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The Relationship Between Acrosome Reaction and Polyunsaturated Fatty acid Composition in Boar Sperm

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Simple Summary: Sperm must undergo various physiological processes to successfully transport paternal genetic information to oocyte, of the processes, acrosome reaction is involved in fusion between sperm and oocyte. Generally, polyunsaturated fatty acids (PUFAs) composition of sperm control the acrosome reaction in mammals, however correlation with acrosome reaction and sperm PUFA composition has not yet been clearly demonstrated in pigs. Therefore, this study investigated fatty acids composition in excessive over the 85% acrosome reacted sperm and analyzed correlation with acrosome reaction and PUFAs composition in pigs. The 60 mM methyl-beta-cyclodextrin (MBCD) was used to induce acrosome reaction and fatty acid composition was analyzed using gas chromatography. The PUFAs composition dramatically decreased, otherwise saturated fatty acids (SFAs) composition increased in acrosome reacted boar sperm, there were closely negatively correlated with acrosome reaction and PUFAs in boar sperm. Therefore, acrosome reaction induced loss of PUFAs in boar sperm, the results suggest that provide knowledge for relation between PUFAs and the acrosome reaction in pigs.

Abstract: This study investigated the relationship of acrosome reactions and fatty acid composition on fertility in boar sperm. The acrosome reaction of sperm was induced by MBCD, and acrosome reaction and plasma membrane integrity were analyzed using flow cytometry, and fertility were verified via *in vitro* fertilization assay. The fatty acid composition of sperm was determined via gas chromatography. The results showed that the acrosome reaction was induced over 85% by 60 mM MBCD, and the plasma membrane integrity was significantly ($p < 0.05$) decreased and was dependent on the MBCD level. The PUFAs composition was significantly higher in non-acrosome reaction group than acrosome reacted group, on the other hand, SFAs composition was significantly lower ($p < 0.05$). Moreover, the acrosome reacted sperm from 60 mM MBCD significantly decreased *in vitro* fertility and blastocyst formation relative to non-acrosome reacted sperm ($p < 0.05$), and the acrosome reaction was positively correlated with SFAs and negatively correlated with PUFAs. Of these fatty acids, C22:5n-6 (docosapentaenoic acid [DPA]) and C22:6n-3 (docosahexaenoic acid [DHA]) were directly negatively correlated with the acrosome reaction (r = -0.982 and -0.947, respectively). In conclusion, the acrosome reactions may excessively occur by reducing the PUFAs, which may then dramatically decrease sperm fertility in pigs.

Keywords: Acrosome reaction; Polyunsaturated fatty acid; Sperm; Pigs

1. Introduction

Sperm are the male germ cells, which are produced in the testes and are then excreted through the vas deferens, after which they acquire capacitation [1]. Unlike oocytes, sperm have motility, and

they transport paternal genetic information to the oocyte for the creation of new organisms [2]. The sperm primarily consists of a head, neck and tail, with the head containing paternal DNA and the front of the head containing enzymes that dissolves the cummus cells and zona pellucida of the oocyte, and mitochondria of neck produces energy for tail motility [3,4]. When the sperm meets the oocyte in the female reproductive tract, the sperm releases the acrosome that is contained in the front of the head for successful fertilization, and this chemical reaction is defined as the acrosome reaction [5].

The acrosome reaction does not occur in the testis but is caused by various environmental changes after injection of semen [5]. Generally, the acrosome reaction occurs in the female reproductive tract, such as the uterine and the oviduct, when the sperm reaches these organs, decapitation factors are physically deteriorated via sperm motility, after which the plasma membranes are damaged which is due to higher HCO_3^- levels that are found within the female reproductive environment than in a normal environment [5]. Finally, outer acrosomal membranes are fused with damaged plasma membranes, and the acrosome is released from the sperm head [5]. During this process, hyaluronidase is released outside of the acrosome region, while calcium ion (Ca^{2+}) enters into the acrosome to activate acrosin [6]. Of these enzymes, hyaluronidase plays a role in the weakening of the gap junctions of the cumulus cells, and acrosin dissolves the zona pellucida to allow sperm penetration, only the sperm head can enter the oocyte [7,8].

The acrosome is generated from the acrosomal granules of the spermatogonia stem cells and moves to the anterior part of the sperm head during spermiogenesis [9]. It exists as a cap shape above the sperm head and contains various factors for penetrating the oocyte [9]. For this reason, the acrosome reaction must occur at the timing in the encounter with oocyte in oviduct, therefore, the acrosome should be safely wrapped around the outer acrosomal membrane during moving to oviduct and preserved until fertilization with oocyte [10]. In the aspect of reproductive cell biology, the acrosome reaction is initiated by phospholipid scrambling via HCO_3^- [11], G-protein activation via progesterone and its receptor binding in plasma membrane [12], pH changes inside of the cell [6], plasma membrane lipid peroxidation via influx of reactive oxygen species (ROS), capacitation by Ca^{2+} [13], and physical damage to the plasma membrane [10]. Finally, the events induce a fusion of the plasma membrane and the outer acrosomal membrane which triggered to release acrosome from sperm head. Specifically, the phospholipid makeup of the plasma membrane is scrambled, and the fatty acids of the phospholipid bilayer are modified, and consequentially, the acrosome reaction is very closely related to the fatty acid activity in mammalian sperm [10].

Fatty acids are composed of long carbon chains, with a carboxyl group (-COOH) situated at one end and a methyl group (-CH$_3$) situated at the other end [14]. Every cell contains fatty acids, and its metabolism of animal cells not only plays an important role in energy production but also regulates the binding of other lipids, which allows for the resistance of physical and chemical stress, for the maintenance of cellular balance, and for the contribution to cell homeostasis [15]. Fatty acids play an important role in the survival, maturation, and fertilization of sperm, especially in regard to boar sperm, which contain a higher proportion of unsaturated fatty acids (PUFAs) in the plasma membrane, compared to other species [16,17]. The plasma membrane is the first sperm cell organelle to meet with the oocyte, and it is necessary for membrane fluidity and for the successful fusion with the oocyte surface, and PUFAs of the plasma membrane are related with the fluidity of the membrane [17]. Due to the specific fluidity of the membrane structures, the fusion of the plasma membrane and the outer acrosomal membrane for the acrosome reaction easily occurs, therefore, the ratio of PUFAs of the plasma membrane is directly involved with sperm viability and fertility [18,19]. Due to these characteristics, the fatty acids of sperm have been studied over a long time periods for their potential to function as predictive markers for semen quality, with the idea that they can be used as therapies for infertility and for the selection of superior species, based on predictions of motility, viability, semen volume, and sperm concentration [20,21]. However, studies on the changes in fatty acid composition in excessive acrosome-reacted sperm have not yet been conducted, and correlation with acrosome reaction and sperm PUFA composition have not yet been clearly demonstrated in pigs. Therefore, this study aimed to investigate the influence of the acrosome reaction on fatty acid

composition, plasma membrane integrity, and *in vitro* fertilization (IVF) ability in >85% acrosome-reacted boar sperm.

2. Materials and Methods

2.1. Animals and Semen Collection

All experiment procedure that included animals followed the scientific and ethical regulations proposed by the European Animal Experiment Handling License Textbook [22] and approved from Animal Experiment Ethics Committee in Kangwon National University, Republic of Korea (No: KIACUC-09-0139). Fresh semen samples from pigs (Duroc), which had an average age of 34.0 ± 2.5 months, were collected via a glove-hand method from five pigs. Collected sperm was diluted with a semen extender (Modena B; 30.0 g/L glucose, 2.25 g/L EDTA, 2.50 g/L sodium citrate, 1.00 g/L sodium bicarbonate, 5.00 g/L Tris, 2.50 g/L citric acid, 0.05 g/L cysteine, and 0.30 g/L gentamicin sulfate) until a concentration of 1.5×10^7 sperm/mL was obtained, after which the samples were transported to a laboratory within 2 h at 18°C before experiment.

2.2. Acrosome Reaction using Methyl-Beta-Cyclodextrin (MBCD)

The diluted semen samples were centrifuged at 410 g for 5 min, after which the supernatant was removed and resuspended with a semen extender until a concentration of 1.5×10^7 sperm/mL was obtained. Afterwards, the semen samples were treated with 0, 10, 20, 30, 40, and 60 mM MBCD (Sigma, MO, USA) for 30 min at room temperature, and the supernatant was removed via centrifugation. Additionally, 60 mM MBCD was added as treatments in the semen based on the following concentrations: 3.5×10^7, 7.0×10^7, 3.5×10^8, and 1.0×10^9 sperm/mL. These concentrations allowed for the examination of the acrosome reactional influence of MBCD on sperm concentration.

2.3. Acrosome Reaction Detection

Sperm samples were diluted with extender until a concentration of 7.5×10^6 sperm/mL was obtained, after which 2 μM lectin from arachis hypogagea (FITC-PNA; Sigma) and 2 μM propidium iodide (PI; Sigma) were added to each of the samples for 5 min. After the incubation, samples were centrifuged at 410 g for 5 min, the supernatants were removed. Samples were resuspended with PBS, and a total of 10,000 sperm were analyzed via flow cytometry (BD Biosciences, CA, USA). FITC-PNA positive sperm were evaluated, in order to detected acrosome-reacted sperm.

2.4. Sperm Plasma Membrane Integrity Assay

Sperm plasma membrane integrity assays were performed according to methods from a previous study [23]. Forty nM SYBR-14 (Molecular probes, NY, USA) and 2 μM PI were added to 1.5×10^6 sperm/mL at 38°C for 5 min, and these samples were centrifuged to remove supernatants at 410 g for 5 min. Samples were resuspended with PBS, and a total of 10,000 sperm were analyzed via flow cytometry.

2.5. In Vitro Fertilization (IVF)

All IVF methods and processes were performed according to methods from a previous study [23]. Ovaries were collected from prepubertal gilts at a slaughter house (Pocheonfarm, Pocheon, Republic of Korea) and transported to the laboratory within 2 h. Oocytes were then aspirated from the antral follicles. Intact cumulus–oocyte complexes (COCs) were selected, washed in PBS-PVA, and incubated in maturation medium that consisted of modified TCM-199 (Sigma) with 10 ng/ml of epidermal growth factor (EGF; Sigma), 10 IU/ml of human chorionic gonadotropin (hCG; Sigma), and 10% (v/v) porcine follicular fluid (pFF) with luteinizing hormone (LH; Sigma) and follicle stimulating hormone (FSH; Sigma) for 22 h at 38.5°C and 5% CO_2. Afterwards, COCs were transported in the maturation medium without hormonal supplements and incubated for 22 h at 38.5°C and 5% CO_2. After 44 h of culturing, all of the oocytes were separated from the enclosing

cumulus cells via gentle pipetting in a maturation medium containing 0.1% hyaluronidase (Sigma). The fertilization medium was based on a modified Tris-buffered medium (mTBM) with caffeine, and mature oocytes were washed and transferred to a 25 μL droplet of mTBM covered with paraffin oil. To investigate the IVF ability, 25 μL of 6.0×10^5 sperm/mL in mTBM aliquots were added to samples that contained maturated oocytes. The sperm-oocytes were incubated for 6 h at 38.5°C under 5% CO_2. After fertilization, extra spermatozoa were removed from the oocytes by repetitive pipetting, and oocytes were washed in culture media (porcine zygote medium: PZM-3 with 0.3% BSA) and incubated at 38.5°C for 192 h under 5% CO_2 in humidified air. The oocyte cleavage and blastocyst formation rates were assessed via microscopy after 192 h of culture.

2.6. Gas Chromatography

To induce excessive acrosome reactions, the 3.5×10^7 sperm/mL samples were treated with 60 mM MBCD for 30 min at room temperature. Afterwards, samples were washed twice at 410 g for 5 min, then centrifuged at 3,700 g, 4°C for 10 min. Samples were diluted with semen extender, then centrifuged at 13,000 g, 4°C for 20 min and were stored at -80°C before the experiment. Sperm pellets were completely diluted with 1 mL semen extender, which were then diluted with 20 mL chloroform-methanol (2:1) and 0.88% NaCl solution, after which they were mixed for 5 min. After incubation for 36 h at 4°C, samples were centrifuged at 1,700 g for 30 min, and the 10 mL bottom layer was transferred to a new tube, and the solvents were removed using nitrogen air. Afterwards, 1 mL of 0.5 N methanolic NaOH was added and incubated at 100 °C for 15 min. After cooling, 2 mL of 14% Boron trifluoride (BF_3)-methanol was added and incubated at 100°C for 15 min, after cooling, 1 mL of heptane and 2 mL of NaCl were diluted and incubated for 40 min at room temperature. The supernatant was analyzed via gas chromatography (Agilent Technologies Co., CA, USA).

2.7. Statistical Analysis

Statistical analyses were conducted by using SAS v. 9.4 (SAS Institute, USA). Data were evaluated using analysis of variance (ANOVA) and Duncan's multiple-range tests via the use of general linear models. The differences were considered to be statistically significant at $p < 0.05$. Correlation tests were conducted between the analysis of the acrosome reaction, the plasma membrane integrity, the IVF ability, and the fatty acid composition.

3. Results

3.1. Effect of MBCD Level on Acrosome Reaction

The influence of MBCD on acrosome reactions in boar sperm are shown in Figure 1A. The 10 mM MBCD group exhibited no significant differences compared to the 0 mM MBCD group, in regard to the boar sperm acrosome reaction; however, the 20 and 30 mM MBCD treatments significantly induced acrosome reactions, compared to the 0 mM MBCD treatment ($p < 0.05$). Additionally, acrosome reactions were significantly increased in the 40 and 60 mM MBCD groups, compared to the other treatment groups ($p < 0.05$). Furthermore, over 85% of the sperm were induced to have acrosome reactions (n=3, 87.16, 88.32, and 85.65%) via the 60 mM MBCD treatment.

### 3.3. Effect of Sperm Concentrations on Acrosome Reaction

Based on previous experiments, we conducted tests examining the effect of 60 mM MBCD on the acrosome reactions of sperm concentrations, and the results are shown in Figure 1B. Acrosome reactions were induced by 60 mM MBCD in over 85% of the sperm in all of the treatments (3.5×10^7, 7.0×10^7, 3.5×10^8, and 1.0×10^9 sperm/mL), and acrosome reactions were significantly increased in the 3.5×10^7 sperm/mL ($89.31 \pm 3.15\%$) group than in the 3.5×10^8 sperm/mL ($83.64 \pm 1.94\%$) and 1.0×10^9 sperm/mL ($81.29 \pm 1.73\%$) groups ($p < 0.05$).

a

b
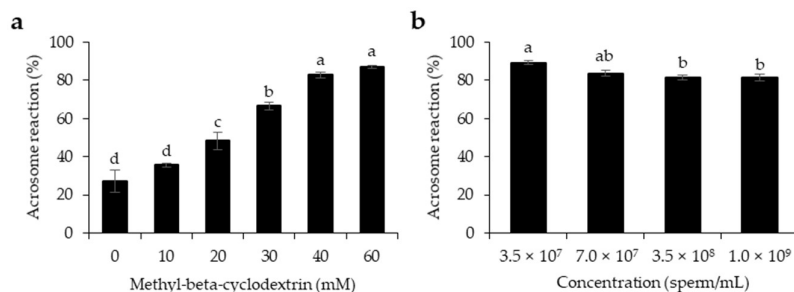
Figure 1. Change of acrosome reaction by methyl-beta-cyclodextrin (MBCD) level (**a**) and sperm concentration (**b**) in boar semen. The MBCD were treated in 1.5×10^7 sperm/mL of semen (**a**) and 60 mM MBCD was treated in each other concentration sperm (**b**). [a-d] Values with differents uperscripts in the same column with sperm are significantly different ($p < 0.05$, n=3)

3.3. Plasma Membrane Integrity

The influence of MBCD on the plasma membrane integrity in boar sperm is shown in Figure 2. The plasma membrane integrity was significantly decreased, according to the MBCD level ($p < 0.05$). In particular, treatments greater than 30 mM MBCD dramatically decreased the plasma membrane integrity in boar sperm, and the plasma membrane integrity was significantly lower at 60 mM MBCD ($20.68 \pm 0.12\%$) than in the other groups ($p < 0.05$).
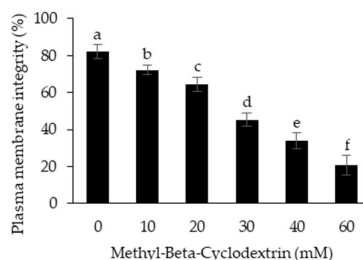
Figure 2. Effect of plasma membrane integrity by methyl-beta-cyclodextrin in fresh boar sperm. [a-f] Values with differents uperscripts in the same column with sperm are significantly different ($p < 0.05$, n=3).

3.3. Fatty Acid Composition

Changes in fatty acid composition, according to the acrosome reactions in boar sperm, are shown in Table 1. C16:0 (palmitic acid), C18:0 (stearic acid), and C18:1n-9 (oleic acid) ratios were significantly increased in the acrosome reaction groups (over 85% of the acrosome-reacted sperm) compared to the non-acrosome reaction group ($p < 0.01$). Conversely, the C22:5n-6 (docosapentaenoic acid [DPA]) and C22:6n-3 (docosahexaenoic acid [DHA]) ratio was decreased in the acrosome reaction group compared to the non-acrosome reaction group ($p < 0.01$). The saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) ratio was significantly increased in the acrosome reaction group, conversely, the unsaturated fatty acid (UFA) and polyunsaturated fatty acid (PUFA) ratio was significantly decreased in the acrosome reaction group ($p < 0.01$). In addition, the SFA/UFA ratio was significantly increased in the acrosome reaction group ($p < 0.05$).

3.4. Fertility Assay

The influence of excessive acrosome-reacted boar sperm on *in vitro* fertility is shown in Table 2. Embryo cleavage was significantly decreased in the acrosome reaction group, compared to the non-acrosome reaction group, and blastocyst formation rates were significantly decreased in the acrosome reaction group, compared to the non-acrosome reaction group ($p < 0.01$). In particular, no blastocysts were formed in the acrosome reaction group, and the degeneration ratios were increased in the acrosome reaction group compared to the non-acrosome reaction group.

Table 1. Comparison of relative fatty acids composition between non-acrosome reacted and excessive acrosome reacted by methyl-beta-cyclodextrin in pigs.

Fatty acids	Sperm type	
	Non-acrosome reacted [1]	Acrosome reacted [2]
C14:0 [3]	13.91 ± 0.24	13.66 ± 0.33
C16:0 [4]	21.58 ± 0.27	27.56 ± 0.24 *
C18:0 [5]	12.55 ± 0.20	21.63 ± 0.15 *
C18:1n-9 [6]	3.90 ± 0.09	5.74 ± 0.19 *
C18:2n-6 [7]	3.39 ± 0.07	4.60 ± 0.46
C20:0 [8]	0.94 ± 0.13	0.94 ± 0.13
C20:3n-6 [9]	0.97 ± 0.05	0.68 ± 0.12
C20:4n-6 [10]	3.40 ± 0.18	2.82 ± 0.13
C22:5n-6 [11]	26.50 ± 0.29	13.94 ± 0.35 *
C22:6n-3 [12]	12.85 ± 0.47	8.42 ± 0.15*
SFA [13]	48.99 ± 0.61	63.80 ± 0.48 *
UFA [14]	51.01 ± 0.61	36.20 ± 0.48 *
MUFA [15]	3.90 ± 0.09	5.74 ± 0.19 *
PUFA [16]	47.12 ± 0.52	30.46 ± 0.45 *
SFA/UFA	0.96 ± 0.02	1.76 ± 0.04 *

[1] fresh sperm, [2] 60 mM methyl-beta-cyclodextrin treated sperm, [3] myristic acid, [4] palmitic acid, [5] stearic acid, [6] oleic acid, [7] linoleic acid, [8] arachidic acid, [9] dihomo-y-linolenic acid, [10] eicosatetraenoic acid, [11] docosapentaenoic acid (DPA), [12] docosahexaenoic acid (DHA), [13] saturated fatty acid, [14] unsaturated fatty acid, [15] mono unsaturated fatty acid, [16] poly unsaturated fatty acid, values with different superscripts within rows show significant difference, mean ± SEM, *$p < 0.01$, n=3

Table 2. *In vitro* development of oocytes of 168 h after fertilized with non-acrosome reacted and acrosome reacted sperm in pigs.

Sperm type	No. of oocytes examined	Cleavage (%)	No. of embryo development (%)		Degeneration (%)
			2 cell to morula	Blastocyst	
Non-acrosome reaction [1]	270	223 (82.2±1.2)	173 (63.7±1.0)	50 (18.5±0.2)	47 (17.8±1.2)
Acrosome reaction [2]	266	138 (51.9±0.5) *	138 (51.9±0.5) *	0 (0.0±0.0) *	128 (48.1±0.5) *

[1] fresh sperm, [2] 60 mM methyl-beta-cyclodextrin treated sperm, values in the same column with different superscripts are significantly different, mean ± SEM, *$p < 0.05$, n=3.

3.5. Relationship between Acrosome Reactions and Fatty Acids Composition

Correlations between the composition of fatty acids and the acrosome reaction, plasma membrane integrity, *in vitro* fertility, and blastocyst formation in boar sperm are shown in Tables 3 and 4. The acrosome reactions were highly negatively correlated with plasma membrane integrity ($p = 0.0007$), *in vitro* fertility ($p = 0.0012$), and blastocyst formation ($p = 0.0003$) (Table 3). The C16:0 ($p = 0.0021$), C18:0 ($p = 0.0003$), C18:1n-9 ($p = 0.0079$), SFA ($p = 0.0006$), MUFA ($p = 0.0079$), and SFA/UFA ($p = 0.0004$) ratios were positively correlated with acrosome reactions and were also negatively correlated with the plasma membrane integrity, *in vitro* fertility, and blastocyst formation (Table 4). Additionally, the C20:4n-6 ($p = 0.0332$), C22:5n-6 ($p = 0.0005$), C22:6n-3 ($p = 0.0042$), UFA ($p = 0.0003$),

and PUFA (p = 0.0004) ratios were negatively correlated with acrosome reactions and were also positively correlated with the plasma membrane integrity, *in vitro* fertility, and blastocyst formation (Table 4).

Table 3. Correlations among the acrosome reaction versus plasma membrane integrity, *in vitro* fertility, and blastocyst formation in boar sperm

Items	Plasma membrane integrity		*In vitro* fertility		Blastocyst formation	
	r	p value	r	p value	r	p value
Acrosome reaction	-0.978	0.0007	-0.972	0.0012	-0.980	0.0006

4. Discussion

The biochemical mechanisms in acrosome reacted sperm during fertilization have been studied to understand causes of male infertility for decades [10,24]. Generally, in female reproductive tract, sperm acquire capacitation at this time, and the fluidity of sperm is increased due to scrambling of the plasma membrane, since then, the acrosome of sperm head is extracted to extracellular by the fusion of the plasma membrane and the outer acrosomal membrane [10]. The sperm membrane is composed of glycoproteins [25], which contains high levels of PUFAs [18] and low levels of cholesterol [26], with their interaction being necessary for maintaining the survival and motility of sperm [27]. The acrosome reaction is an essential phenomenon for the acquisition of fertility in sperm, for this reason, many studies on acrosome reaction mechanism have been investigated [10]. Especially, many studies reported that sperm fatty acids composition is changed during oxidative stress [20,28] and freezing [16] because these events induce plasma membrane damage and acrosome reactions. The studies on acrosome reaction and fatty acids composition in pigs have been mostly conducted that freezing-thawed sperm [16,29] and ejaculated sperm from PUFAs supplemented pigs [30,31] and difference breed [32], in the studies, fatty acids composition had been analyzed in acrosome reacted sperm with 8-16% [16], 25-60% [29], 45-50% [30], 8-13% [31], and 10-55% [32]. However, the studies could not be clearly demonstrated whether changes of exactly fatty acids composition according to acrosome reaction in boar sperm, therefore, we investigated changes in fatty acids composition in extremely acrosome-reacted sperm (over the 85%) in pigs. The majority of cells contain cholesterol in the plasma membrane. If there is an efflux of cholesterol from the plasma membrane, the membrane is damaged, involving events such as phospholipid scrambling, membrane structure disruption, and PUFAs oxidation [26]. The MBCD represents the cholesterol efflux material from the plasma membrane, and several studies have reported that not only is 89% of cholesterol reduced, but P4R is also activated, when MBCD was added to semen for 24 h [33]. In this study, over 85% of sperm were induced to having acrosome reactions by 60 mM MBCD in 3.5×10^7 sperm/mL semen for 30 min, and excessive acrosome-reacted sperm was used to investigate the fatty acid composition and *in vitro* fertility.

Sperm is covered with lipoproteins, and the cell membrane is involved in fertilization when the sperm meets the oocyte [34]. Moreover, the reproductive mechanism is one of the major areas of interest in sperm physiology and pathology [34]. In addition, membrane fusion with the oocyte and the normal morphology of sperm are predominant conditions that primarily depend on the lipid composition [10]. In practice, previous studies have reported that mammalian and nonmammalian sperm are composed of triglycerides, cholesterol, phospholipids, and glycolipids [10]. In particular, phospholipids are the most abundant types of lipids in the sperm plasma membrane and are classified as phosphatidylcholine and phosphatidylethanolamine [35]. It has been reported that many UFAs are composed of fatty acids from plasmalogen, specifically, C18:3n-3, C18:2n-6, and C18:1n-6 in the sperm are converted to PUFAs via fatty acid metabolism [36]. The highly PUFAs (HUFAs), such as C22:5n-6 (DPA) and C22:6n-3 (DHA), were found in boar sperm, and the composition of the fatty acids protects the sperm from oxidative stress [36]. However, the PUFAs in the plasma membrane are damaged during the fertilization process, which then leads to fusion with the outer

acrosomal membrane [10]. Thus, cholesterol efflux and PUFA damage in the sperm plasma membrane is directly linked to the acrosome reaction, and the results also confirmed that excessive acrosome reactions (over 85%) were induced by high concentrations of MBCD.

Table 4. Correlations between composition of fatty acids and acrosome reaction, plasma membrane integrity, *in vitro* fertility, and blastocyst formation in boar sperm

Items	Acrosome reaction		Plasma membrane integrity		*In vitro* fertility		Blastocyst formation	
	r	p value	r	p value	r	p value	r	p value
C14:0 [1]	-0.137	0.7962	0.299	0.5654	0.316	0.5423	0.290	0.5766
C16:0 [2]	0.962	0.0021	-0.994	<.0001	-0.987	0.0003	-0.993	<.0001
C18:0 [3]	0.986	0.0003	-0.998	<.0001	-0.992	<.0001	-0.999	<.0001
C18:1n-9 [4]	0.927	0.0079	-0.975	0.001	-0.979	0.0007	-0.973	0.0011
C18:2n-6 [5]	0.741	0.0922	-0.792	0.0606	-0.771	0.073	-0.791	0.0607
C20:0 [6]	0.138	0.7943	-0.012	0.9826	-0.007	0.9893	-0.018	0.9726
C20:3n-6 [7]	-0.753	0.0837	0.731	0.0987	0.737	0.0946	0.732	0.098
C20:4n-6 [8]	-0.847	0.0332	0.793	0.0596	0.828	0.042	0.797	0.0578
C22:5n-6 [9]	-0.982	0.0005	0.997	<.0001	0.991	0.0001	0.998	<.0001
C22:6n-3 [10]	-0.947	0.0042	0.977	0.0008	0.960	0.0024	0.976	0.0008
SFA [11]	0.987	0.0003	-0.995	<.0001	-0.987	0.0002	-0.995	<.0001
UFA [12]	-0.987	0.0003	0.995	<.0001	0.987	0.0002	0.995	<.0001
MUFA [13]	0.927	0.0079	-0.975	0.001	-0.979	0.0007	-0.973	0.0011
PUFA [14]	-0.984	0.0004	0.997	<.0001	0.991	0.0001	0.997	<.0001
SFA/UFA	0.985	0.0004	-0.994	<.0001	-0.989	0.0002	-0.995	<.0001

[1] myristic acid, [2] palmitic acid, [3] stearic acid, [4] oleic acid, [5] linoleic acid, [6] arachidic acid, [7] dihomo-y-linolenic acid, [8] eicosatetraenoic acid, [9] docosapentaenoic acid (DPA), [10] docosahexaenoic acid (DHA), [11] saturated fatty acid, [12] unsaturated fatty acid, [13] mono unsaturated fatty acid, [14] poly unsaturated fatty acid

The sperm plasma membrane is a thin layer that covers the surface, and it contains phospholipids, proteins, and PUFAs, in particular, pigs contain a higher proportion of PUFAs in the sperm plasma membrane than other species [36]. The PUFA is a fatty acid containing a double bond, and it is chemically unstable compared to SAFs, because it is easily oxidized and has a low melting point [37]. In the aspect of dietetics, PUFA is an essential fatty acid, not only for the removal of ROS, the regulation of blood cholesterol, and the regulation of blood lipoproteins but also for the maintenance of the retina, brain, and cell structure in mammals [37]. DPA and DHA are omega-3 PUFAs that maintain cellular structure and regulate metabolism in mammals [38]. They also play various roles, such as inhibition of the blood epithelium, promotion of bone formation, reduction of cholesterol and triglycerides, and improvement of circulatory system function [39]. On the other hand, SFAs have high chemical stability and a high melting point because they do not have double bonds [39]. Moreover, PUFA intake is known to be directly related to sperm function, in fact, previous studies have reported that PUFA intake improved sperm concentration, viability, and motility in domestic animals, and it also increased the properties of PUFAs in human [17,40]. In addition, a reduction of PUFA in the sperm plasma membrane induces oxidative stress, which indicates that lipid peroxidation is closely related to sperm motility, survival rate, and normal morphology [41]. Infertile male sperm contains lower levels of DHA than normal male sperm, and DHA is associated with tail motility in sperm [42]. However, PUFAs are vulnerable to the production of ROS by oxides, which affects not only lipid peroxidation but also midpiece abnormalities [26]. Finally, it also induces

the reduction of motility, plasma membrane damage, low characteristics, and negative influences on fusion with the oocyte [43]. Our results demonstrated that the plasma membrane integrity was reduced with increases of the acrosome reaction in boar sperm, and the results suggest that plasma membrane disruption is necessary for the efflux of the acrosome from the sperm head. In addition, the study showed that DPA and DHA were decreased in excessive acrosome-reacted sperm and exhibited highly negative correlations with the acrosome reaction. Overall, the PUFAs, DPA and DHA may be used as accurate, predictive fatty acid markers for excessive acrosome reactions in boar sperm.

Generally, the acrosome reaction is an essential process for successful fertilization for the meeting of sperm and oocytes, however, if the acrosome reaction is induced at an inappropriate time, such as before the penetration of the female reproductive tract and fusion with the oocyte, sperm fertility is dramatically decreased [24]. This indicates that acrosome-reacted sperm cannot be fertilized with oocytes, and that the ROS that is generated from dead sperm produces negative effects on the viable sperm, such as lipid peroxidation and acrosome reactions, subsequently, death occurs [13]. Therefore, the development of embryos and the formation rate of blastocysts were both significantly reduced when artificial insemination or IVF was performed by using semen with a high content of acrosome-reacted sperm [44]. Our results demonstrated that excessive acrosome-reacted sperm significantly decreased embryo cleavage and blastocyst formation when fertility assays utilized IVF and that DPA and DHA have highly positive correlations with acrosome reactions, but highly negative correlations with *in vitro* fertility and blastocyst formation. These results suggest that DPA and DHA are significantly involved in the acrosome reaction and that the fatty acids could be used as predictive markers for semen quality.

In this study, over 85% of the sperm experienced acrosome reactions from MBCD treatment (which is a cholesterol efflux substance), and this resulted in increased plasma membrane damage, reduced PUFA ratios, such as DPA and DHA, and markedly decreased fertilization abilities in pigs. Therefore, the PUFAs, especially DPA and DHA, may be directly associated with the acrosome reaction in boar sperm, and studying PUFAs may help to understand the relationship between PUFAs and the acrosome reaction during sperm capacitation and fertilization in mammals.

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References

1. Kidd, S.A.; Eskenazi, B.; Wyrobek, A.J. Effects of male age on semen quality and fertility: a review of the literature. *Fertil. Steril.* **2001**, *75*, 237-248.
2. Coetzee, K.; Kruge, T.F.; Lombard, C.J. Predictive value of normal sperm morphology: a structured literature review. *Hum. Reprod. Update* **1998**, *4*, 73-82.
3. Evenson, D.P.; LARSON, K.L.; Jost, L.K. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J. Androl.* **2002**, *23*, 25-43.
4. Brewis, I.A.; Boerke, A.; Tsai, P.-s.; Gadella, B.M. Sperm head membrane reorganisation during capacitation. *Int. J. Dev. Biol.* **2004**, *52*, 473-480.
5. Abou-haila, A.; Tulsiani, D.R. Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. *Arch. Biochem. Biophys.* **2009**, *485*, 72-81.
6. Darszon, A.; Nishigaki, T.; Wood, C.; Treviño, C.L.; Felix, R.; Beltrán, C. Calcium channels and Ca^{2+} fluctuations in sperm physiology. *Int. Rev. Cytol.* **2005**, *243*, 79-172.
7. Boerke, A.; Tsai, P.; Garcia-Gil, N.; Brewis, I.; Gadella, B. Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. *Theriogenology* **2008**, *70*, 1188-1196.
8. Galindo, B.E.; Vacquier, V.D.; Swanson, W.J. Positive selection in the egg receptor for abalone sperm lysin.

Proc. Natl. Acad. Sci. **2003**, *100*, 4639-4643.

9.   Grootegoed, J.A.; Siep, M.; Baarends, W.M. Molecular and cellular mechanisms in spermatogenesis. *Best Pract. Res., Clin. Endocrinol. Metab.* **2000**, *14*, 331-343.

10.   Tulsiani, D.R.; Abou-Haila, A.; Loeser, C.R.; Pereira, B.M. The biological and functional significance of the sperm acrosome and acrosomal enzymes in mammalian fertilization. *Exp. Cell Res.* **1998**, *240*, 151-164.

11.   Boatman, D.E.; Robbins, R.S. Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. *Biol. Reprod.* **1991**, *44*, 806-813.

12.   Sabeur, K.; Edwards, D.P.; Meizel, S. Human sperm plasma membrane progesterone receptor (s) and the acrosome reaction. *Biol. Reprod.* **1996**, *54*, 993-1001.

13.   Awda, B.J.; Mackenzie-Bell, M.; Buhr, M.M. Reactive oxygen species and boar sperm function. *Biol. Reprod.* **2009**, *81*, 553-561.

14.   Horton, J.D.; Goldstein, J.L.; Brown, M.S. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **2002**, *109*, 1125-1131.

15.   Guerzoni, M.E.; Lanciotti, R.; Cocconcelli, P.S. Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in Lactobacillus helveticus. *Microbiology* **2001**, *147*, 2255-2264.

16.   Cerolini, S.; Maldjian, A.; Pizzi, F.; Gliozzi, T. Changes in sperm quality and lipid composition during cryopreservation of boar semen. *Reproduction* **2001**, *121*, 395-401.

17.   Strzezek, J.; Fraser, L.; Kuklinska, M.; Dziekonska, A.; Lecewicz, M. Effects of dietary supplementation with polyunsaturated fatty acids and antioxidants on biochemical characteristics of boar semen. *Reprod. Biol.* **2004**, *4*, 271-287.

18.   Kubagawa, H.M.; Watts, J.L.; Corrigan, C.; Edmonds, J.W.; Sztul, E.; Browse, J.; Miller, M.A. Oocyte signals derived from polyunsaturated fatty acids control sperm recruitment *in vivo*. *Nat. Cell Biol.* **2006**, *8*, 1143-1148.

19.   Lenzi, A.; Gandini, L.; Picardo, M.; Tramer, F.; Sandri, G.; Panfili, E. Lipoperoxidation damage of spermatozoa polyunsaturated fatty acids (PUFA): scavenger mechanisms and possible scavenger therapies. *Front. Biosci.* **2000**, *5*, 1-15.

20.   Lenzi, A.; Picardo, M.; Gandini, L.; Dondero, F. Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. *Hum. Reprod. Update* **1996**, *2*, 246-256.

21.   Khosrowbeygi, A.; Zarghami, N. Fatty acid composition of human spermatozoa and seminal plasma levels of oxidative stress biomarkers in subfertile males. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2007**, *77*, 117-121.

22.   Close, B.; Banister, K.; Baumans, V.; Bernoth, E.-M.; Bromage, N.; Bunyan, J.; Erhardt, W.; Flecknell, P.; Gregory, N.; Hackbarth, H. Recommendations for euthanasia of experimental animals: Part 2. *Lab. Anim.* **1997**, *31*, 1-32.

23.   Lee, S.-H.; Park, C.-K. Effect of magnetized extender on sperm membrane integrity and development of oocytes *in vitro* fertilized with liquid storage boar semen. *Anim. Reprod. Sci.* **2015**, *154*, 86-94.

24.   Bleil, J.D.; Greve, J.M.; Wassarman, P.M. Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs. *Dev. Biol.* **1988**, *128*, 376-385.

25.   Manjunath, P.; Thérien, I. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. *J. Reprod. Immunol.* **2002**, *53*, 109-119.

26.   Cross, N.L. Role of cholesterol in sperm capacitation. *Biol Reprod* **1998**, *59*, 7-11.

27.   Foote, R.H.; Parks, J.E. Factors affecting preservation and fertility of bull sperm: a brief review. *Reprod. Fertil. Dev.* **1993**, *5*, 665-673.

28.   Ollero, M.; Powers, R.D.; Alvarez, J.G. Variation of docosahexaenoic acid content in subsets of human spermatozoa at different stages of maturation: implications for sperm lipoperoxidative damage. *Mol. Reprod. Dev.* **2000**, *55*, 326-334.

29.   Bathgate, R.; Maxwell, W.; Evans, G. Studies on the effect of supplementing boar semen cryopreservation media with different avian egg yolk types on *in vitro* post-thaw sperm quality. *Reprod. Domest. Anim.* **2006**, *41*, 68-73.

30.   Rooke, J.; Shao, C.; Speake, B. Effects of feeding tuna oil on the lipid composition of pig spermatozoa and *in vitro* characteristics of semen. *Reproduction* **2001**, *121*, 315-322.

31.   Yeste, M.; Barrera, X.; Coll, D.; Bonet, S. The effects on boar sperm quality of dietary supplementation with omega-3 polyunsaturated fatty acids differ among porcine breeds. *Theriogenology* **2011**, *76*, 184-196.

32.   Waterhouse, K.; Hofmo, P.; Tverdal, A.; Miller, R. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. *Reproduction* **2006**, *131*, 887-894.

33.   Cross, N.L. Effect of methyl-β-cyclodextrin on the acrosomal responsiveness of human sperm. *Mol. Reprod.*

Dev. **1999**, *53*, 92-98.

34. Talevi, R.; Barbato, V.; Fiorentino, I.; Braun, S.; Longobardi, S.; Gualtieri, R. Protective effects of *in vitro* treatment with zinc, d-aspartate and coenzyme q10 on human sperm motility, lipid peroxidation and DNA fragmentation. *Reprod. Biol. Endocrinol.* **2013**, *11*, 81.

35. Boscarato, A.; Martins, L. Use of cholesterol in sperm cryopreservation and fertility: a review. *Arq. Cienc. Mar.* **2014**, *17*, 145-150.

36. delBarco-Trillo, J.; Roldan, E.R. Effects of metabolic rate and sperm competition on the fatty-acid composition of mammalian sperm. *J. Evol. Biol.* **2014**, *27*, 55-62.

37. Carrillo, C.; del Mar Cavia, M.; Roelofs, H.; Wanten, G.; Alonso-Torre, S.R. Activation of human neutrophils by oleic acid involves the production of reactive oxygen species and a rise in cytosolic calcium concentration: a comparison with N-6 polyunsaturated fatty acids. *Cell Physiol. Biochem.* **2011**, *28*, 329-338.

38. Ueshima, H.; Stamler, J.; Elliott, P.; Chan, Q.; Brown, I.J.; Carnethon, M.R.; Daviglus, M.L.; He, K.; Moag-Stahlberg, A.; Rodriguez, B.L. Food omega-3 fatty acid intake of individuals (total, linolenic acid, long-chain) and their blood pressure: INTERMAP study. *Hypertension* **2007**, *50*, 313-319.

39. Wassall, S.R.; Brzustowicz, M.R.; Shaikh, S.R.; Cherezov, V.; Caffrey, M.; Stillwell, W. Order from disorder, corralling cholesterol with chaotic lipids: The role of polyunsaturated lipids in membrane raft formation. *Chem. Phys. Lipids* **2004**, *132*, 79-88.

40. Safarinejad, M.R.; Hosseini, S.Y.; Dadkhah, F.; Asgari, M.A. Relationship of omega-3 and omega-6 fatty acids with semen characteristics, and anti-oxidant status of seminal plasma: a comparison between fertile and infertile men. *Clin. Nutr.* **2010**, *29*, 100-105.

41. Rurangwa, E.; Kime, D.; Ollevier, F.; Nash, J. The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture* **2004**, *234*, 1-28.

42. Gulaya, N.; Margitich, V.; Govseeva, N.; Klimashevsky, V.; Gorpynchenko, I.; Boyko, M. Phospholipid composition of human sperm and seminal plasma in relation to sperm fertility. *Arch. Androl.* **2001**, *46*, 169-175.

43. Sanocka, D.; Kurpisz, M. Reactive oxygen species and sperm cells. *Reprod. Biol. Endocrinol.* **2004**, *2*, 12.

44. Morris, I.; Ilott, S.; Dixon, L.; Brison, D. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Hum. Reprod.* **2002**, *17*, 990-998.