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Effect of bicarbonate and progesterone on plasma membrane integrity, acrosome reaction and proportion of fatty acids in boar sperm

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ABSTRACT This study investigated the influence of sodium bicarbonate ($NaHCO_3$) and progesterone on acrosome reaction and proportion of polyunsaturated fatty acid (PUFA) composition boar sperm. The sperm were diluted with semen extender and incubated with $NaHCO_3$ and progesterone at 38℃, 5% CO_2 for 6 h. Plasma membrane integrity and acrosome reaction were analyzed using SYBR14/propidium iodide (PI) and FITC-PNA/PI doubling staining method, and proportion of PUFA was analyzed using gas chromatography. In results, Plasma membrane integrity was significantly decreased in 50 mM $NaHCO_3$ group and acrosome reaction was significantly increased by over the 100 mM $NaHCO_3$ group compared to control group ($p < 0.05$). In addition, progesterone significantly increased decreased plasma membrane integrity at 100 mM progesterone and acrosome reaction at over the 5.0 μM progesterone ($p < 0.05$), but there was no difference among the 5.0 to 100 μM groups. PUFAs were significantly decreased in 100 mM $NaHCO_3$ and 50 μM progesterone treatments compared to control group. In summary $NaHCO_3$ and progesterone induce acrosome reaction and reduce PUFA composition in boar sperm, therefore, the results maybe help to understand basically knowledge for the acrosome reaction and PUFA composition in boar sperm.

Keywords: acrosome reaction, progesterone, PUFA, sodium bicarbonate, sperm

INTRODUCTION

Sperm is largely composed of head, midpiece, and tail, the head contains paternal DNA, and enzymes that dissolve the cumulus cells and zona pellucida of oocyte are present in the front of head (Evenson, et al., 2002; Gadella et al., 2004). Acrosome of the sperm head is released during the fertilization when sperm arrive in the oocyte, this biochemical reaction is defined as acrosome reaction (Abou-haila and Tulsiani, 2009). The acrosome are special organelles that do not exist in normal somatic cells. Acrosomal granules present in the cytoplasm of spermatogonial cells migrate to the front of the sperm head during spermiogenesis and it contains various substances for the sperm to invade into the oocyte during fertilization (Grootegoed et al., 2000). Because the acrosome reaction should occur at the time of meeting with the oocyte for successful fertilization, after ejaculation, the acrosome of the sperm is protected in the outer acrosomal membrane before meeting of the oocytes (Tulsiani et al., 1998).

Generally, acrosome reaction of sperm occurs in the female reproductive tract when sperm approach the cumu-

lus cell-oocytes complexes (COCs) (Boatman and Robbins, 1991). In this time, phospholipid and membrane proteins are changed by bicarbonate (HCO_3^-) and progesterone which lead to increasing of lipid peroxidation, calcium ion, reactive oxygen species in sperm (Sabeur et al., 1996; Awda et al., 2009). A common phenomenon of physiological events is that the plasma membrane is damaged, after this process, the sperm is fused with the plasma membrane and the acrosomal outer membrane, consequently, acrosome is released from sperm head (Tulsiani et al., 1998). In other words, it can be defined as a phenomenon that phospholipids of plasma membrane are entangled accompany with oxidation of fatty acids due to physical and chemical impact on the plasma membrane, for this reason, the acrosome reaction of sperm is closely related to plasma membrane damage and fatty acid oxidation (Tulsiani et al., 1998).

The sperm head contains a large amount of PUFAs, which play an important role in the fluidity of the plasma membrane and the exchange of substances inside and outside the sperm (Tapia et al., 2012). In particular, the PUFAs proportion of sperm is higher in pigs compared to other species, it is known that the proportion of PUFAs of sperm decreases following increasing of the acrosome reaction in pigs (Lee et al,. 2020). Although HCO_3^- and progesterone of the female reproductive tract are directly cause of acrosome reaction, fundamental studies on the acrosome reaction and changes in PUFAs by HCO_3^- and progesterone have not yet been reported in boar sperm. Therefore, we observed change of the acrosome reaction and proportion of PUFAs by HCO_3^- and progesterone in boar sperm.

MATERIALS AND METHODS

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 (Close et al., 1997) and approved from Animal Experiment Ethics Committee in Kangwon National University, Republic of Korea (KIACUC-09-0139) (Close et al., 1997). The semen was collected from boars (Duroc, n = 3, average ages; 32.6 ± 8.9 months) via a glove-hand method (Gumbo Artificial Insemination Industry, Wonju, Republic of Korea) and the samples were diluted with a semen extender (Lee and Park, 2015) until a concentration of 1.5×10^7 sperm/mL, after which were transported to a laboratory within 2 h at 18℃ before experiment. Only ejaculates with more than 80% motility, 70% plasma membrane integrity and 20% acrosome membrane damage were used for experiment (Lee and Park, 2015). Visual motility was evaluated under 400× magnification with a phase contract microscope.

Treatment of $NaHCO_3$ and progesterone

$NaHCO_3$ (Sigma, NY, USA) were added until 0, 10, 20, 30, 40, 50, 100, 300, 500, 700, and 1,000 mM, after $NaHCO_3$ treated semen extenders were adjusted to pH 7.0 to 7.1. The progesterone was solved in ethanol until 1.0 M, after then the progesterone stock solution was diluted with semen extender for experiment. The stock solution was diluted until 0, 5, 15, 25, 35, 50, 65, 75, 85, and 100 μM in sperm samples. All samples were incubated at 38℃ for 6 h, after incubation, centrifuged at 410 g for 5 min. After, supernatant was removed and resuspended with semen extender.

Detection of plasma membrane integrity

The plasma membrane integrity of sperm was evaluated using sperm SYBR14 and PI doubling staining method (Kim et al., 2020). The sperm samples of 7.5×10^6 sperm/mL were diluted with 40 nM SYBR-14 and 2.0 μM PI which were incubated at 38℃ for 5 min at dark room and then, and these samples were centrifuged to remove supernatants at 410 g for 5 min. After incubation, stained 10,000 count sperm were measured using flow cytometry (FACSCaliber, BD Biosciences, CA, USA). Viability was analyzed using dot-plot method (CELLQuest, version 6.0 software, BD Biosciences) (Jo et al., 2021).

Detection of acrosome reaction

Analysis of acrosome reaction were carried out according to previous study (Lee et al., 2020). Sperm samples were diluted with boar semen extender (Lee and Park, 2015) until a concentration of 7.5×10^6 sperm/mL, then 2.0 μM lectin from Arachis hypogagea (FITC-PNA; Sigma) and 2.0 μM PI were treated to each of the samples for 5 min. After incubation, the samples were centrifuged at 410 g for 5 min, and then the supernatants were removed. The samples were resuspended with phosphate buffered saline (PBS) until a concentration of 7.5×10^6 sperm/mL, and a

total of 10,000 sperm were analyzed via flow cytometry (BD Biosciences) using argon laser tuned to 488 nm. Flow cytometry data was analyzed from CELLQuest software (Jo et al., 2021).

Detection of polyunsaturated fatty acid composition

Detection of polyunsaturated fatty acid composition were carried out according to previous study (Lee et al., 2020). Untreated with $NaHCO_3$ and progesterone sperm, 100 mM $NaHCO_3$, and 50 μM progesterone treated samples were washed twice at 410 g for 5 min, followed by centrifugation at 3,700 g at 4℃ for 10 min. The samples were diluted with semen extender, centrifuged at 13,000 g at 4℃ for 20 min, and stored at -80℃ before the experiment. Sperm pellets were completely diluted with 1.0 mL of semen extender, which were then diluted with 20 mL of chloroform-methanol (2:1) and 0.88% NaCl solution and subsequently mixed for 5 min. After incubation for 36 h at 4℃, the samples were centrifuged at 3,700 g for 30 min, the 10 mL bottom layer was transferred to a new tube, and the solvents were removed using nitrogen air. Next, 1.0 mL of 0.5 N methanolic NaOH was added, followed by incubation at 100℃ for 15 min. After samples were cooled in 18℃ water in 20 min, 2.0 mL of 14% BF_3-methanol was added and incubated at 100℃ for 15 min; after cooling, 1.0 mL of heptane and 2.0 mL of NaCl were diluted and incubated for 40 min at room temperature. The supernatant was analyzed via gas chromatography (Shimadzu-17A, Shimadzu, Kyoto, Japan).

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 general linear models was used to analyze acrosome reaction detection and PUFA composition. Data were shown to mean ± standard error means (SEM).

RESULTS

Effect of $NaHCO_3$ on plasma membrane integrity and acrosome reaction

Change of plasma membrane integrity and acrosome reaction following $NaHCO_3$ level in boar sperm are shown in Fig. 1. Plasma membrane integrity was not significantly difference among the 0, 10, 20, 30, and 40 mM $NaHCO_3$ treatment groups, however, above 50 mM $NaHCO_3$ significantly reduced plasma membrane integrity of boar sperm ($p < 0.05$). Similarly, there was not significantly difference among the 0, 10, 20, 30, 40, and 50 mM $NaHCO_3$ treatment groups, 100 mM $NaHCO_3$ significantly increased acrosome reaction in boar sperm ($p < 0.05$).

Effect of progesterone on plasma membrane integrity and acrosome reaction

The influence of plasma membrane integrity and acrosome reaction following progesterone level in boar sperm are shown in Fig. 2. Plasma membrane integrity was not significantly difference among the 0, 5, 15, 25, 35, 50,

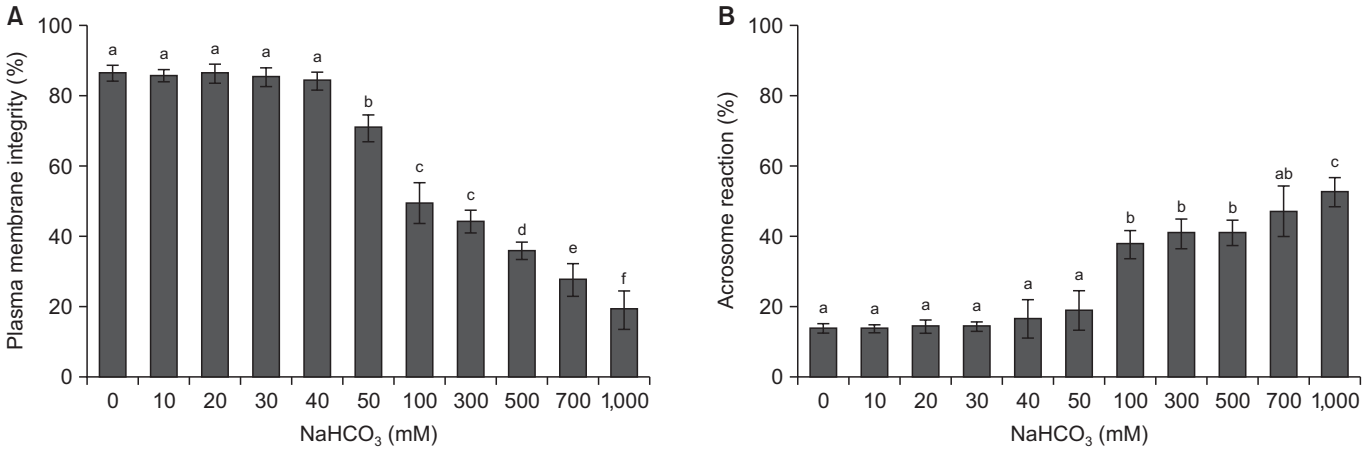

Fig. 1. Effect of $NaHCO_3$ on plasma membrane integrity in boar sperm (A). $^{a-f}$Values with differents uperscripts in the same column with sperm are significantly different ($p < 0.05$). Influence of $NaHCO_3$ on acrosome reaction in boar sperm (B). $^{a-c}$Values with differents uperscripts in the same column with sperm are significantly different ($p < 0.05$). Boar sperm was treated with $NaHCO_3$ at 38℃, 5% CO_2 for 6 h.

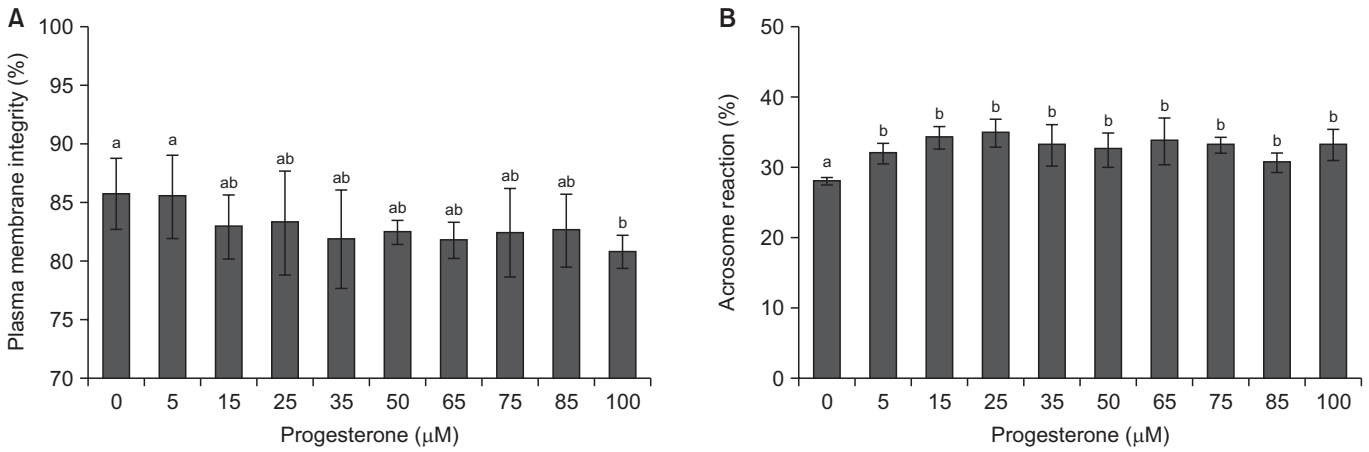

Fig. 2. Effect of progesterone on plasma membrane integrity in boar sperm (A). Influence of progesterone on acrosome reaction in boar sperm (B). [a,b]Values with differents uperscripts in the same column with sperm are significantly different ($p < 0.05$). Boar sperm was treated with progesterone at 38℃, 5% CO_2 for 6 h.

65, 75, and 85 μM progesterone treatment groups, however, 100 μM progesterone significantly reduced plasma membrane integrity of boar sperm ($p < 0.05$). In addition, more than 5.0 μM progesterone significantly increased acrosome reaction in boar sperm ($p < 0.05$).

Change of fatty acids proportion by $NaHCO_3$ and progesterone

Change of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), PUFA proportions by $NaHCO_3$ and progesterone in boar sperm are shown in Fig. 3. The proportion of SFA was increased in 100 mM $NaHCO_3$ and 50 μM progesterone treatment groups compared to control groups ($p < 0.05$). However, proportions of UFA and PUFA were significantly decreased by 100 mM $NaHCO_3$ and 50 μM progesterone in boar sperm ($p < 0.05$).

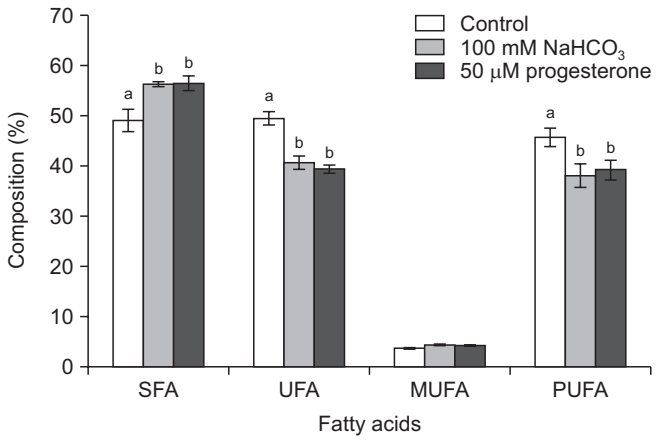

Fig. 3. Changes of saturated fatty acid (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) proportions, boar sperm were treated with 100 mM $NaHCO_3$ and 50 μM progesterone, [a,b]Values with differents uperscripts in the same fatty acid column ($p < 0.05$).

DISCUSSION

Understanding of biochemical mechanism between the ejaculated sperm and the matured oocytes during the fertilization process can be applied to identify cause of infertility and improvement of pregnancy. In general, the fluidity of the plasma membrane is increased by albumin for sperm acquire capacitation, which triggers fusion of plasma membrane and outer acrosomal membrane (Tulsiani et al., 1998). The acrosome reaction is a physiological characteristic of sperm that is not found in other somatic cells, in this reason, studies on the mechanism of acrosome reaction in the uterine environment and suppression of acrosome reaction for semen preservation are widely conducted (Tulsiani et al., 1998). In this study, we conducted focusing on changes in $NaHCO_3$ and progesterone, which are representative substances that induce acrosome reactions in female reproductive tracts. In general, the concentration of HCO_3^- in ejaculated semen is known to be less than 5 mM and is known to be 23 mM in the oviduct of female reproductive tract (Silva and Gadella, 2006). Although our study did not show that plasma membrane integrity and acrosome reaction were not change in 20 to 30 mM $NaHCO_3$, high concentration

of HCO_3^- (100 mM $NaHCO_3$) induced acrosome reaction of boar sperm (Silva and Gadella, 2006). This result show that boar sperm do not occur acrosome reaction in 20 to 30 mM HCO_3^- environment when sperm is exposed in only HCO_3^- and 100 mM HCO_3^- induces acrosome reaction. We suggest that acrosome reaction not only need pro HCO_3^- environment but also other stimulation such as progesterone, oxidative stress. As such, the sperm acquires capacitation due to the effusion of UFA from the plasma membrane when HCO_3^- is entered into the sperm (Silva and Gadella, 2006). Efflux of UFA and cholesterol induces dissolving of plasma membrane which induces acrosome reaction of sperm in hamster (Visconti et al., 1999), pig (Flesch et al., 2001), and cows (Rathi et al., 2001). In practice, acrosome reaction of boar sperm decrease proportion of PUFA such as DHA and DPA in all fatty acids. It is considered that the acrosome reaction is induced by high concentration of HCO_3^- level in the uterine environment, and then the UFAs are released outside from plasma membrane, finally, proportion of these fatty acids decrease in sperm. Substances such as caffeine, theophylline, IBMX and papaverine are used to increase the concentration of HCO_3^- in sperm cells, generally, caffeine is used to induces sperm capacitation and acrosome reaction during *in vitro* fertilization (Silva and Gadella, 2006). In this study, the results of acrosome reaction according to a wide range of concentrations of HCO_3^- may be useful for fundamental research on acrosome reaction in boar sperm.

Progesterone is one of the sex hormones, which secrete from corpus luteum and play a role increasing of endometrium thickness (Niswender, 2002; Lamy et al., 2017). Progesterone-progesterone receptor activate protein tyrosine kinase, which induce phosphatidylinositol 4,5-bisphosphate and diacylglycerol. The physiological signal extends calcium ion channel of plasma membrane, it leads to influx of calcium ion and acrosome reaction (Witte and Schäfer-Somi, 2007). The acrosome reaction regarding of calcium ion signaling is widely studied, methodology on suppression of calcium ion into sperm intracellular and plasma membrane is used to preservation of sperm (Sabeur and Meizel, 1995). In general, many studies have been conducted using progesterone at 10 μg/mL to induce an acrosome reaction in boar sperm and it is known that acrosome reaction occurs actively in sperm exposed for more than 2 h (Wu et al., 2006). In horses and dogs, there is also a report that an acrosome reaction occurred by 10 μg/mL progesterone level (Witte and Schäfer-Somi, 2007). We found that the acrosome reaction increased when 15 μM or more of progesterone were treated for 30 min, these results are similar to the acrosome reaction in boar sperm treated with 10 μg/mL (about 31 μM) progesterone for 2 to 6 h (Wu et al., 2006). In addition, our study was confirmed that the acrosome reaction occurs more than 40% even with a high concentration (100 μM) of progesterone, and accordingly, it was confirmed that the proportion of UFA and PUFA were decreased compared to control. These results suggest that the acrosome reaction of boar sperm is induced by high progesterone in the intrauterine environment of luteal phase, as a result, the proportion UFA and PUFA is decreased in boar sperm.

The boar sperm plasma membrane has PUFAs, which performs in supporting fluidity to plasma membrane (Tapia et al., 2012). The PUFAs are chemically unstable less than the SFAs because they are easily reacted with free radicals and reactive oxygen species and have a low melting point (Carrillo et al., 2011). On the other hand, the SFAs have high chemical stability and a high melting point because they do not have double bonds (Wassall et al., 2004). In this experiment, proportion of SFA was increased in acrosome reacted boar sperm by HCO_3^- and progesterone. We suggest that even though HCO_3^- and progesterone induced acrosome reaction, they did not influence changes of SFAs in boar sperm. Breaking of fatty acids double bonds by HCO_3^- in phospholipid lead to phospholipid scrambling in sperm plasma membrane (Breitbard and Finkelstein 2015, Tulsiani et al. 1998) and calcium ion activation by progesterone and its receptors bring about combination of outer acrosomal membrane and plasma membrane. Activation of calcium ion down-regulate PUFAs, otherwise, calcium ion had no effect on MUFAs and SFA (Cooray et al., 2022). We suggest that phospholipid scrambling by HCO_3^- and combination of outer acrosomal and plasma membrane by progesterone break fatty acids double bonds in phospholipid of plasma membrane, eventually, HCO_3^- and progesterone decreased proportion of PUFAs. Generally, proportion of UFA and PUFA are negative correlation with the acrosome reaction (Lee et al., 2020). In practice, it was confirmed that the proportion of PUFAs such as unsaturated fatty acids and docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) decreased in boar sperm in which 90% or more of

the acrosome reaction occurred, whereas the proportion of SFAs were increased (Lee et al., 2020). We suggest that

Our results also confirmed that the proportion of UFAs in boar sperm was decreased by $NaHCO_3$ and progesterone. It is considered that the plasma membrane was damaged by $NaHCO_3$ and progesterone, eventually, the proportion of PUFA decreased by the acrosome reaction in boar sperm.

CONCLUSION

Substances that control the acrosome reaction of sperm and signaling mechanisms are continuously being actively studied. However, in the case of livestock sperm except for humans or experimental animals, it is mainly focused on improving preservation by suppressing the acrosome reaction, in results, there are few studies on plasma membrane integrity, acrosome reaction, and proportion of fatty acids following the various conditions for HCO_3^- and progesterone. In this study, we found change of plasma membrane integrity, acrosome reaction, and proportions of SFA, UFA, and PUFA following wide range of HCO_3^- and progesterone. It may provide basic knowledge about the signaling mechanism of the acrosome reaction in boar sperm.

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