sperm exhibiting motility and hyperactivity.

### Acrosome reaction

The AR was evaluated by chlortetracycline (CTC) assay as shown in our previous study in boar sperm [15] with slight modification of method by Ward and Storey [16]. The CTC stock solution containing 750 µM CTC–HCl (Sigma Chemical Co.), 130 mM NaCl, 5 mM L-cysteine and 20 mM Tris acid (pH 7.8) was prepared daily, wrapped in foil to protect against light, and stored at 4°C until use. Sperm were incubated with the fatty acids mixture or arachidonic acid in the presence or absence of Chelerythrine chloride (3, 6 µM), Calphostin C (25, 50 nM), KT 5720 (50, 100 µM) and H-89 (10, 20 µM). Ten

microliters of sperm suspension was mixed with 15 μL of CTC solution on a glass slide at room temperature. Then, 0.3 μL of 12.5% glutaraldehyde in 2.5 M Tris base was added as a fixative. Duplicated samples were covered with coverslips and stored in the dark at 4°C. Sperm were observed within 24 h under a phase contrast microscope (Nikon, Tokyo, Japan) with epifluorescence optics under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm). Situation of capacitation of sperm was evaluated according to CTC staining patterns [17]: fluorescence staining with over the entire head was evaluated as precapacitated cells (pattern F), fluorescence-free band in the postacrosomal region evaluated as capacitated cells (pattern B) and low fluorescence over the entire head except for a thin bright fluorescent band along the equatorial segment was as acrosome-reacted cells (pattern AR). The percentage of AR was determined at 1 and 3 h of incubation, and at least 200 spermatozoa were counted in each sample.

### Incorporation and oxidation

To assess the metabolic activity of spermatozoa treated with fatty acids, 18.5 kBq/ml of $^{3}$H-linoleic (specific activity 370 MBq/mmol) and $^{14}$C-oleic acid (specific activity 385 MBq/mmol) were added to 1–100 μl of the sperm sample and incubated for 3 h. The assessment and determination procedures for metabolic activity were the same as our previous report [4]. The value of incorporation was expressed directly by counts per minute (cpm).

### Lipid extraction and thin layer chromatography

Radioactive fatty acids were added to the spermatozoa and incubated for 30 min and then 19 volumes of chloroform–methanol (2:1, v/v) was added. Lipids were extracted using the procedure of Folch et al. [18] and subjected to the thin layer chromatography (TLC). The TLC was carried out using aluminum sheet silica gel 60 thin-layer plates (2.5 × 7.5 cm; Merck, Darmstadt, Germany). The extracted lipids were fractionated into PL, free cholesterol (FC), TG, free fatty acids (FFA), and CE using a solvent system of hexane: diethyl ether: formic acid (80:20:1, v:v:v) at room temperature. Location of bands of these lipids was visualized under ultraviolet light (320 nm) after being sprayed with a 0.1% (w:v) solution of 2,7-dichlorofluorescein (Sigma) in methanol. Lipid bands were scraped from the plates and collected separately in scintillation vials. The radioactivities of the samples were determined by a liquid scintillation counter (LS-6500, Beckman Instruments, Inc., Fullerton, CA, USA). Data were shown in pmol, which is converted from the cpm value by multiplying by $16.68 \times 10^{-3}$Bq.

### Determination of cholesterol concentration

Sperm were incubated in the medium with various conditions (see detail in "Results"). After the completion of the specified incubation period, each 1 ml sperm suspension with $5 \times 10^{6}$ sperm/ml was centrifuged for 10 min at $10{,}000 \times g$. Cholesterol contents were measured in both supernatant and sperm pellet by a spectrophotometer (APEL Co., Saitama, Japan) at 600 nm using the cholesterol determination kit following the manufacturer's instructions as described previously [15]. The cholesterol contents were expressed as the amount of cholesterol (ng/dl) per $5 \times 10^{6}$ cells.

### Statistical analysis

Data were subjected to the protected Fisher's least significant difference test. The NCSS (Number Crunchier Statistical System; NCSS Statistical Software, Kaysville, UT, USA) Version 5.01 computer software package was used for all statistical analysis. Each experiment was repeated four times and differences were considered significant for $P < 0.05$.

## Results

### Incorporation and oxidation of alone and combine radiolabeled fatty acids

Incorporation of alone and combined $^{3}$H-linoleic and $^{14}$C-oleic acids into the boar sperm is shown in Table 1. The $^{3}$H-linoleic and $^{14}$C-oleic acids were steadily incorporated into the sperm lipids, and the incorporation increased in accordance with incubation time in both alone and combination of radiolabels. Preferential highest incorporation was observed at 3 h of incubation. Incorporation of $^{3}$H-linoleic acid and $^{14}$C-oleic acid were significantly higher in combination of radiolabel than alone radiolabel in all incubation periods. The incorporation of $^{14}$C-oleic acid was higher than that of $^{3}$H-linoleic acid.

Oxidation of alone and combination of radiolabeled $^{14}$C-oleic acid is shown in Table 2. The oxidation was higher in combined radiolabeling than that of the alone radiolabeling one except at 1 h of incubation.

### Distribution of incorporated fatty acids in the sperm

The distribution of alone $^{3}$H-linoleic and $^{14}$C-oleic acids in the lipid fractions from sperm extract is shown in Table 3a. Large amounts of $^{3}$H-linoleic and $^{14}$C-oleic acids were recovered in PL and TG, followed by CO, CE and FFA. The radioactivity of $^{14}$C-oleic acid in the PL class was

**Table 1** Compares alone and combination of $^{3}$H-linoleic and $^{14}$C-oleic acid incorporation into total lipid of boar spermatozoa

| Incubation period (h) | Fatty acid | Alone radiolabel | Combined radiolabel |
|---|---|---|---|
| 0.5 | $^{3}$H-linoleic | 1.70 ± 0.09$^{a}$ | 3.06 ± 0.11$^{b}$ |
| | $^{14}$C-oleic | 2.61 ± 0.10$^{a}$ | 5.01 ± 0.13$^{b}$ |
| 1 | $^{3}$H-linoleic | 2.58 ± 0.08$^{a}$ | 5.28 ± 0.07$^{b}$ |
| | $^{14}$C-oleic | 5.87 ± 0.10 | 7.89 ± 0.12 |
| 2 | $^{3}$H-linoleic | 6.32 ± 0.14$^{a}$ | 13.90 ± 0.11$^{b}$ |
| | $^{14}$C-oleic | 10.70 ± 0.09$^{a}$ | 16.20 ± 0.08$^{b}$ |
| 3 | $^{3}$H-linoleic | 12.66 ± 0.14$^{a}$ | 17.20 ± 0.12$^{b}$ |
| | $^{14}$C-oleic | 15.60 ± 0.13$^{a}$ | 19.50 ± 0.11$^{b}$ |

Superscript values between the single and double label column differ significantly ($P < 0.05$). Values are mean ± SEM of four independent experiments; $n = 4$. Values are expressed as pmol/1 × $10^{8}$ spermatozoa. Sperm in the alone radiolabel was treated separately with $^{3}$H-linoleic acid and $^{14}$C-oleic acid, and values are obtained as alone radiolabel. Sperm in the combined radiolabel was treated in combination with $^{3}$H-linoleic and $^{14}$C-oleic, after that values were separated by detecting $^{3}$H and $^{14}$C content in the sperm extract

**Table 2** Compare alone and combined radiolabel $^{14}$C-oleic acid oxidation into boar spermatozoa

| Incubation period (h) | Single | Double |
|---|---|---|
| 0.5 | 0.14 ± 0.01 | 0.18 ± 0.01 |
| 1 | 0.25 ± 0.02 | 0.24 ± 0.03 |
| 2 | 0.32 ± 0.04 | 0.36 ± 0.02 |
| 3 | 0.42 ± 0.02 | 0.45 ± 0.03 |

The value for double ($^{14}$C-oleic and $^{3}$H-linoleic) is expressed except the $^{3}$H-linoleic acid. Values were compared between columns for alone and combined were not significant. Values are mean ± SEM of four independent experiments; $n = 4$. Values are expressed as pmol/1 × $10^{8}$ spermatozoa

significantly ($P < 0.05$) higher than the $^{3}$H-linoleic acid at 2 h of incubation. A significantly higher level of radioactivity in the TG class was observed in $^{14}$C-oleic acid treated sperm lipid fraction when compared with the $^{3}$H-linoleic acid at 0.5 and 1 h of incubation ($P < 0.05$). The CO class showed no significant difference between $^{3}$H-linoleic and $^{14}$C-oleic acids during the total incubation period except at 1 h incubation. Only in the CO class, $^{14}$C-oleic acid showed a lower incorporation than that in the $^{3}$H-linoleic acid. Very small amounts of both $^{3}$H-linoleic and $^{14}$C-oleic acids appeared in CE and FFA during the incubation periods.

The distribution of combined $^{3}$H-linoleic and $^{14}$C-oleic acids into the lipid fractions is shown in Table 3b. Both $^{3}$H-linoleic and $^{14}$C-oleic acids were mainly recovered in TG, followed by PL, CO, CE and FFA. In the PL fraction, recovery of $^{14}$C-oleic acid was significantly higher than the $^{3}$H-linoleic at 1 and 3 h of incubation ($P < 0.05$). In the TG class, recovery of $^{14}$C-oleic acid and $^{3}$H-linoleic acid was almost the same in almost all incubation periods ($P < 0.05$). $^{14}$C-oleic acid was more significantly incorporated into the FFA fraction than the $^{3}$H-linoleic acid ($P < 0.05$). The lowest radioactivity was recovered in the CE class, and there was no significant difference between $^{3}$H-linoleic and $^{14}$C-oleic acid. Only in the CO class, $^{14}$C-oleic acid showed lower incorporation than that in the $^{3}$H-linoleic acid except at 3 h of incubation. Increased ratio of recovery in $^{14}$C-oleic acid and $^{3}$H-linoleic acid in combined radiolabeling is higher than alone radiolabeling in each lipid fraction, and difference was the highest in the TG and PL fractions.

## Effect of fatty acids on the CO/PL ratio

As shown in Table 4, when compared to alone label, ratio of CO/PL for $^{3}$H-linoleic acid alone and $^{3}$H-linoleic + $^{14}$C-oleic acid significantly decreased in combined label at 3 h incubation ($P < 0.05$). The ratio in $^{14}$C-oleic acid treated sperm is lower than the $^{3}$H-linoleic acid treated sperm.

## Cholesterol efflux and AR induction by fatty acid treatment

Effects of BSA bound fatty acids on AR and cholesterol efflux are shown in Table 5. Compared to the control, BSA-V, PVA-FAM and arachidonic acid caused significant increase in both AR and cholesterol efflux into the medium. Concomitant with the increase in cholesterol content in sperm, AR significantly decreased at 1 and 3 h incubation ($P < 0.05$).

BSA-V, PVA-FAM, oleic acid and arachidonic acid significantly increased the rate of AR, whereas BSA-FAF showed a low rate induction of AR. BSA-V is the best inducer of AR and cholesterol efflux, and it became almost the same as PVA-FAM.

## PKA and PKC pathway and acrosome reaction

Effects of PKA and PKC inhibitors on BSA bound fatty acids-induced AR are shown in Table 6. The combination of PKA and PKC inhibitors inhibited PVA-FAM induced AR ($P < 0.05$). H-89, KT 5720 (PKA inhibitor) and Calphostin C and Chelerythrine chloride (PKC inhibitor) significantly inhibited the arachidonic acid-induced AR ($P < 0.05$). The inhibition of AR by the PKA inhibitors was higher than the PKC inhibitors. Combinations of PKA and PKC inhibitors caused the highest inhibition of arachidonic acid-induced AR ($P < 0.05$).

**Table 3** Distribution of single and combined $^{3}$H-linoleic acid and $^{14}$C-oleic acid in lipid fraction at different incubation period of boar spermatozoa

| Fraction | PL | | TG | | CO | | CE | | FFA | |
|---|---|---|---|---|---|---|---|---|---|---|
| Isotope | $^{14}$C-OA | $^{3}$H-LA | $^{14}$C-OA | $^{3}$H-LA | $^{14}$C-OA | $^{3}$H-LA | $^{14}$C-OA | $^{3}$H-LA | $^{14}$C-OA | $^{3}$H-LA |
| (a) Single | | | | | | | | | | |
| 0.5 h | 0.29 ± 0.02 | 0.25 ± 0.02 | 2.01 ± 0.09[a] | 0.98 ± 0.06[b] | 0.08 ± 0.00 | 0.12 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.00 | 0.04 ± 0.00 | 0.03 ± 0.00 |
| 1 h | 0.45 ± 0.03 | 0.35 ± 0.03 | 4.97 ± 0.11[a] | 1.86 ± 0.03[b] | 0.16 ± 0.01 | 0.22 ± 0.01 | 0.03 ± 0.00 | 0.02 ± 0.02 | 0.03 ± 0.02 | 0.02 ± 0.01 |
| 2 h | 2.28 ± 0.06[a] | 0.89 ± 0.05[b] | 7.17 ± 0.04 | 5.40 ± 0.02 | 0.35 ± 0.04 | 0.38 ± 0.01 | 0.09 ± 0.01[a] | 0.05 ± 0.01[b] | 0.09 ± 0.00 | 0.08 ± 0.01 |
| 3 h | 2.41 ± 0.16 | 2.23 ± 0.16 | 11.99 ± 0.17 | 9.36 ± 0.26 | 0.60 ± 0.02 | 0.67 ± 0.02 | 0.14 ± 0.01[a] | 0.10 ± 0.01[b] | 0.11 ± 0.01[a] | 0.06 ± 0.02[b] |
| (b) Double | | | | | | | | | | |
| 0.5 h | 0.40 ± 0.02[a] | 0.28 ± 0.02[b] | 4.45 ± 0.13[a] | 2.59 ± 0.02[b] | 0.09 ± 0.01 | 0.18 ± 0.03 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.00 | 0.00 ± 0.00 |
| 1 h | 0.65 ± 0.04 | 0.52 ± 0.02 | 6.81 ± 0.22[a] | 3.98 ± 0.14[b] | 0.09 ± 0.00[a] | 0.22 ± 0.01[b] | 0.01 ± 0.00 | 0.00 ± 0.01 | 0.05 ± 0.01[a] | 0.01 ± 0.01[b] |
| 2 h | 2.06 ± 0.08 | 1.62 ± 0.09 | 13.62 ± 0.11 | 11.67 ± 0.06 | 0.41 ± 0.03 | 0.56 ± 0.05 | 0.03 ± 0.02 | 0.03 ± 0.02 | 0.06 ± 0.01[a] | 0.02 ± 0.00[b] |
| 3 h | 2.92 ± 0.05[a] | 2.02 ± 0.05[b] | 15.60 ± 0.14 | 14.23 ± 0.25 | 0.52 ± 0.03 | 0.46 ± 0.27 | 0.04 ± 0.02[a] | 0.05 ± 0.01[b] | 0.04 ± 0.02 | 0.03 ± 0.01 |

Superscript values within the same lipid fraction between $^{14}$C-OA and $^{3}$H-LA differ significantly from each other ($P < 0.05$). $^{14}$C-OA and $^{3}$H-LA mean $^{14}$C-oleic acid and $^{3}$H-linoleic acids, respectively. Values are mean ± SEM of four independent experiments; $n = 4$. Values are expressed as pmol/1 × $10^{8}$ spermatozoa

*PL* Phospholipids, *TG* triglycerides, *CO* cholesterol, *CE* cholesterol ester, *FFA* free fatty acids

### Effects of fatty acids on sperm motility and hyperactivity

Effects of oleic acid and linoleic acid on motility and hyperactivity of boar sperm are shown in Table 7. Compared to the control, motility increased by both oleic acid and linoleic acid ($P < 0.05$), while hyperactivity increased only by oleic acid in boar spermatozoa ($P < 0.05$).

## Discussion

Intracellular signal transduction in the sperm capacitation and AR is known to be established with a multistep process including bicarbonate ion-mediated cAMP production and following cAMP-dependent activation of PKA, $Ca^{2+}$-dependent activation of PKC and phosphotyrosin phosphorylation (PTP) on a subset of proteins [19]. However, knowledge on the up-stream signaling occurring at the plasma membrane is still restricted. Only the occurrence of cholesterol efflux [20], loss of transbilayer PL asymmetry [21], membrane hyperpolarization [22], activation of voltage-gated $Ca^{2+}$ channels [23], protein-lipid restructuring [24] and energy balances are events worth mentioning. Fatty acids may play important roles as an effective factor triggering the up-stream events since oleic, linoleic and arachdonic acids were reported to modulate AR [5].

Here, we showed that these fatty acids as well as arachidonic acid, along with cellular energy supply, stimulate efflux of membrane cholesterol and PKA and PKC pathways to enhance PTP in the boar sperm. It was also revealed that boar sperm, in in vitro condition, gain energy via differential utilization of exogenous oleic and linoleic acid by synthesizing sperm lipid fractions. Furthermore, incorporation of the highest amounts of radioactive oleic and linoleic acid into the TG fraction was found, although the localization of these fatty acids in the TG fraction of boar sperm has not been reported previously. The preferential incorporation of oleic acid is higher than the linoleic acid, and the result is partly in accordance with the knowledge of the fact that high content of this fatty acid is present in several types mammalian sperm including boar sperm [25].

Difference of particular stereochemistry and the degree of unsaturation in the two fatty acids, oleic acid and linoleic acid might be critical for the difference of incorporation rate into the sperm cells. Oleic acid other than linoleic acid has a double bond between the 9th and 10th carbons in the *cis*-configuration and this configuration of the fatty acid as well as other ones with similar configuration, have a better neutral lipid synthesizing ability [26], resulting in higher incorporation of the oleic acid than linoleic acid in the boar sperm as shown in the present study. The series of 18 carbon *cis*-unsaturated oleic acid has an effectiveness

**Table 4** Cholesterol/phospholipids ratio of $^{3}$H-linoleic and $^{14}$C-oleic acid treated boar sperm at 3 h of incubation

| Label | Fatty acid | CO/PL ratio |
|---|---|---|
| Single | $^{3}$H-linoleic | $0.30 \pm 0.01^{a}$ |
| | $^{14}$C-oleic | $0.24 \pm 0.01^{b}$ |
| Double | $^{3}$H-linoleic (alone) | $0.22 \pm 0.03^{b}$ |
| | $^{14}$C-oleic (alone) | $0.21 \pm 0.03^{bc}$ |
| | $^{3}$H-linoleic + $^{14}$C-oleic | $0.18 \pm 0.01^{c}$ |

Superscript values between alone and combination of radiolabels within the same column differ significantly from each other ($P < 0.05$). Values are mean ± SEM. Values are expressed as pmol/$1 \times 10^{8}$ spermatozoa

CO and PL values were obtained from the Table 3 and calculated for CO/PL ratio

**Table 5** Effect of BSA-V bound fatty acids on cholesterol efflux and AR in boar spermatozoa

| Treatment | 1 h | | | 3 h | | |
|---|---|---|---|---|---|---|
| | Cholesterol efflux | | | Cholesterol efflux | | |
| | AR (%) | Sperm | Medium | AR (%) | Sperm | Medium |
| Control | $12.5 \pm 1.32^{a}$ | $419.5 \pm 19.0^{a}$ | $26.2 \pm 6.6^{a}$ | $27.2 \pm 2.17^{a}$ | $392.2 \pm 20.1^{a}$ | $53.7 \pm 9.3^{a}$ |
| BSA-V | $24.2 \pm 2.42^{b}$ | $351.7 \pm 18.6^{b}$ | $80.2 \pm 10.3^{b}$ | $49.0 \pm 4.04^{b}$ | $245.5 \pm 20.8^{b}$ | $186.5 \pm 13.5^{b}$ |
| BSA-FAF | $15.2 \pm 1.49^{ab}$ | $386.0 \pm 34.5^{ab}$ | $48.0 \pm 8.5^{b}$ | $35.2 \pm 5.13^{ab}$ | $344.5 \pm 24.0^{ab}$ | $90.0 \pm 8.8^{b}$ |
| PVA-FAM | $23.7 \pm 2.13^{b}$ | $354.0 \pm 20.9^{b}$ | $75.2 \pm 11.1^{b}$ | $47.7 \pm 4.90^{b}$ | $266.0 \pm 30.8^{b}$ | $165.7 \pm 11.6^{b}$ |
| OA | $20.0 \pm 1.87^{b}$ | $377.0 \pm 24.7^{ab}$ | $55.5 \pm 12.8^{b}$ | $39.2 \pm 3.59^{b}$ | $349.0 \pm 29.2^{ab}$ | $83.5 \pm 4.5^{b}$ |
| LA | $17.4 \pm 1.6^{ab}$ | $363.7 \pm 15.4^{b}$ | $68.5 \pm 8.5^{b}$ | $33.1 \pm 4.2^{ab}$ | $283.7 \pm 31.8^{b}$ | $150.5 \pm 22.4^{b}$ |
| AA | $22.7 \pm 2.05^{b}$ | $363.0 \pm 14.4^{b}$ | $67.2 \pm 10.3^{b}$ | $43.0 \pm 3.24^{b}$ | $281.7 \pm 31.9^{b}$ | $147.2 \pm 20.0^{b}$ |

Values within a column with different superscript letters were significantly different ($P < 0.05$). Values are mean ± SEM of four independent experiments; $n = 4$. Spermatozoa were incubated for 1 and 3 h; values are expressed as ng/$10^{6}$ spermatozoa

*BSA-V* Bovine serum albumin fraction five, *BSA-FAF* bovine serum albumin-fatty acid free, *PVA-FAM* polyvinyl alcohol-fatty acid mixture, *OA* oleic acid, *LA* linoleic acid, *AA* arachidonic acid

**Table 6** Effect of PKA and PKC inhibitors on fatty acids induced AR at 3 h of incubation in boar spermatozoa

| Inducer | PKA Inhibitor | | PKC Inhibitor | | % AR |
|---|---|---|---|---|---|
| | H 89 | KT 5720 | Calphostin C | Chelerythrine Chloride | |
| No treatment | – | – | – | – | $19.0 \pm 1.3^{c}$ |
| Fatty acids mix | – | – | – | – | $46.1 \pm 1.4^{a}$ |
| Fatty acids mix | – | 50 μM | – | 3 μM | $20.6 \pm 1.0^{c}$ |
| Arachidonic acid | – | – | – | – | $44.0 \pm 1.7^{a}$ |
| Arachidonic acid | 10 μM | – | – | – | $25.0 \pm 2.2^{bc}$ |
| Arachidonic acid | 20 μM | – | – | – | $24.0 \pm 1.5^{bc}$ |
| Arachidonic acid | – | 50 μM | – | – | $24.2 \pm 1.5^{bc}$ |
| Arachidonic acid | – | 100 μM | – | – | $23.5 \pm 2.2^{bc}$ |
| Arachidonic acid | – | – | 25 nM | – | $30.0 \pm 2.3^{b}$ |
| Arachidonic acid | – | – | 50 nM | – | $28.7 \pm 0.6^{b}$ |
| Arachidonic acid | – | – | – | 3 μM | $32.5 \pm 1.6^{b}$ |
| Arachidonic acid | – | – | – | 6 μM | $31.0 \pm 3.2^{b}$ |
| Arachidonic acid | 10 μM | – | 25 nM | – | $20.6 \pm 1.1^{c}$ |
| Arachidonic acid | 10 μM | – | – | 3 μM | $20.7 \pm 1.6^{c}$ |
| Arachidonic acid | – | 50 μM | 25 nM | – | $19.5 \pm 1.3^{c}$ |
| Arachidonic acid | | 50 μM | – | 3 μM | $18.0 \pm 2.1^{c}$ |

Values within a column with different superscript letters were significantly different ($P < 0.05$) Values are mean ± SEM of four independent experiments; $n = 4$

*PKA* Protein kinase A, *PKC* protein kinase C

**Table 7** Effect of oleic and linoleic acid on the motility and hyperactivity of boar spermatozoa

| Treatment | Motility (%) | | Hyperactivity (%) | |
|---|---|---|---|---|
| | 1 h | 3 h | 1 h | 3 h |
| Control | 69.04 ± 3.6 | $59.23 \pm 3.3^{a}$ | $8.20 \pm 1.2^{A}$ | $10.25 \pm 2.2^{x}$ |
| Oleic acid | 74.41 ± 2.8 | $70.22 \pm 4.5^{b}$ | $14.18 \pm 2.4^{B}$ | $29.62 \pm 3.9^{y}$ |
| Linoleic acid | 72.36 ± 4.1 | $64.61 \pm 2.8^{ab}$ | $12.23 \pm 3.7^{AB}$ | $17.36 \pm 2.5^{xy}$ |

Values within a column with different superscript letters were significantly different ($P < 0.05$). Values are mean ± SEM of four independent experiments; $n = 4$

Spermatozoa were incubated for 1 and 3 h

that decreased as their degree of saturation increase; where as linoleic acid has two double bond at 9th and 12th carbon position. It may be relevant that Klausner et al. [27] have suggested that oleic acid enter different lipid domains in natural and artificial membranes than do other unsaturated or saturated fatty acids. This result is in agreement with Mita et al. [28] showing that $^{14}$C-oleic acid was mainly recovered in the TG fraction in sea urchin sperm. Lahnsteiner [29] observed in rainbow trout that TG is the major substrate for energy production in spermatozoa. Aziz et al. [30] found that incorporation of fatty acids into TG are utilized as substrate of energy supply in mammalian sperm. He also mentioned that not only fatty acids but also fructose contribute to the energy supply. However, boar sperm have few carbohydrates [31], therefore it seems that endogenous TG derived from fatty acids may be the main substrate of metabolic energy for boar sperm functions. In the present experiments, $^{14}$C-oleic acid was rapidly oxidized to $CO_2$. This finding is in agreement with our previous results that fatty acids are required for active boar sperm function such as motility, viability and AR [5], and that the fatty acid derived from TG is oxidized to produce ATP through $\beta$-oxidation and the Krebs cycle [28], although possible contribution of carbohydrate to energy metabolism in boar sperm is considerable.

Glycolysis is generally considered as main substrate for ATP production in mammalian sperm. We previously reported that fructose not glucose; potentially enhance both progressive motility [32] and rate of in vitro fertilization in boar sperm [33]. Energy in the form of ATP is necessary for hyperactivating motility of boar sperm [34]. Therefore, boar sperm may have the potentiality of ATP utilization

from diverse substrates, fatty acids and fructose. Namely, boar sperm seem to have complex system to optimize energy levels by simultaneous control of separate metabolic pathways, like $\beta$-oxidation and fructolysis for regulating intracellular ATP levels and ATP/ADP ratio.

We also showed here that the second highest level of radioactivity was recovered in the PL fraction. Lardy and Phillips [35] and Lardy et al. [36] observed in the bull that spermatozoa rely on intracellular PL for energy substrate. Thus, the second highest rate of PL synthesis in boar sperm is in agreement with the finding of these reports [35, 36]. Moreover, Mann [37] reported that mammalian sperm contain less intracellular glycogen, thus sperm must rely on PL as well as TG and fructose to support respiration and oxidative phosphorylation in sperm activity.

The higher incorporations of combined radiolabeled fatty acids into boar sperm indicates that the sperm utilize more than one fatty acid at a time. Although similar concentration of $^{14}$C-oleic and $^{3}$H-linoleic acid were used in the combined radiolabel and alone radiolabel, the turnover rate in combined radiolabel was much higher than the alone radiolabel separate use, this phenomena indicates that utilization rate of fatty acids increased when they were applied synergistically. This result is in accordance with our previous study [5], indicating that higher induction of AR is obtained by addition of combination of fatty acids to the boar sperm as compared with the addition of the single fatty acid.

When BSA was used as a sterol acceptor, 20% of membrane cholesterol decreased in the mouse spermatozoa [25]. A similar magnitude of cholesterol efflux and increase in the rate of AR were observed here in the presence of BSA bound fatty acids especially with linoleic acid, suggesting that boar sperm AR and cholesterol efflux are closely related. BSA-V caused higher cholesterol efflux than the BSA-FAF and again became almost same when FAM was added in the medium, indicating that cholesterol efflux by BSA-V is mainly due to the effects of fatty acids included in the BSA. Similar observation on cholesterol efflux from mammalian sperm by BSA-V was reported by Langlais and Roberts [38]. Furthermore, oleic acid failed to cause cholesterol efflux, and the performance of arachidonic acid in inducing AR is the same as that of PVA plus FAM. These findings suggest that cholesterol efflux by FAM is mainly due to the arachidonic acid. Due to the higher number of double bonds in arachidonic acid, it has a higher scope to remove cholesterol from the sperm plasma membrane by altering the bulk biophysical properties of the membrane through changing membrane fluidity, resulting in high rate induction of AR.

Inhibitors of PKA and PKC pathways suppressed AR induced by fatty acid mixture or arachidonic acid. These findings indicate that fatty acids might exert a direct effect on AR by the regulation of both protein kinase-dependent pathways in boar sperm. It has been reported that arachidonic acid and related unsaturated fatty acids cause $Ca^{2+}$ entry into cells, activation of PKA, inhibition of Ras-GTPase protein and activation of a GTP binding protein [39], suggesting that arachidonic acid may act as a second messenger [40, 41]. Since second messengers generally activate specific protein kinases (PKA, PKC) [42], and arachidonic acid also activates protein kinase (PKx) contained in goat testis cytoplasm [39], unsaturated fatty acid, especially arachidoinc acid, may activate PKA and PKC to induce boar sperm AR. Inhibition of arachidonic acid-induced AR by PKA or PKC inhibitor alone was not complete, and the presence of both inhibitors was required for complete inhibition, suggesting that both pathways participate in AR. However, the inhibition of the AR was stronger in PKA inhibitor than that by PKC inhibitor, indicating that the cAMP dependant signaling pathway is superior to the $Ca^{2+}$ dependant pathway in induction of boar sperm AR. Cooperation of cAMP-dependent and $Ca^{2+}$-dependent pathways in cell signaling has been demonstrated in a number of cell types including spermatozoa [43]. It was also suggested that the PKA and PKC pathways cross-regulate AR in human sperm [44]. Similar interaction of PKA and PKC signaling pathways after the activation of arachidonic acid-specific receptors may be involved in arachidonic acid-induced AR in boar sperm. The new insight in the present study suggesting the regulation of fatty acids induction through PKA and PKC pathways in boar sperm may open the way for clarifying signal transduction underlying induction of AR.

## References

1. Chen RF. Removal of fatty acids from serum albumin by charcoal treatment. J Biol Chem. 1967;242:173–81.
2. Harrison RAP, Dott HM, Foster GC. Bovine serum albumin, sperm motility and the dilution effect. J Exp Zool. 1982;222:81–8.
3. Ravnik SE, Albers JJ, Muller CH. A novel view of albumin supported sperm capacitation: role of lipid transfer protein-1. Fertil Steril. 1993;59:629–38.
4. Hossain MS, Hyeong LJ, Miah AG, Tsujii H. Effect of fatty acids bound to bovine serum albumin-V on acrosome reaction and utilization of glucose in boar spermatozoa. Reprod Med Biol. 2007;6:109–15.
5. Hossain MS, Tareq KMA, Hamano K, Tsujii H. Effect of fatty acids on boar sperm motility, viability and acrosome reaction. Reprod Med Biol. 2007;6:235–9.
6. Meizel S, Turner KO. Stimulation of an exocytotic event, the hamster sperm acrosome reaction, by *cis*-unsaturated fatty acids. FEBS Lett. 1983;161:315–8.
7. Mukai C, Okuno M. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biol Reprod. 2004;71:540–7.

8. Mita M, Nakamura M. Energy metablism of sea Urchin spermatozoa: An approach based on echinoid phylogeny. Zool Sci. 1998;15:1–10.
9. Pratt JH, Longcope C. Effect of adrenocorticotropin on production rates and metabolic clearance rates of testosterone and estriol. J Clin Endocrinol Metab. 1978;47:307–13.
10. Voet D, Voet JG. Lipids and membranes. In: Rose N, editor. Biochemistry. 2nd ed. New York: Wiley; 1995. p. 277–314.
11. Meizel S. The mammalian sperm acrosome reaction, a biochemical approach. In: Johnson MH, editor. Development in mammals. Amsterdam: North-Holland Publishing; 1978. p. 1–64.
12. Johnson LA, Aalbers JG, Grooten HJG. Artificial insemination of swine: fecundity of boar semen stored in Beltsville TS (BTS), modified Modena (MM), or MR-A and inseminated on one, three and four days after collection. Reprod Domest Anim. 2007; 23:49–55.
13. Parrish JJ, Parrish SJ, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod. 1988;38:1171–80.
14. Hiroshi H, Masashi M. A cyclic adenosine 3′, 5′-monophosphate-dependent protein kinase C activation is involved in the hyperactivation of boar spermatozoa. Mol Reprod Dev. 2006;73:1169–78.
15. Miah AG, Salma U, Takagi Y, Kohsaka T, Hamano K, Tsujii H. Effect of relaxin and IGF-I on capacitation, acrosome reaction, cholesterol efflux and utilization of labeled and unlabeled glucose in porcine spermatozoa. Reprod Med Biol. 2008;7:29–36.
16. Ward CR, Storey BT. Determination of the time course of capacitation in mouse sperm using a chlortetracycline fluorescence assay. Dev Biol. 1984;104:287–96.
17. Fraser LR, Abeydeera LR, Niwa K. $Ca^{2+}$ regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Mol Reprod Dev. 1995;40:233–41.
18. Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissue. J Biol Chem. 1957;226:497–509.
19. Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf G. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development. 1995;121:1129–37.
20. Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, et al. Cholesterol efflux-mediated signal transduction in mammalian sperm. -cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosin phosphorylation and capacitation. J Biol Chem. 1999;274:3235–42.
21. Flesch FM, Brouwers JF, Nievelstein PF, Verkleij AJ, van Golde LM, Colenbrander B, et al. Bicarbonate stimulated phospholipids scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. J Cell Sci. 2001;114:3543–55.
22. Zeng Y, Clark EN, Florman HM. Sperm membrane potential: hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. Dev Biol. 1995;171:554–63.
23. Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CM. A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels. Biol Reprod. 1998;59:12–6.
24. Lin Y, Kan FW. Regionalization and redistribution of membrane phospholipids and cholesterol in mouse spermatozoa during in vitro capacitation. Biol Reprod. 1996;55:1133–46.
25. Ahluwalia B, Holman RT. Fatty acid composition of lipids of bull, boar, rabbit and human semen. J Reprod Fertil. 1969;18:431–7.
26. Creutz CE. *Cis*-unsaturated fatty acids induce the fusion of chromaffin granules aggregated by synex. J Cell Biol. 1981;91:247–56.
27. Klausner RD, Kleinfeld AM, Hoover RL, Karnovsky MJ. Lipid domains in membranes: evidence derived from structural perturbations induced by free fatty acids and lifetime heterogeneity analysis. J Biol Chem. 1980;255:1286–95.
28. Mita M, Yasumasu I, Nakamura M. Energy metabolism of spermatozoa of the sand dollar *Clypeaster japonicus*: the endogenous substrate and ultrastructural correlates. J Biochem. 1994;116:108–13.
29. Lahnsteiner F, Patzner RA, Weismann T. Energy resources of spermatozoa of the rainbow trout (*Oncorhynchus mykiss*). Reprod Nutr Dev. 1993;33:349–60.
30. Aziz MTA, El-Haggar S, Tawadrous GA, Hamada T, Shawky MA, Amin KS. Seminal lipids as energy substrate for the spermatozoa. Andrologia. 1983;15:259–63.
31. Medrano A, Pena A, Rigau T, Rodrıguez-Gil JE. Variations in the proportion of glycolytic/non-glycolytic energy substrates modulate sperm membrane integrity and function in diluted boar samples stored at 15–17°C. Reprod Dom Anim. 2005;40:448–53.
32. Tsujii H, Ohata E, Miah AG, Hossain MS, Salma U. Effect of fructose on motility, acrosome reaction and in vitro fertilization capability of boar spermatozoa. Reprod Med Biol. 2006;5:255–61.
33. Tsujii H, Lee JH, Hossain MS, Tareq KMA, Hamano K, Sawada T. The beneficial effect of fructose and glucose on in vitro maturation and the fertilization of porcine oocytes. Reprod Med Biol. 2009;8:19–24.
34. Schmidt H, Kamp G. Induced hyperactivity in boar spermatozoa and its evaluation by computer-assisted sperm analysis. Reproduction. 2004;128:171–9.
35. Lardy HA, Phillips PH. Phospholipids as a source of energy for motility of bull spermatozoa. Am J Physiol. 1941;134:542–8.
36. Lardy HA, Hansen RG, Phillips PH. The metabolism of bovine epididymal spermatozoa. Arch Biochem. 1945;6:41–51.
37. Mann T. The biochemistry of semen and of the male reproductive tract. New York: Wiley; 1964. p.100–6.
38. Langlais J, Roberts KD. A molecular membrane model of sperm capacitation and acrosome reaction of mammalian spermatozoa. Gamate Res. 1985;12:183–224.
39. Roy K, Mandal AK, Sikdar R, Majumdar S, Ono Y, Sen PC. Unsaturated fatty acid-activated protein kinase (PKx) from goat testis cytosol. Biochim Biophys Acta. 1999;1434:161–9.
40. Naor Z. Is arachidonic acid a second messenger in signal transduction? Mol Cell Endocrinol. 1991;80:C181–6.
41. Jones PM, Persaud SJ. Arachidonic acid as a second messenger in glucose-induced insulin secretion from pancreatic $\beta$-cells. J Endocrinol. 1993;137:7–14.
42. Harrison DE, Ashcroft SJ, Christie MR, Lord JM. Protein phosphorylation in the pancreatic B-cell. Experientia. 1984;40:1075–84.
43. Tollner TL, Overstreet JW, Vandevoort CA. Effect of protein kinase C stimulators on zona pellucida binding and the acrosome reaction of Mocaque sperm. Biol Reprod. 1995;52:1418–25.
44. Doherty CM, Tarchala SM, Radwanska E, De Jonge CJ. Characterization of two-second second messenger pathways and their interactions in eliciting the human sperm acrosome reaction. J Androl. 1995;16:36–46.