

**Studies on the Effect of Magnesium and Polymyxin B on
Liquid-stored Boar Spermatozoa**

**(ブタ液状保存精子へ及ぼすマグネシウム及びポリミキシン B
の効果に関する研究)**

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List of Abbreviations	
AI	Artificial Insemination
AR	Acrosome Reaction
ANOVA	Analysis of Variance
A23187	Calcium Inophore A23187
BTS	Beltsville Thawing Solution
BSA	Bovine Serum Albumin
CAM	Calmodulin
cAMP	Cyclic adenosine monophosphate
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
LPS	Lipopolysaccharides
PMB	Polymixin B sulphate
PVA	Polyvinyl alcohol
PDE	Phosphodiesterase
PEG	Polyethylene glycol

PKC	Protein Kinase C
PI	Propidium iodide

Chapter 1

General Introduction

Artificial Insemination in porcine industry

During the last decades, the use of porcine semen for artificial insemination (AI) by means of fresh diluted semen has increased considerably (Maes et al., 2011; Riesenbeck et al., 2015). Compared to natural mating, AI reduces the risk of disease transmission (Maes et al., 2011). By allowing the introduction of superior genes into sow herds and additionally it leads to a better profitability of each boar ejaculate. Therefore, AI has become a very useful tool in countries with intensive pig production.

Liquid preservation and semen dilution

In different studies, the effect of short versus long term extenders on semen quality and sow fertility has been investigated. According to in vitro and in vivo studies, most extenders in the market provide an acceptable sperm vitality protection during the first 72 hours of storage, although some differences were observed regarding fertility of semen stored during more than 4 days (Kuster and Althouse, 1999; Vyt et al., 2004). The media used for liquid storage are necessary to prolong sperm survival by providing energy to the cells, buffering the pH of the suspension and avoiding the growth of bacteria (Vyt et al., 2004)

Sperm Characteristics:

Different parameters can be used to evaluate the characteristics of boar semen. Sperm motility is an indication of an active metabolism and the integrity of membranes (Johnson et al. 2000) and is considered to be of great importance for fertilizing. Sperm morphology gives an indication of sperm viability and fertility and can be routinely examined (Menkveld et al., 2011). Progressive motility is also an essential parameter in the evaluation of sperm as it is indicative of the vitality of the cells (that is directly correlated to viability) (Maroto Martin et al., 2010).

Acrosomal exocytosis and its importance on fertilization

At fertilization, the spermatozoa undergoes an exocytotic process (the so-called 'acrosome reaction'), which releases enzymes to allow the sperm cell to penetrate the egg vestments and also primes the sperm for fusion with the egg itself (Roldan and Harrison, 1992). Thus, capacitation and the subsequent acrosome reaction (AR) are important events in the process of fertilization. They are essential for spermatozoa to achieve the abilities to bind to and also to penetrate the zona pellucida (ZP) and to subsequently fuse with the oocyte plasma membrane (Yanagimachi, 1994).

Role of divalent cations on acrosomal exocytosis

Initiation of sperm exocytosis takes place in response to oocyte-derived signals, such as progesterone or zona pellucida (Florman and First, 1988). *In vitro*, the AR can be induced artificially by incubation with solubilized ZP proteins or with other molecules known as AR inducers such as progesterone or Ca^{2+} ionophores. Influx of extracellular Ca^{2+} seems to be an early response evoked by progesterone and zona pellucida (Florman et al., 1989; Thomas and Meizel, 1989). Molecular mechanisms underlying acrosomal exocytosis, which ends in membrane fusion of the outer acrosomal membrane and the plasma membrane, have been extensively investigated. Studies have revealed that after Ca^{2+} entry, a variety of pathways are sequentially activated leading to membrane fusion.

Calcium is an important modulator for capacitation and AR and is probably the key messenger in the information exchange between sperm and egg (Darszon et al., 2006). The control of sperm Ca^{2+} channel function seems to be responsible for minimizing the rate of premature AR (Florman and First, 1988). Capacitation and AR have been shown to occur in the absence of oocytes outside the female genital tract (spontaneous AR) and under *in vitro*

conditions mimicking the physiological environment (Breitbart, 2002).

Summer infertility and male factor:

So called ‘summer infertility’, which causes nadir in fertility during summer, has long been known worldwide and is threatening to production and stable supply of pork to human beings. It is thought that infertility may arise at a rate of 40-70% by the male factor. Boar spermatozoa collected during summer showed hypersensitivity to the agonist of the AR (Murase et al., 2007). The premature acquisition of the fertilizing ability (capacitation and the AR of spermatozoa) well before sperm penetration of the oocyte takes place is one of the major causes for failure in fertilization leading to infertility. Preventing the premature capacitation/AR might lead to increase in the chance of fertilization, potentially leading to a higher farrowing rate. Control of pathways leading to AR may be one of the most powerful ways. There are still unknown pathways leading to the acrosome reaction.

Bacterial contamination and polymixin B (PMB):

Semen is an ideal medium for the establishment and growth of many microorganisms including bacteria. Bacteria appear to exert their spermicidal effect directly upon the sperm cell. Microorganisms have a deleterious effect on sperm function, both directly by altering the structure of the sperm, by affecting its motility (Depuydt et al., 1998; Diemer et al., 1996) or by provoking a premature acrosome reaction (Köhn et al., 1998).

PMB is a polycationic peptide antibiotic, active against most strains of gram-negative bacteria (Storm et al., 1977), isolated from *Bacillus Polymyxa*. Polymyxins interact with lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria. It is known that PMB neutralizes endotoxin of bacteria with which semen may be contaminated and that addition of PMB to chilled stored boar spermatozoa is beneficial for maintaining motility

(Okazaki et al. 2010). In somatic cells, it has been shown that PMB is an inhibitor of protein kinase C (Mazzei et al. 1982; Wise et al. 1982), also has been reported to inhibit CaM (Hegemann et al., 1991) and Ca^{2+} -ATPase (Ktenas et al., 1989), K^{+} -channel (Harding et al., 1994).

General Objectives:

Sperm collected during summer season, when summer infertility takes place, show hypersensitivity. This implies that any treatment that decreases hypersensitivity would be useful to alleviate summer infertility of boar spermatozoa. The study focused on magnesium ion for this purpose.

PMB has been shown to be useful for neutralizing LPS in boar semen (Okazaki et.al, 2010). However, PMB has been used as inhibitor of signalling molecules in somatic cells. Therefore, it was hypothesized that PMB would have direct effect on boar spermatozoa. Thus, the main objectives of the study were:

1. Involvement of magnesium ion (Mg^{2+}) in the AR was investigated. This ion has been shown to have an action to prevent excess occurrence of the acrosome reaction and therefore by clarifying its involvement, it was examined whether or not this ion might be beneficial to maintain the fertilizing ability of spermatozoa.
2. In order to prevent premature acquisition of the fertilizing ability, boar spermatozoa stored in the presence of added Mg^{2+} and then the sperm characteristics and acrosomal exocytosis were observed.
3. It is unknown whether or not PMB might affect the acrosomal exocytosis of spermatozoa directly. The effect of PMB on acrosomal exocytosis triggered by Ca^{2+} /A23187 was examined.

4. To know the effect of PMB added during sperm storage on the sperm characteristics and acrosomal exocytosis.

Chapter 2

Section I

Effect of Magnesium on Exocytosis of Boar Sperm Acrosome Stimulated by Calcium and the Calcium Ionophore A23187

Introduction

The sperm acrosomal exocytosis is an extracellular Ca^{2+} -dependent exocytosis of acrosomal contents and morphologically characterized by partial fusions between plasma membrane and outer acrosomal membrane at the multiple parts (Yanagimachi, 1994). Acrosomal exocytosis can be triggered by sperm contact with natural stimuli, such as progesterone or the zona pellucida, but it can also be induced by in vitro treatment with Ca^{2+} and ionophores (Roldan and Murase, 1994) such as A23187 which exchanges Ca^{2+} for 2H^+ . In case of ionophore induced acrosome reaction this signal transduction cascade up to the elevation of intracellular calcium is bypassed, but the resulting acrosome reaction is morphologically indistinguishable from that induced by zona pellucida (Spungin et al., 1995). The importance for sperm function of membrane Ca^{2+} -channels and Ca^{2+} -influx is well-established (Darszon et al., 2011). In the regulation of mammalian sperm function and the mechanisms by which sperm Ca^{2+} signals are generated, considerable progress has also been made about the important role of Ca^{2+} signalling. Mg^{2+} , a divalent cation, has been shown to antagonize the actions of calcium in the acrosome reaction (Rogers and Yanagimachi, 1976). Although magnesium, is essential to regulate numerous cellular functions and enzymes, including ion channels, metabolic cycles, and signaling pathways. Johnson (1975) has stated that magnesium counteracts with calcium in the interaction of the acrosome reaction in guinea pig spermatozoa. Magnesium is competitive with calcium and the magnesium/calcium ratio in the medium controls the initiation of the acrosome reaction of guinea pig spermatozoa (Rogers and Yanagimachi, 1976). This study examined the effect of magnesium in the presence of millimolar levels of calcium on acrosomal exocytosis triggered by A23187 in boar spermatozoa.

Materials and Methods

Semen collection and dilution

The semen was collected by the gloved-hand method from 3 mature fertile boar (Landrace and Large White), aged 18-24 months kept at Fujinozo service Fujinomiya, Shizuoka, Japan. For storage of semen, the sperm rich fraction was diluted with Beltsville Thawing Solution (BTS) extender to yeild a concentration of 1.5×10^8 sperm/ml at room temperature and stored in refrigerator at 17°C for up to 3 days until use.

Table 1. Composition of BTS

Ingredient	Amount (g/L)
Glucose	37.0
EDTA	1.25
Sodium Citrate	6.00
Sodium bicarbonate	1.25
Potassium chloride	0.75
Gentamicin sulphate	0.30
pH	7.2
Osmatic pressure(mOsm)	330

Beltsville thawing solution: (Johnson et al., 1988)

Media

Routine chemicals and reagents used in this study were of high purity grade and obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Wako Pure Chemicals Industries (Osaka, Japan) unless otherwise specified.

Throughout the experiments (for washing and incubation of spermatozoa), the standard saline medium used consisted of 142 mM NaCl, 2.5 mM KOH, 10 mM glucose and 20 mM Hepes, adjusted to pH 7.55 at 20 °C with NaOH. Sucrose medium consisting of 222 mM sucrose in place of NaCl was used for washing spermatozoa. Saline and sucrose media also contained 0.1% (w/v) polyvinyl alcohol (average molecular weight of 30,000–70,000; Sigma Chemical, St. Louis, MO, USA) and 0.1% (w/v) polyethylene glycol (consists of 2 moles of polyethylene glycol, 7000-9000 Sigma Chemical). A stock solution of 30 μ M ionophore A23187 (Free acid; Calbiochem- Novabiochem/EMD Biosciences, La Jolla, CA, USA) was prepared in dimethyl sulfoxide (DMSO) and diluted in saline medium to give a final concentration of 0.3 μ M.

Table 2. Composition of Saline medium

Ingredient	Amount (mM)
NaCl	142
KOH	2.5
Glucose	10
Hepes	20

Adjusted pH to 7.55 (20°C) with 4M NaOH or 1M NaOH

Table 3. Composition of Sucrose medium

Ingredient	Amount (mM)
Sucrose	222
KOH	2.5
Glucose	10
Hepes	20

Adjusted pH to 7.55 (20°C) with 4M NaOH or 1M NaOH
(Roldan and Harrison, 1989)

Preparation of spermatozoa

A portion of the stored semen (2.5 ml) was placed in two test tubes and left standing for 10 minutes to sediment of large cell clumps. A supernatant containing spermatozoa was overlaid onto a sucrose medium and centrifuged at 126 g for 5 min followed by 263 g for 7 min at room temperature. The supernatant was aspirated, and 200 μ l of the loose pellets was mixed with 2 ml of saline medium. Spermatozoa were then centrifuged at 263 g for 5 min, resuspended in saline medium containing 3 mM CaCl_2 .

Induction of acrosomal exocytosis by calcium ionophore A23187

Spermatozoa were washed through sucrose medium followed by saline medium and resuspended in saline medium. Exocytosis of the sperm acrosome was induced by Ca^{2+} /A23187 and incubation was done in air at 37°C in the presence of different concentration of MgSO_4 (0.01, 0.05, 0.1, 0.5, 1, and 3 mM) or MgCl_2 (0.1, 0.5, 1, and 3 mM) with or without 0.3 μ M A23187. Incubation was carried out up to 15 min for experiment 1 and 2 and in order to obtain a time-course of acrosomal exocytosis (experiment 3), incubation was done up to 60 min. At various intervals subsamples were taken (0, 5, 10, 15, 30 and 60 min) and fixed with 1% glutaraldehyde. Spermatozoa were then examined under a phase contrast microscope (400 x), and the acrosomal exocytosis was monitored as described by Shams-Borhan and Harrison (1981). Spermatozoa showing a head with a dense apical ridge were considered to be acrosome-intact, and all others were considered to have undergone the acrosomal exocytosis (Figure 1). The results were expressed as an acrosomal exocytosis percentage (using 4 replicates for each experiment).

Experiment design:

Experiment no: 1 Effect of MgSO₄ on boar sperm acrosomal exocytosis at 10 min and 15 min of incubation.

Experiment no: 2 Effect of MgCl₂ on boar sperm acrosomal exocytosis at 10 min and 15 min of incubation.

Experiment no: 3 Effect of MgSO₄ on the time course of acrosomal exocytosis induced by Ca²⁺ and A23187 in boar spermatozoa.

Statistical Analysis:

The results are presented as the mean ± standard error of the mean (SEM). The obtained data were subjected to two-way ANOVA followed by the Bonferroni's multiple comparison test. Values of P < 0.05 were considered to be statistically significant. All analyses were carried out using a statistical software program (GraphPad Prism Version 6.0; Graph Pad Software, San Deigo, CA, USA).



Acrosome intact

Vesiculated

Acrosome reacted

Showing a head with a dense apical ridge

Vesiculation takes place between plasma and acrosome membranes in the course of the acrosome reaction

Dispersal of fused membranes

Figure 1. Photographs of acrosome reaction of boar sperm.

Results

Effect of MgSO₄ and MgCl₂ on boar sperm acrosomal exocytosis (Experiment 1 and 2):

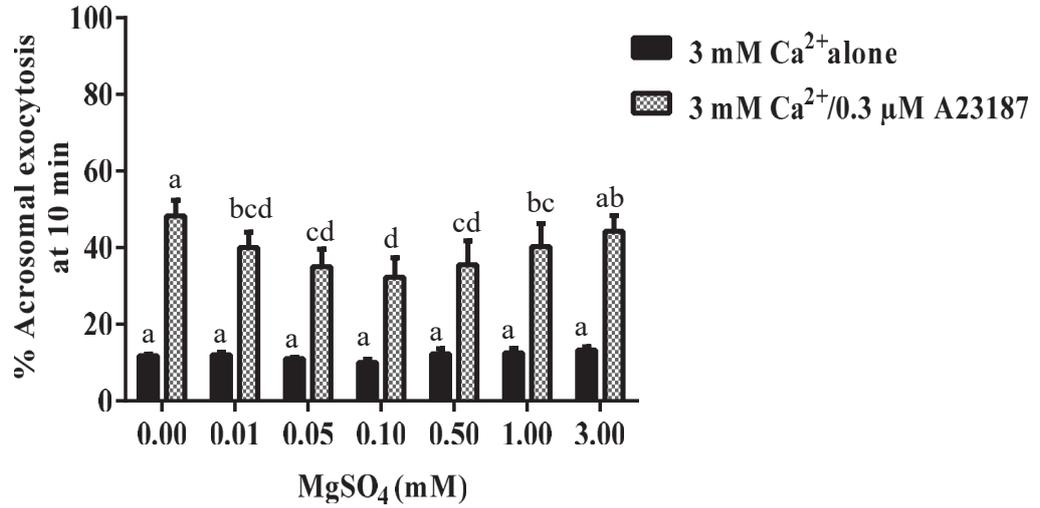
Figure 1 and 2 show that the percentages of acrosomal exocytosis in presence of MgSO₄ and MgCl₂ triggered by Ca²⁺/A23187.

When spermatozoa were stimulated with Ca²⁺/A23187 in presence of different concentrations of MgSO₄ and MgCl₂ there was concentration dependent effects of magnesium on exocytosis. In this study, boar spermatozoa were incubated with 0, 0.01, 0.05, 0.1, 0.5, 1 and 3 mM MgSO₄ (Fig.2 A and B) or MgCl₂ (Fig. 3 A and B) in presence of Ca²⁺/A23187 for 10 and 15 min, the percentage of acrosomal exocytosis was significantly lower with 0.1 mM of both MgSO₄ and MgCl₂ compared with the other concentrations and control at both intervals (Two-way ANOVA, Bonferroni's multiple comparison test).

Effect of MgSO₄ on boar sperm time course changes in acrosomal exocytosis (Experiment -3):

Spermatozoa were stimulated with Ca²⁺/A23187 in the presence of 0~0.1 mM MgSO₄ for up to 60 min, a clear dose-dependent inhibition was observed with the maximal inhibition at 0.1 mM (Fig. 4 C, Two-way ANOVA, time after stimulation × concentrations; p < 0.0001). When spermatozoa were similarly treated with higher concentrations of MgSO₄ (0.1~3 mM), % acrosomal exocytosis was the lowest again at 0.1 mM (Fig. 4 D). Treatment of boar spermatozoa with Ca²⁺/A23187 in presence of Mg²⁺ resulted in a time-dependent enhancement in exocytosis. Acrosomal exocytosis was observed a maximum value (~90%) 30 min after the beginning of treatment (the onset of exocytosis was noted 10 min after stimulation and reached maximal values by 30 min). Regardless of the concentration used, the effect of Mg²⁺ was seen clearly at 10 min and up to 60 min but was not observed at 5 min.

(A)



(B)

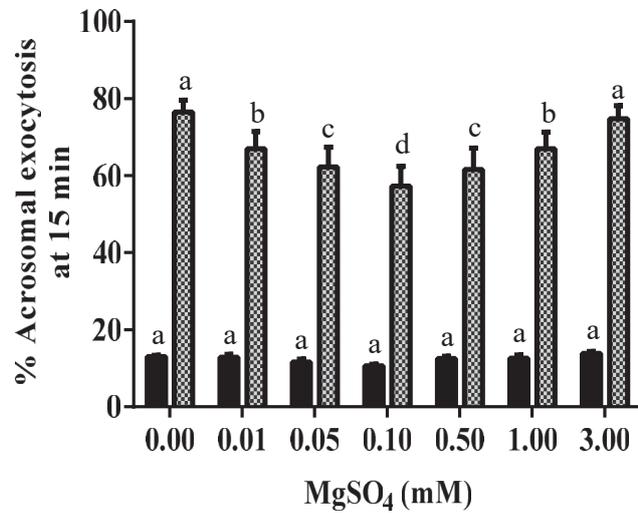


Figure 2. Effect of different concentration of MgSO₄ on boar sperm acrosomal exocytosis at 10 min (A) and 15 min (B) of incubation triggered by Ca²⁺/A23187; (a-d) indicates significant differences from control and among the concentrations ($P < 0.05$). All values percentages are expressed as mean \pm SEM ($n = 4$).

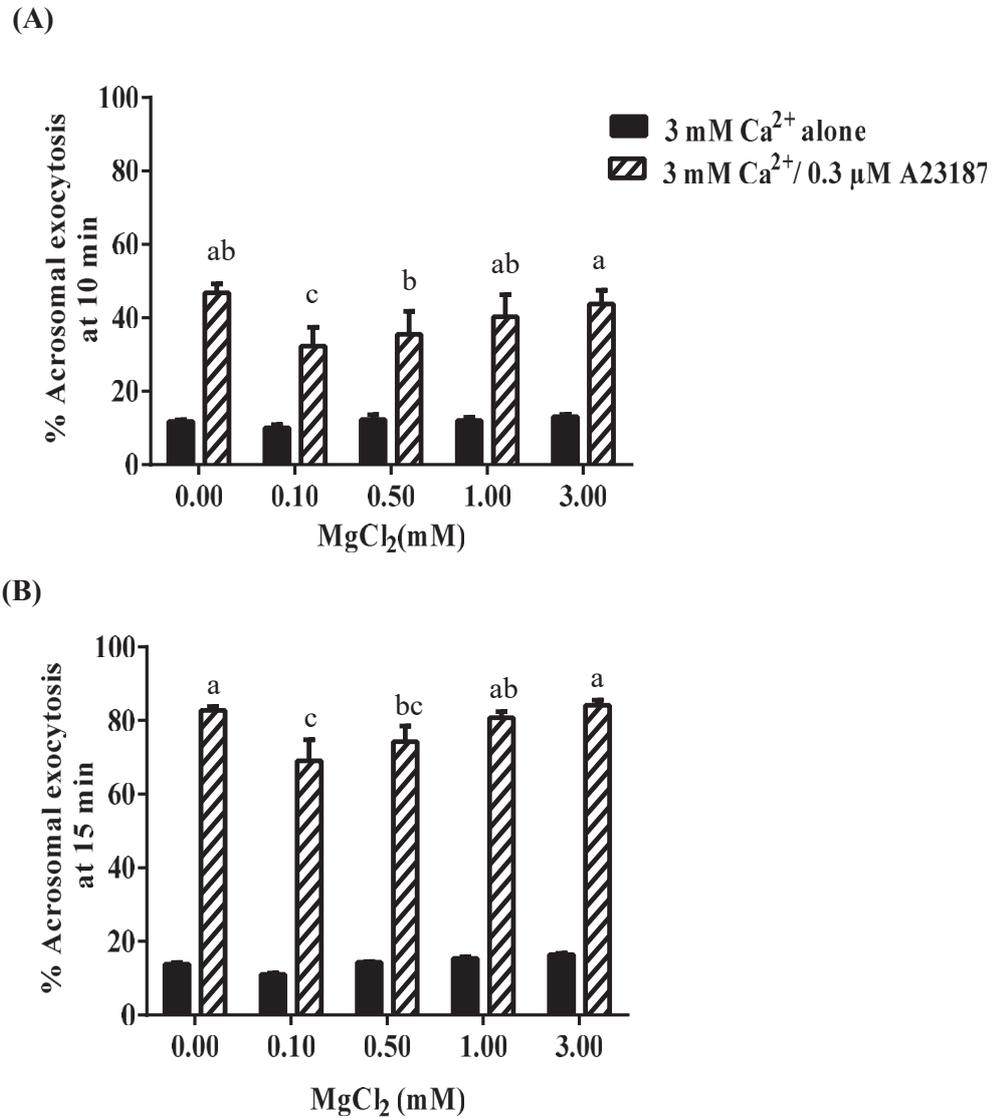


Figure 3. Effect of different concentration of MgCl₂ on boar sperm acrosomal exocytosis at 10 min (A) and 15 min (B) of incubation triggered by Ca²⁺/A23187; (a-c) indicates significant differences from control and among the concentrations (P < 0.05). All values percentages are expressed as mean ± SEM (n = 4).

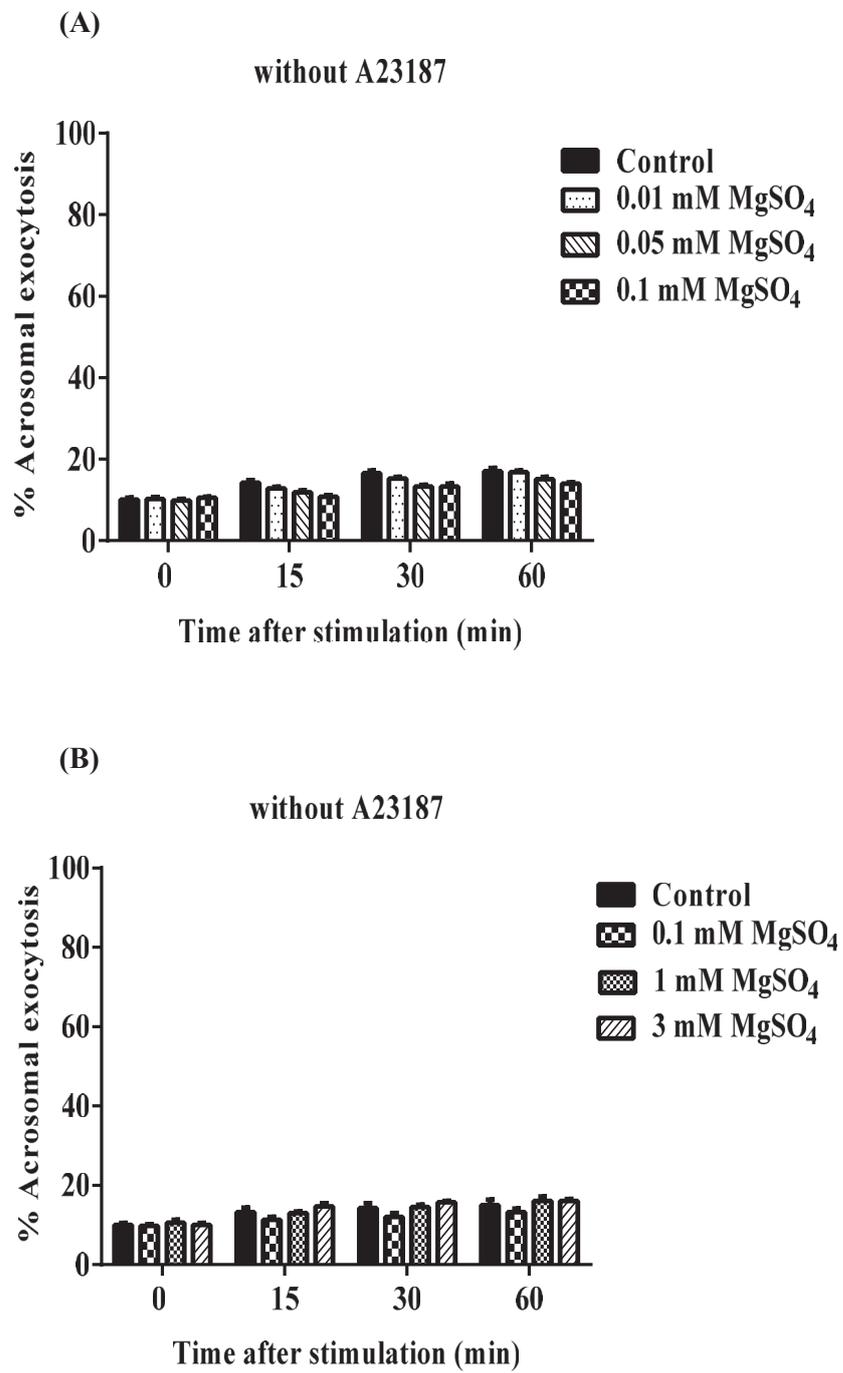


Figure 4.

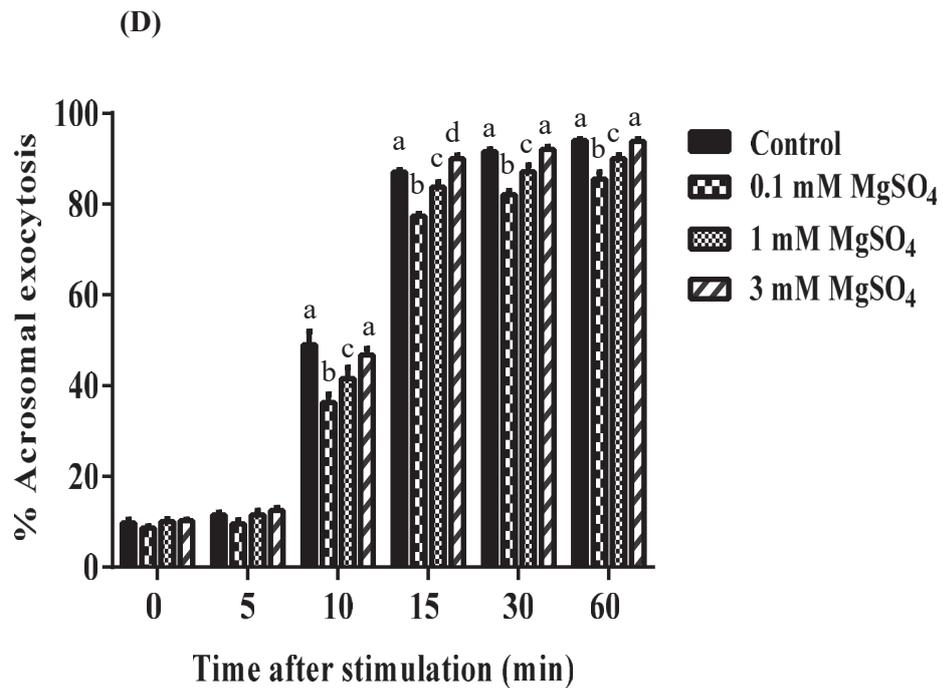
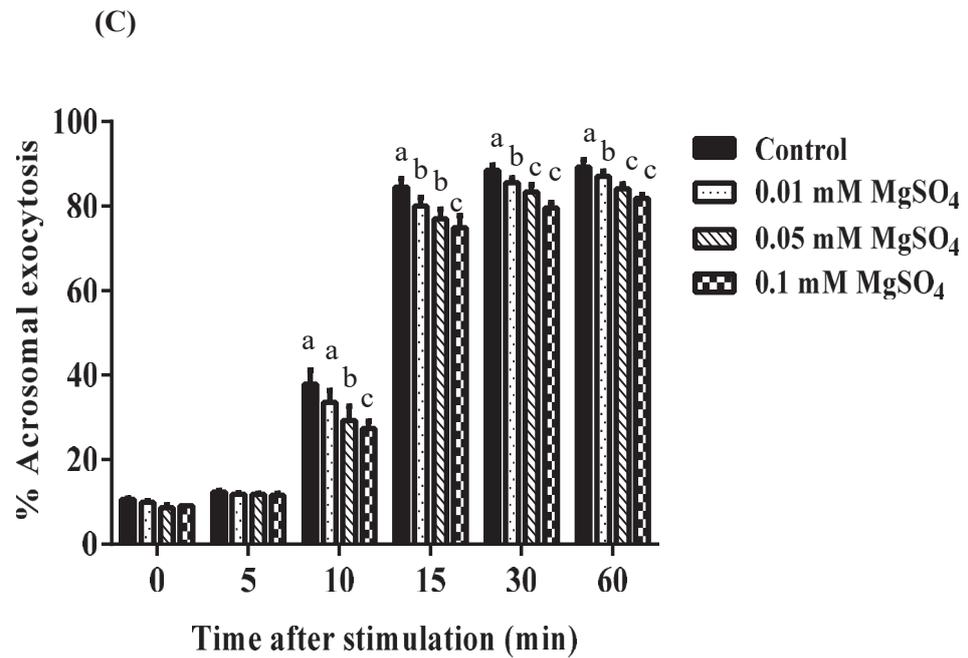


Figure 4. Effect of MgSO₄ on the time course of acrosomal exocytosis in boar spermatozoa.

Spermatozoa stimulated with Ca²⁺/A23187 and incubated with different concentrations of MgSO₄ (A and C) 0, 0.01, 0.05 and 0.1 mM and (B and D) 0, 0.1, 1 and 3 mM upto 60 min. (a-d) indicates significant differences from control and among the concentrations (P < 0.05). All values percentages are expressed as mean ± SEM (n = 4).

Discussion

In this study, the occurrence of the acrosomal exocytosis was monitored in boar spermatozoa which were incubated with different concentrations of Mg^{2+} and Ca^{2+} /A23187. The inhibitory effect of Mg^{2+} (highest inhibition with 0.1mM) was observed in presence of extracellular Ca^{2+} /A23187 on exocytosis of boar sperm acrosome. According to Talbot (1975), the incidence of the acrosomal exocytosis of guinea pig spermatozoa decreases when magnesium is present in the medium. Magnesium acts as a competitive inhibitor of the calcium-stimulated acrosome reaction of guinea pig spermatozoa in vitro and the magnesium may simply be competing with calcium for sites on a carrier in the plasma membrane whose function is to transport extra cellular calcium into the cell (Rogers and Yanagimachi, 1976). Interestingly, in this study, a concentration of 3 mM of $MgSO_4$ and $MgCl_2$ in presence of 3 mM Ca^{2+} and 0.3 μM A23187 was not shown to inhibit the acrosomal exocytosis. Rogers and Yanagimachi (1976) found that a calcium concentration of 3 or 5 mM in the presence of 1 mM magnesium was shown to overcome the inhibitory effect of magnesium on the acrosomal exocytosis in guinea pig spermatozoa. Results suggest the important role of Mg^{2+} that also found in pig spermatozoa, in presence of Mg^{2+} at concentration of 10 μM are capable of enhancing the acrosomal exocytosis induced by glycosaminoglycans (Delgado et al., 1985). The present study clearly showed that micromolar levels of Mg^{2+} could inhibit acrosomal exocytosis in boar spermatozoa in the presence of millimolar levels of Ca^{2+} . It could be due to the different conditions, including stimulus, media compositions and species, that spermatozoa were stimulated. The findings might become useful information to develop a novel method to reduce the excess induction of acrosomal exocytosis taking place in boar spermatozoa collected in summer, characteristic to summer infertility.

Section II

Effects of Magnesium Added during Liquid Storage on Sperm Characteristics and Acrosomal Exocytosis in Boar Spermatozoa

Introduction

Artificial insemination (AI) is a highly efficient breeding technology in the pig industry. AI with liquid-stored semen is widely used for pig production. Summer infertility in pigs has been explored in previous studies, (Murase et al., 2007; Trudeau and Sanford, 1986), the lowest fertility rate was observed during summer and summer infertility was more prevalent in herds with a low overall reproduction performance. However, boar spermatozoa collected during summer showed hypersensitivity to the agonist (calcium and A23187) of the acrosome reaction (Murase et al., 2007). A study by Holt et al., (1997) reported that the response of boar spermatozoa to calcium and A23187 which correlate boar sperm traits with fertility after artificial insemination and also observed that the higher rate of acrosome reaction induced by Ca^{2+} /A23187 is related to smaller litter size. Therefore, preventing the excessive occurrence of acrosome reaction might lead to increase the chances of fertilization.

However, many semen extenders have been developed to store boar ejaculates in liquid state for 3 days or more longer period (Johnson et al., 2000), dilution with an extender reduces the concentrations of proteins present in seminal plasma by destabilizing the membrane and functional capacitation can be reserved in boar spermatozoa (Maxwell and Johnson, 1999). Although, storage of boar semen with BTS for up to 3 days has no significant influence on fertility (Johnson et al., 1988).

It is well known that Ca^{2+} is an essential bivalent cation for the occurrence of acrosome reactions (Roldan and Harrison, 1989) and the presence of this ion during storage time could be an indicator of boar fertility. In previous study by Murase et al., (2007), low Mg^{2+} concentration in seminal plasma was found in boar seminal plasma collected during summer season. The purpose of this study was to determine the changes in sperm characteristics, when boar semen was extended and stored at 17°C for up to 72 h post-collection with added

MgSO₄ and also its involvement in the acrosomal exocytosis was investigated in diluted and stored boar spermatozoa.

Materials and Methods:

Boars, ejaculates and seminal plasma:

Ejaculates were collected using the gloved-hand method from healthy and mature boars from 4 Landrace boars undergoing regular semen collection for commercial AI (Fujinozo service, shizuoka, Japan). All ejaculates used fulfilled the standards of quantity and sperm quality (collected at the end of September) The sperm-rich fractions of 4 boars were diluted 6-fold with BTS. After dilution the various concentrations of MgSO₄ (0, 2, 4 and 8 mM) added to samples. Then samples were protected and transported to the laboratory, stored in a refrigerator at 17°C for 72 hr.

Seminal plasma (SP) was obtained from the sperm-rich fraction of each boar (4 Landrace) and stored frozen at -30 C until assayed. Concentrations of calcium and magnesium were measured using automated equipments (DRI-CHEM 3500V for calcium and magnesium; Fuji Film, Tokyo, Japan) according to the manufacturer's instructions. For analyses, the SP samples were thawed at room temperature.

Washing of spermatozoa:

Boar spermatozoa were washed according to Murase et al., (2004). Briefly, a portion of the stored semen was placed for sedimentation of large cell clumps by leaving standing for 10 minutes. The upper phase containing spermatozoa was overlaid onto a sucrose medium and centrifuged. The supernatant was aspirated and the loose pellets of spermatozoa was

mixed with the saline medium and then centrifuged and resuspended in the saline medium. Sperm concentration was 2.4×10^7 sperm/ ml during incubation.

Assessment of motility in BTS:

After 3 days of storage, 0.5 ml of sperm from each treatment (0, 2, 4 and 8 mM MgSO₄) was heated in water bath at 38.5 °C for 15 min, and examined at 20× magnification under a phase contrast microscope at 38.5°C. Subjective assesment of motile spermatozoa (% Total motility) and progressively motile spermatozoa (% Progressive motility) was carried out.

Assessment of sperm charateristics:

To determine the effect of Mg²⁺ after 72 hr storage, boar spermatozoa were subjected to visual assessment of percentages of Total motility and Progressive motility. The spermatozoa washed and then incubated without A23187 at 37°C in air for 30 min, were examined at 20× magnification under a phase contrast microscope at 38.5°C for subjective assesment of motile spermatozoa (% Total motility) and % Progressive motility. Sperm viability was measeured by staining with propidium iodide (PI) according to Harrison and Vickers, (1990).

Induction of acrosomal exocytosis by calcium ionophore A23187:

Spermatozoa were incubated for stimulation (for 30 min) with 3 mM calcium and with or without (control) 0.3 µM of A23187 at 37°C in air as described by Murase et al, (2007). At various intervals of stimulation (5, 10, 15 and 30 min) subsamples were taken and fixed by mixing with an equal volume of 2% glutaraldehyde/0.165 M cacodylate buffer (pH 7.3). Spermatozoa were then examined under a phase contrast microscope (400x), and the acrosomal exocytosis was examined (Murase et al., 2004; Shams-Borhan and Harrison, 1981) as described in Chapter 2, Section I (using 4 replicates for each experiment).

After incubation, sperm motility and viability were examined. Motility was examined as above and sperm viability was measured by staining with propidium iodide (PI) according to Harrison and Vickers (Harrison and Vickers, 1990).

Statistical analysis:

A one-way ANOVA was performed to investigate differences on % Total motility, % Progressive motility, and % Viability. Percentage of acrosomal exocytosis was analysed by two-way ANOVA (time after stimulation \times MgSO₄ concentrations). The Bonferroni test was used for post-hoc analyses where appropriate. Values of $P < 0.05$ were considered to be statistically significant. Data are shown as means \pm SEM. All analyses were carried out using a statistical software program (GraphPad Prism Version 6.0; GraphPad Software, San Diego, CA, USA).

Results

Effects of MgSO₄ on sperm motility and viability:

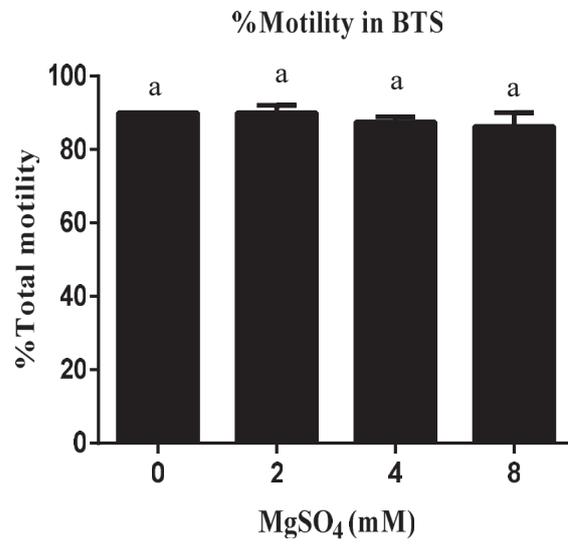
Fig. 1 shows that the percentages of Total motility (A), Progressive motility (B) in BTS and Fig. 2 shows that the percentages of Total motility (A), Progressive motility (B) and % Viability (C) of washed spermatozoa and incubated for 30 min after 72 hr of storage with different concentrations of MgSO₄. One way-Anova revealed that addition of MgSO₄ (0, 2, 4 and 8 mM) did not significantly affect % Total motility, % Progressive motility and Viability with increasing concentrations of MgSO₄ compared to control after 30 min of incubation in presence of 3 mM of CaCl₂ ($P > 0.05$, One-way ANOVA, Fig. 2).

Table 1 shows measured the concentrations of Mg²⁺ and Ca²⁺ in seminal plasma and the remaining Mg²⁺ after adding MgSO₄.

Effect of MgSO₄ on %Acrosomal Exocytosis:

Fig. 3 shows the % acrosomal exocytosis in boar spermatozoa stimulated with Ca²⁺/A23187 after storage with MgSO₄. The Two-way ANOVA revealed that there was a significant interaction between incubation time and MgSO₄ concentrations ($P < 0.0001$). When spermatozoa were stored with 4 mM MgSO₄ added, % the acrosomal exocytosis induced by Ca²⁺/A23187 at 10 and 15 min of incubation was decreased significantly (Fig. 3 B, Two-way ANOVA, Bonferroni post-test $P < 0.05$) but there was no significant differences with MgSO₄ compared to control ($P > 0.05$) at 30 min of incubation. When spermatozoa stored in the presence of MgSO₄ and subsequently incubated without A23187, there was no significant main effect of incubation time and MgSO₄ nor interaction (2-way ANOVA, $P > 0.05$, Fig. 3, A).

(A)



(B)

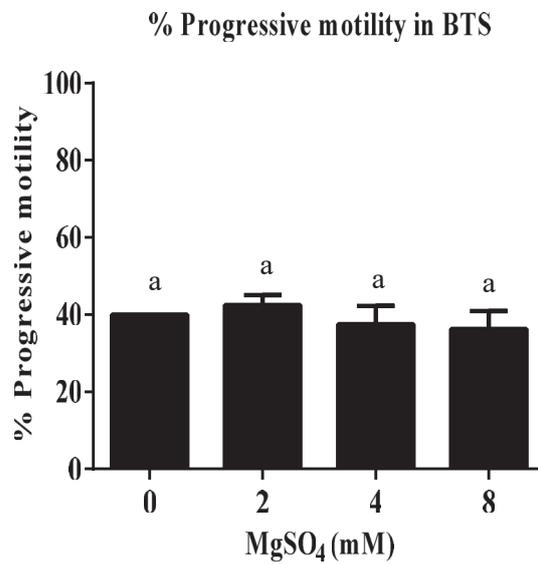


Figure 1. % Total motility (A) and % Progressive motility (B) of boar spermatozoa in BTS after storage for 72 hr with MgSO₄ (0-8 mM). Values are mean \pm SEM from 4 replicates (One-way ANOVA).

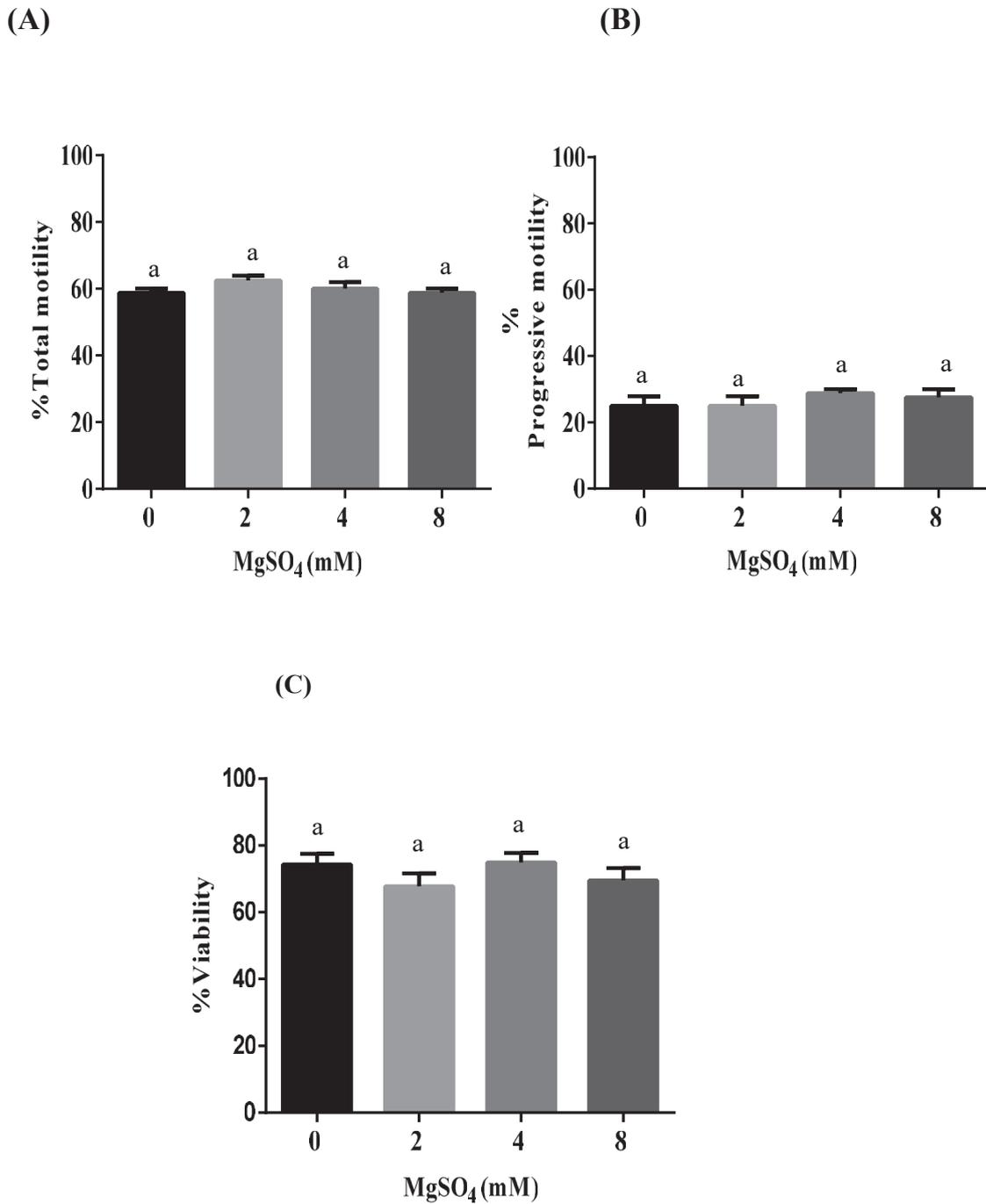


Figure 2. % Total motility (A), % Progressive motility (B) and %Viability (C) of boar spermatozoa washed, and incubated for 30 min after storage for 72 hr with MgSO₄ (0-8 mM). Values are mean \pm SEM from 4 replicates (One-way ANOVA).

Table:1 Concentrations of Mg^{2+} and Ca^{2+} in boar seminal plasma and extended semen after addition of $MgSO_4$.

Boar	Ca^{2+} (mM)	Mg^{2+} (mM)	1/6 Ca^{2+}	1/6 Mg^{2+}	Sum of 1/6 Ca^{2+} and 1/6 Mg^{2+} (α)	Remaining EDTA (mM) in BTS after subtracting α from 2.8 mM ($3.36 \times$ $5/6$)	Actual Mg^{2+} concentration in extended semen after adding $MgSO_4$:			
							0 mM	2 mM	4 mM	8 mM
A	0.925	4.03	0.154	0.672	0.826	1.974	0	0.026	2.026	6.026
B	0.775	4.60	0.129	0.767	0.896	1.904	0	0.096	2.096	6.096
C	1.025	5.26	0.171	0.877	1.048	1.752	0	0.248	2.248	6.248
D	1.025	6.56	0.171	1.093	1.264	1.536	0	0.464	2.464	6.464
Range	0.775 ~1.025	4.03 ~6.56	0.129 ~0.171	0.672 ~1.093	0.826 ~1.264	1.536~1.974	0	0.026 ~0.464	2.026 ~2.464	6.026 ~6.464

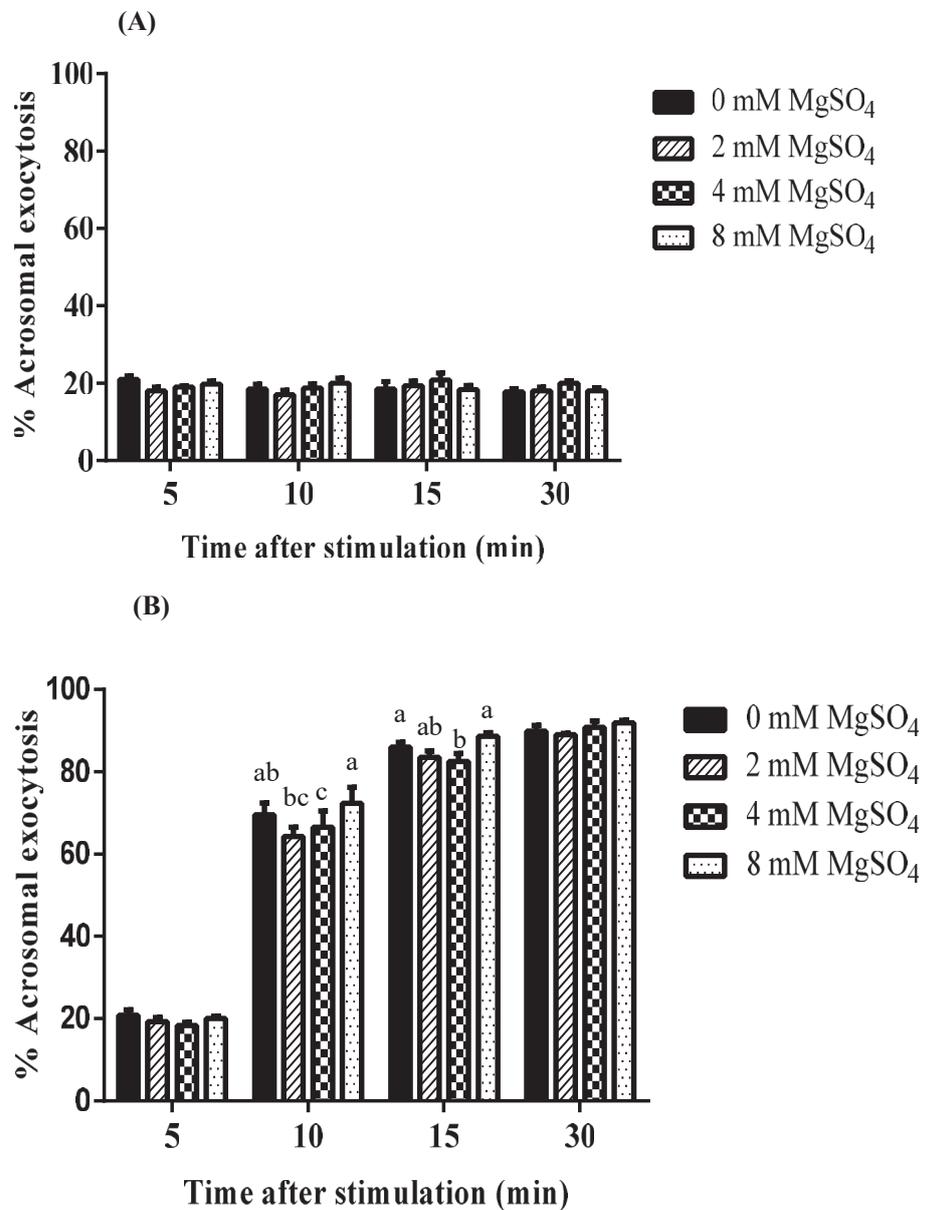


Figure 3: Effect of MgSO₄ on boar sperm acrosomal exocytosis induced by calcium and A23187. Boar spermatozoa from ejaculates diluted with BTS and stored for 72 hr with MgSO₄ (0-8 mM), washed and then incubated in the absence (A) or presence (B) of 0.3 μM A23187 for 5, 10, 15 and 30 min. Interaction between incubation time and concentrations of MgSO₄ was significant (Two-way ANOVA, $P < 0.0001$). a-c: Significant differences ($P < 0.05$). Values are mean \pm SEM from 4 replicates.

Discussion

The use of porcine semen for AI by means of fresh diluted semen has increased considerably (Maes et al., 2011) and contribute to the improvement of genetic potential (offers important genetic material or productivity improvement). BTS (Pursel and Johnson, 1975) is a simple extender that is widely used for storage of boar semen for up to 3 days. However, after incubation of 30 min, the motility and viability were not affected by various concentrations of Mg^{2+} . It should be noted that, after 30 min incubation, the percentages of live spermatozoa was higher than the motile one (i.e some of the live spermatozoa did not move), a possible dysfunction of cell mitochondria could be inducing the loss of motility in spermatozoa with intact membranes.

For liquid storage of boar spermatozoa, the inclusion of EDTA in diluents is an important step. EDTA which is used as a chelating substance, (captures divalent cation, especially Ca^{2+} and Mg^{2+}), and prevent premature occurrence of capacitation and acrosome reaction by limiting Ca^{2+} movement across the plasma membrane (Johnson et al., 2000). In this study, the calcium and magnesium concentrations were measured in seminal plasma from 4 boars. When remaining Ca^{2+} and Mg^{2+} concentrations were calculated in diluted semen after adding $MgSO_4$ (0-8 mM), concentration of Ca^{2+} was 0 mM for all and that of Mg^{2+} was 0 mM, 0.0264 ~ 0.464 mM, 2.026 ~ 2.464 mM and 6.026 ~ 6.464 mM with $MgSO_4$ added at 0, 2, 4 and 8 mM. Thus boar spermatozoa were stored in the absence of Ca^{2+} but in presence of Mg^{2+} . Because semen was diluted 6-fold, the original concentration of Mg^{2+} was reduced to 0.826 ~ 1.264 mM (Table. 1), and Mg^{2+} concentrations does not seem to have ranged widely among different boars after dilution and addition of $MgSO_4$. Thus, comparison among different concentrations of add $MgSO_4$ may have been valid.

The % acrosomal exocytosis was significantly decreased at 10 and 15 min of stimulation with A23187 (Fig: 3B) whereas no reduction in motility and viability by addition of MgSO₄ to diluted semen was observed at the concentrations used (MgSO₄ ≤ 8 mM). Previous results showed that in summer, the acrosome reaction in response to A23187 occurred more quickly, and there was a tendency that magnesium concentrations in seminal plasma also decreased in summer (Murase et al., 2007). Therefore, addition of magnesium may be beneficial to alleviate hypersensitive spermatozoa during summer with influence motility.

Chapter 3

Introduction:

AI is an effective and globally accepted method of breeding animals including pigs. Liquid preservation is a method of boar semen storage, in which diluted semen is stored at 15-20°C for several days until it used for AI (Huo et al., 2002). Depending on the composition of the extender, semen can be stored in short-term extenders (for 2–3 days) and up to five days or longer in long-term extenders (Johnson et al., 2000). Semen collected from boars is ultimately diluted in any one of a variety of commercially available boar semen extenders. Among the commercially available boar semen extenders, one widely used is BTS. However, during storage of boar spermatozoa, they undergo several changes including diminished motility, viability, membrane permeability and DNA fragmentation (Bryła and Trzcí, 2015). In addition, Sepúlveda et al. (2014), observed that when bacteria are present at higher concentrations, 3.5×10^3 cfu/mL, there is a significant decreases in the proportion of total and progressive sperm motility, sperm viability and acrosome integrity.

PMB is polycationic antibiotic that binds to the LPS complex by neutralizing its endotoxic activity largely because of its high-affinity binding to lipid A (Moore et al., 1986) . Other than an antibacterial action, PMB has also been used as a modulator of signalling molecules in somatic cells. It inhibits a variety of enzymes such as protein kinase C (LI et al., 2016; Mizumaki et al., 2002; Radallah et al., 1999; Wallin et al., 2003.), a phospholipid-sensitive Ca^{2+} protein kinase (Mazzei et al., 1982), calmodulin (CAM) (Hegemann et al., 1991), CAM-dependent phosphodiesterase from bovine heart *in vitro* (Hegemann et al., 1991), Ca^{2+} /CAM-dependent protein kinases (Depaoli-Roach et al., 1979; Walsh et al., 1979), K^{+} -ATP channels (Harding et al., 1994), Ca^{2+} -ATPase, p-nitrophenyl phosphatase and phosphorylase kinase of rabbit skeletal muscle sarcoplasmic reticulum membranes (Ktenas et al., 1989).

The purposes of this chapter was to examine the possible effect of PMB on boar sperm acrosomal exocytosis triggered by Ca^{2+} /A23187 and PMB added during storage upto 48 hr and then stimulated by A23187 for 30 min to explore the effect of PMB on acrosomal exocytosis after incubation.

Section I

**Effects of Polymyxin B on Exocytosis of the Boar Sperm
Acrosome Stimulated by Calcium and Calcium Ionophore
A23187**

Introduction:

Bacterial contamination is routinely observed in stored semen (Bryła and Trzcińska, 2015) and Gram-negative bacteria release an endotoxin, lipopolysaccharide (LPS) which decreases sperm motility. Okazaki *et al.* (2010), reported that PMB is able to neutralizes endotoxin of bacteria with which boar semen may be contaminated and that addition of PMB (100 µg/ml) to chilled stored boar spermatozoa is beneficial to avoid decrease in motility. In human spermatozoa, PMB suppressed the negative effects of LPS derived from *Chlamydia trachomatis* (Hosseinzadeh et al., 2003).

While PMB is beneficial for boar semen storage via antibacterial action, the direct effect of PMB, not via neutralizing effect of PMB on bacteria, has been totally unknown. Because acrosomal exocytosis is an essential step to fertilization, information on the direct effect is of significance to employ PMB for boar semen storage. This chapter investigated the effect of PMB on acrosomal exocytosis triggered by calcium and the calcium ionophore A23187 in boar spermatozoa that were freed from seminal plasma, extender, and hence from LPS. Because direct effect of PMB on boar spermatozoa has been totally unknown, its effect on motility, viability, sperm vigour and agglutination were first examined. Then the PMB concentrations found to be without effect on these traits were used to investigate the effect of PMB on acrosomal exocytosis in order to avoid false assessment by degenerative acrosome loss.

Materials and Methods:

Media

Saline medium was used for washing and incubation of spermatozoa and consisted of 142 mM NaCl, 2.5 mM KOH, 10 mM glucose and 20 mM Hepes, adjusted to pH 7.55 at 20°C with NaOH (Roldan and Harrison, 1989). Sucrose medium containing 222 mM sucrose in place of NaCl in saline medium was used for washing spermatozoa. Saline and sucrose media also contained 0.1% (w/v) polyvinyl alcohol (average molecular weight of 30,000–70,000) and 0.1% (w/v) polyethylene glycol (consists of 2 moles of polyethylene glycol, 7000-9000). Saline medium contained 3 mM CaCl₂ during sperm pre-incubation and incubation for stimulation. A stock solution of 30 µM ionophore A23187 (Free acid; Calbiochem-Novabiochem/EMD Biosciences, La Jolla, CA, USA) was prepared in dimethyl sulfoxide (DMSO) and diluted in saline medium to give a final concentration of 0.3 µM. Polymixin B Sulfate (EMD Chemicals, San Diego, CA USA) was dissolved in H₂O at 15 mM and kept frozen at –30°C as stock solution. For use in each experiment, a stock solution was allowed to thaw and then diluted with H₂O for 100x stocks of 1, 2.5, 5, 7.5 and 10 mM. The 100x stocks were added to saline medium for the desired final concentrations.

Spermatozoa

Boar semen was purchased from Fuji Nojo Service, a porcine AI center in Japan. The sperm-rich fraction was collected by the gloved-hand method from three mature fertile boars (one Landrace and two Large White) aged 18-24 months and diluted with BTS (Johnson et al., 1988) at a constant of 6-fold and dispatched at 17°C to the laboratory, where semen was stored at 17°C for up to 3 days.

The experiments described here were approved by the Committee for Animal Research and Welfare of Gifu University.

Boar spermatozoa were washed according to Murase et al., (2004) as described in chapter 2. Sperm concentration was adjusted to 2.4×10^7 sperm/ml during pre-incubation (Experiment 2) and incubation (Experiments 1 and 2).

Experiment 1 – Assessment of sperm characteristics

The washed spermatozoa were incubated with various concentrations of PMB (0, 10, 25, 50, 75 and 100 μ M) at 37°C in air for 20 min. After incubation, spermatozoa were examined at 20 \times magnification under a phase contrast microscope at 38.5°C for subjective assessment of motile spermatozoa (% Total motility). Sperm viability was measured by staining with propidium iodide (PI) according to Harrison and Vickers, (1990). Sperm vigor of flagellar beating was also subjectively evaluated on a grade of 0 to 4 (sperm vigor grade); 0, immotile; 1, sluggish movement; 2, movement with lowest speed; 3, sperm flagellum beating as fast as flagellum track is visible; and 4, spermatozoa beating flagellum as fast as unrecognizable track. Agglutination was classified into 4 categories (agglutination score): no spermatozoa agglutinated; 1, $0 < \text{agglutinated} \leq \frac{1}{4}$; 2, $\frac{1}{4} < \text{agglutinated} \leq \frac{1}{2}$; 3, $\frac{1}{2} < \text{agglutinated} \leq \frac{3}{4}$ and 4, $\frac{3}{4} < \text{agglutinated}$ (Agglutination score) (Murase et al., 2007). An ejaculate from each boar was used on day 1, 2 and 3 of storage so that 9 replicates (3 boars \times 3 storage days) were carried out for each experiment.

Experiment 2 – Induction of acrosomal exocytosis by calcium ionophore A23187

Washed spermatozoa from the 3 different boars were pre-incubated with various concentrations of PMB (0, 0.01, 0.05, 0.1 and 0.5 μ M for the experiment with low concentrations of PMB and 0, 1, 10, 25 and 50 μ M for that with high concentrations of PMB)

in saline medium at 37°C in air for 10 min and then incubated for stimulation with 0.3 µM A23187. H₂O and DMSO were added as vehicle control for PMB (0 µM) and A23187, respectively. At various intervals of stimulation (5, 10 and 15 min), subsamples were taken and fixed by mixing with an equal volume of 2% glutaraldehyde/0.165 M cacodylate buffer (pH 7.3). Spermatozoa were then examined under a phase contrast microscope (400x), and acrosomal exocytosis was examined according to Shams-Borhan and Harrison (Shams-Borhan & Harrison, 1981). The percentage of spermatozoa displaying acrosomal exocytosis was obtained (% Acrosomal exocytosis).

Statistical analyses

All data are expressed as the Means ± SEM. % Total motility, % Viability and Sperm vigor grade were analysed by 2-way ANOVA (storage time × PMB concentration) followed by the Tukey's multiple comparison test except for % Viability or Fisher's protected least significant difference test (% Viability). Percentage of acrosomal exocytosis was analysed by 2-way ANOVA (time after stimulation × PMB concentration) followed by Bonferroni multiple comparison test. Values of P < 0.05 were considered to be statistically significant. All analyses were carried out using a statistical software program (GraphPad Prism Version 6.0; GraphPad Software, San Diego, CA, USA)

Results

Experiment -1 (Effect of PMB on sperm characteristics)

Two-way ANOVA did not reveal a significant main effect of storage nor interaction on any of the % Total motility, % Progressive motility, % Viability, Vigor grade and Agglutination score but found a significant main effect of PMB on % Total motility, % Progressive motility, % Viability and Sperm vigor grade. For the parameters on which a significant main effect of PMB was found, data were pooled across the days of storages (day 1, day 2 and day 3) and each parameter was compared among different concentrations of PMB.

Figs. 1 shows % Total motility (A and B), % Progressive motility (C and D), % Viability (E and F), Sperm vigor grade (G and H) and Agglutination score (I and J) in spermatozoa incubated with different concentrations of PMB. The % Total motility and Viability did not significantly differ up to 50 μM PMB with a significant decline at 75 μM and 100 μM (Figs. 1 A, B, E and F) while % Progressive motility significantly decreased at 25 μM PMB and higher concentration (Figs 1 C and D; Tukey's multiple comparison test, $P < 0.0001$). Sperm vigor grade did not differ significantly up to 50 μM but the higher concentrations of PMB (75-100 μM) significantly reduced it (Figs. 1 G and H; $P < 0.0001$). There were no significant differences in the Agglutination scores (Figs. 1 I and J; $P > 0.05$) among any concentrations of PMB examined.

Experiment-2 (Effect of PMB on acrosomal exocytosis)

Figs. 2 shows % Acrosomal exocytosis in boar spermatozoa stimulated with A23187 after preincubated with PMB (Figs. 2 A and B; 0-0.5 μM and Figs 3 A and B; 0-50 μM). The Two-way ANOVA revealed that there was a significant interaction between time and PMB ($P < 0.0001$). Preincubation of boar spermatozoa with PMB (0.01-0.5 μM , Fig 2 B;

1-50 μ M, Fig 3 B) resulted in enhancement of acrosomal exocytosis induced by A23187 at 10 and 15 min. In spermatozoa preincubated in the presence of PMB and subsequently incubated without A23187, there was no significant main effect of PMB and time nor interaction between time and concentrations of PMB (2-way ANOVA; $P > 0.05$, Figs. 2 A and 3 A).

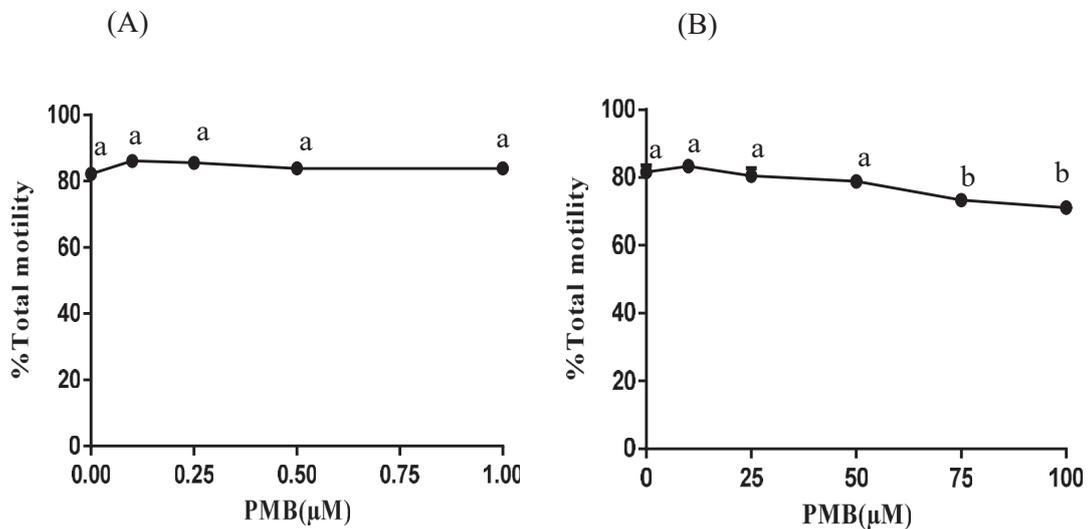


Figure: 1 The effect of different concentrations PMB on the percentages of motility, progressive motility, viability, vigor grade and agglutination score of boar spermatozoa. Sperm incubated for 20 min in presence of 3mM of Ca^{2+} . (A) Total motility (0-1μM), (B) Total motility (10-100μM), (C) Progressive motility (0-1μM), (D) Progressive motility (10-100μM), (E) Viability (0-1μM), (F) Viability (10-100μM), (G) Sperm vigor grade (0-1 μM), (H) Sperm vigor grade (10-100 μM), (I) Agglutination score (0-1 μM), and (J) Agglutination score (10-100 μM) after 20 min of incubation. (a-c) indicates significant differences from the controls ($P < 0.05$). No significant main effect of storage ($P > 0.05$) nor interaction was found for all the parameters, data were pooled across the days of storage (Day-1, 2, 3). A significant main effect of PMB concentration was revealed for all the parameters. Values are mean \pm SEM calculated on the basis of nine experiments (3 days \times 3 boars).

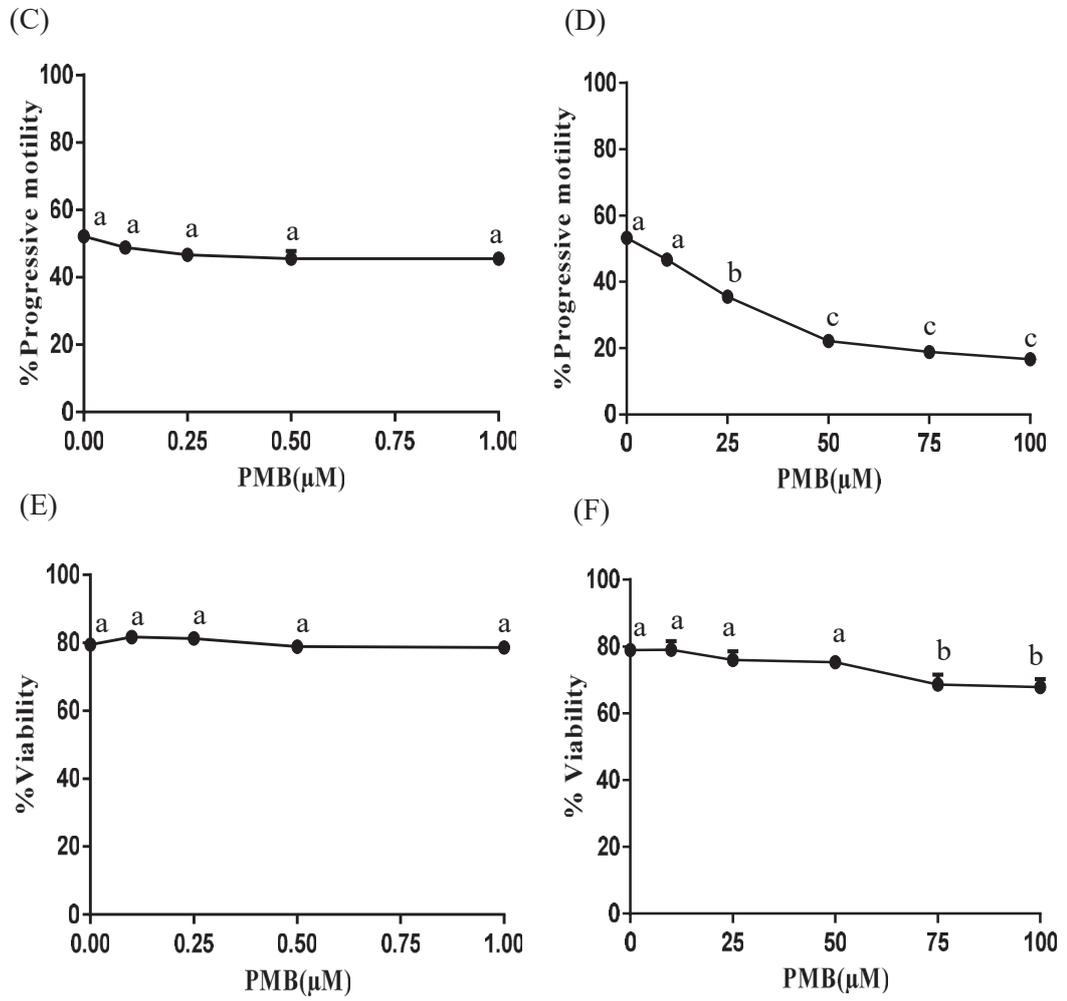


FIGURE 1

Continuing

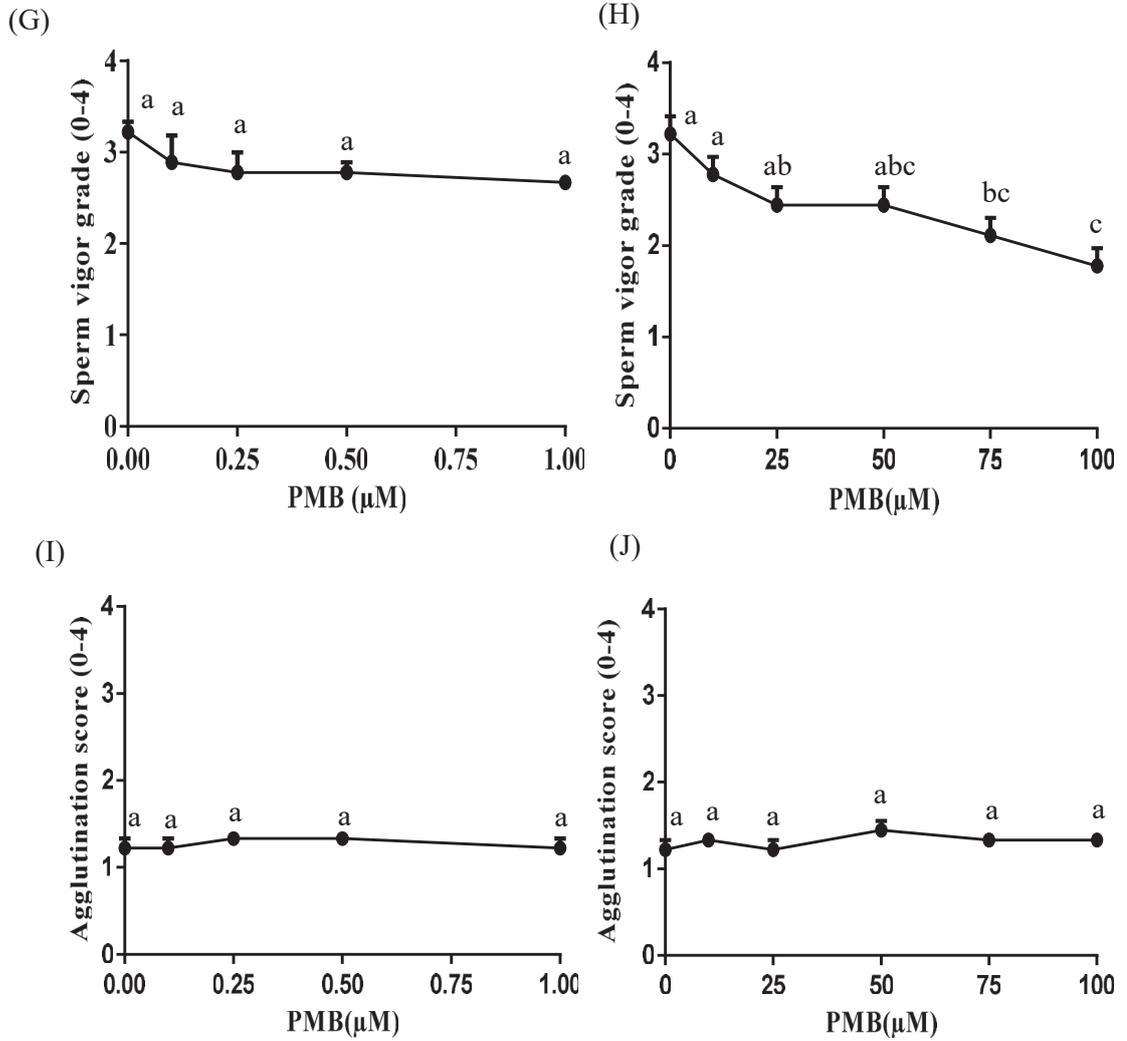


FIGURE-1

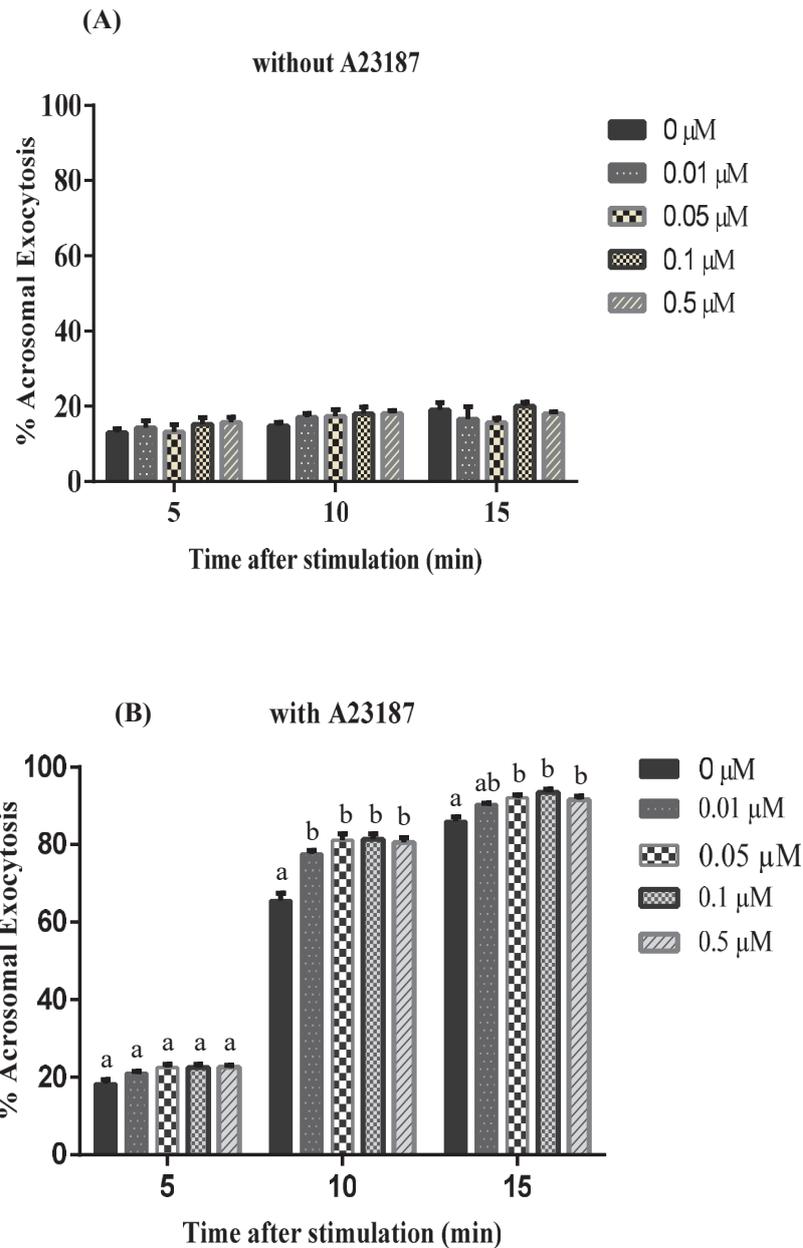


Figure 2. Effect of PMB on boar sperm acrosomal exocytosis induced by calcium and A23187. Acrosomal exocytosis after incubation in the absence (A) or presence (B) of 0.3 μM A23187 for 5, 10 and 15 min of incubation following preincubation for 10 min with different concentrations of PMB (A and B, 0–0.5 μM). Interaction between incubation time and concentrations of PMB was significant. a, b: Significant differences ($P < 0.05$). Values are means \pm SEM from 3 replicates.

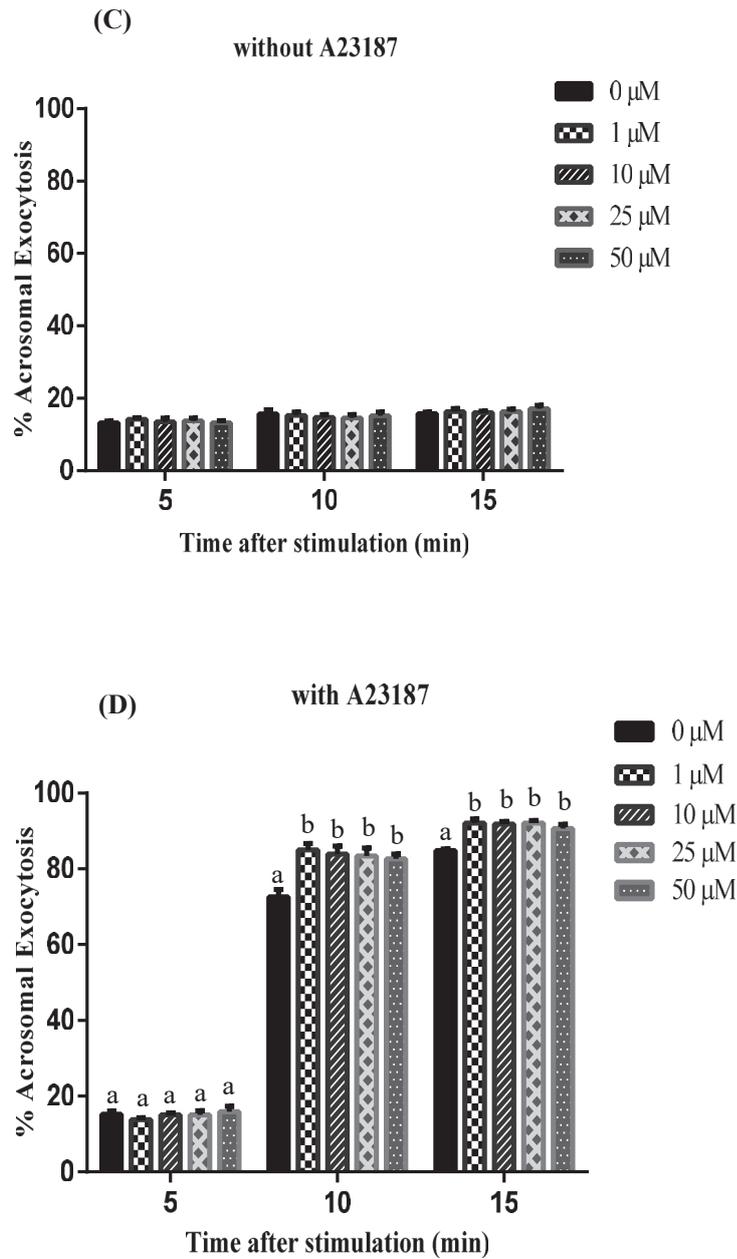


Figure 3. Effect of PMB on boar sperm acrosomal exocytosis induced by calcium and A23187. Acrosomal exocytosis after incubation in the absence (A) or presence (B) of 0.3 μ M A23187 for 5, 10 and 15 min of incubation following preincubation for 10 min with different concentrations of PMB (A and B, 0–50 μ M). Interaction between incubation time and concentrations of PMB was significant (Two-way ANOVA, $P < 0.0001$). a, b: Significant differences ($P < 0.05$). Values are means \pm SEM from 3 replicates.

Discussion:

The present study examined the effect of PMB on boar sperm acrosomal exocytosis in order to provide basic information for utilization of PMB as an additive to semen extender during boar semen storage. The results showed that PMB enhanced acrosomal exocytosis triggered by A23187 at the concentration that did not affect sperm motility and viability, suggesting that PMB can increase the ability of boar spermatozoa to undergo acrosomal exocytosis.

Effect on motility and viability

Among the substrates for inhibition by PMB so far identified in somatic cells, PKC (Bragado et al., 2010), calmodulin (Schlingmann et al., 2007; Lackey & Gray, 2015; Marin-Briggiler, 2005) and endoplasmic reticulum Ca^{2+} -ATPase (Williams & Ford, 2003) are also involved in sperm motility regulation. In somatic cells, PMB was used as a PKC inhibitor at around 10 μM [14 μM , (Li et al., 2016) 10 μM (Wallin et al., 2003)] and as an inhibitor of sarcoplasmic reticulum Ca^{2+} -ATPase at 100 μM of IC_{50} (Ktenas et al., 1989). Unlike the results shown by Okazaki et al., (2010), in which sperm motility was maintained higher with PMB and penicillin G added during storage than penicillin G alone, we observed that after incubation with PMB, sperm motility was decreased by 75–100 μM (Figures 1 B). This discrepancy may be due to binding of PMB to LPS present in the extended semen, resulting in less PMB molecules remaining available to spermatozoa in the report by Okazaki et al. (2010). Thus spermatozoa may have been exposed to less number of available PMB in the extended semen while washed spermatozoa were directly exposed to PMB at high concentrations without binding to LPS in this study. A PKC inhibitor Ro-32-0432 inhibited boar sperm motility under non-capacitating conditions in a medium without CaCl_2 , NaHCO_3 and BSA (Bragado et al., 2010). Although saline medium used to incubate boar spermatozoa

for motility examination contained Ca^{2+} , it did not contain bicarbonate or BSA. Therefore the medium may be recognized as 'non-capacitating' medium, and it is speculated that PMB may have inhibited PKC leading to decreased motility in saline medium in this study. Calmodulin is another possible target to decrease sperm motility. CAM is present in the principal piece of the flagellum in boar spermatozoa and is involved in regulation of motility via calmodulin/CAMK $\alpha\beta$ and cAMP/PKA pathway (Schlingmann et al., 2007) or CAM/CAMK-dependent soluble adenylylase/cAMP/PKA pathway (Lackey and Gray, 2015). It was shown that inhibition of CAM decreases mouse cauda sperm motility (Schlingmann et al., 2007) and human sperm motility (Marin-Briggiler, 2005). Thus it was possible that PMB might have inhibited calmodulin leading to the decrease in sperm motility in this study. Furthermore, because thapsigargin, an inhibitor of endoplasmic reticulum (acrosomal) Ca^{2+} -ATPase, also inhibited human sperm motility (Williams and Ford, 2003), it might be possible that PMB inhibited endoplasmic reticulum Ca^{2+} -ATPase of spermatozoa and decreased motility in this study.

Treatment of boar spermatozoa with high concentrations of PMB ($\geq 50 \mu\text{M}$) resulted in the decrease in viability (Figures 1 F). A recent study showed that addition of PMB to bull semen extender at $100 \mu\text{g/ml}$ (equal to $\sim 75 \mu\text{M}$) resulted in beneficial effect on sperm motility after freezing-thawing while higher concentration ($1,000 \mu\text{g/ml}$, equal to $\sim 750 \mu\text{M}$) of PMB reduced viability (Rashedi et al., 2017). This indicates that the antibiotic is spermicidal depending on the concentrations used. It, however, should be taken into consideration that PMB molecules that bind to LPS is expected to be no longer available to spermatozoa and that thus depending on the LPS content in semen, direct effect of PMB on spermatozoa may be decreased.

Effect on Agglutination:

Ca²⁺-ATPase inhibitor, thapsigargin, promoted head-to-head agglutination of boar spermatozoa in a concentration dependent manner (Marin-Briggiler, 2005). Teijeiro *et al.*, 2017, suggested that boar sperm agglutination is produced by increased membrane fluidity in sperm head via a mechanism mediated by PKC. If acrosomal Ca²⁺-ATPase had been inhibited by PMB in this study, agglutination should have been increased. However, this was not the case and suggests that PMB may not have affected Ca²⁺-ATPase in relation to agglutination. Alternatively, it is considered that PMB may have inhibited PKC and that agglutination may have been unchanged and remained low with different concentrations of PMB.

Effect on acrosomal exocytosis

Among a variety of molecules that are inhibited by PMB, Ca²⁺-ATPase ((Roldan and Fleming, 1989; Meizel & Turner, 1993; Parrish *et al.*, 1999), calmodulin (Ackermann *et al.*, 2009) and calmodulin-dependent phosphodiesterase (PDE) Type-I (Fournier *et al.*, 2003) negatively regulate capacitation and/or acrosomal exocytosis of mammalian spermatozoa. In other words, inhibition of endoplasmic reticulum (acrosomal) Ca²⁺-ATPase by thapsigargin promoted sperm acrosomal exocytosis in human (Meizel and Turner, 1993) and bulls (Parrish *et al.*, 1999), calmodulin inhibitor W7 increased both spontaneous and A23187-induced acrosomal exocytosis in mouse spermatozoa (Ackermann *et al.*, 2009) and promoted human sperm capacitation (Leclere *et al.*, 1998), and calmodulin-dependent phosphodiesterase type I prevents the capacitation taking place prematurely (Fournier *et al.*, 2003). It is speculated that PMB may have inhibited these molecules leading to increased acrosomal exocytosis. However, concentration of PMB used to inhibit Ca²⁺-ATPase was μ M levels as cited above

(Ktenas et al., 1989) while PMB is a much more potent inhibitor of calmodulin with effective concentration at nanomolar levels (Hegemann et al., 1991). Therefore, it is possible that nanomolar levels (0-0.5 μ M) of PMB may have inhibited calmodulin while higher concentration of PMB may have inhibited Ca²⁺-ATPase, both resulting in enhanced acrosomal exocytosis. If this is the case, the interpretation of the mechanisms would be that these lower concentrations of PMB may inhibit calmodulin which in turn inhibits CAM dependent PDE type 1 (Fournier et al., 2003), to accumulate cAMP resulting in enhancement of acrosomal exocytosis. This notion is supported by the findings that cAMP enhances acrosomal exocytosis triggered by A23187 in ram spermatozoa (Garde and Roldan, 2000; Garde and Roldan, 1996). It, however, was unclear whether the mechanisms regulating motility and acrosomal exocytosis via Ca²⁺-ATPase, respectively, were independent from one another.

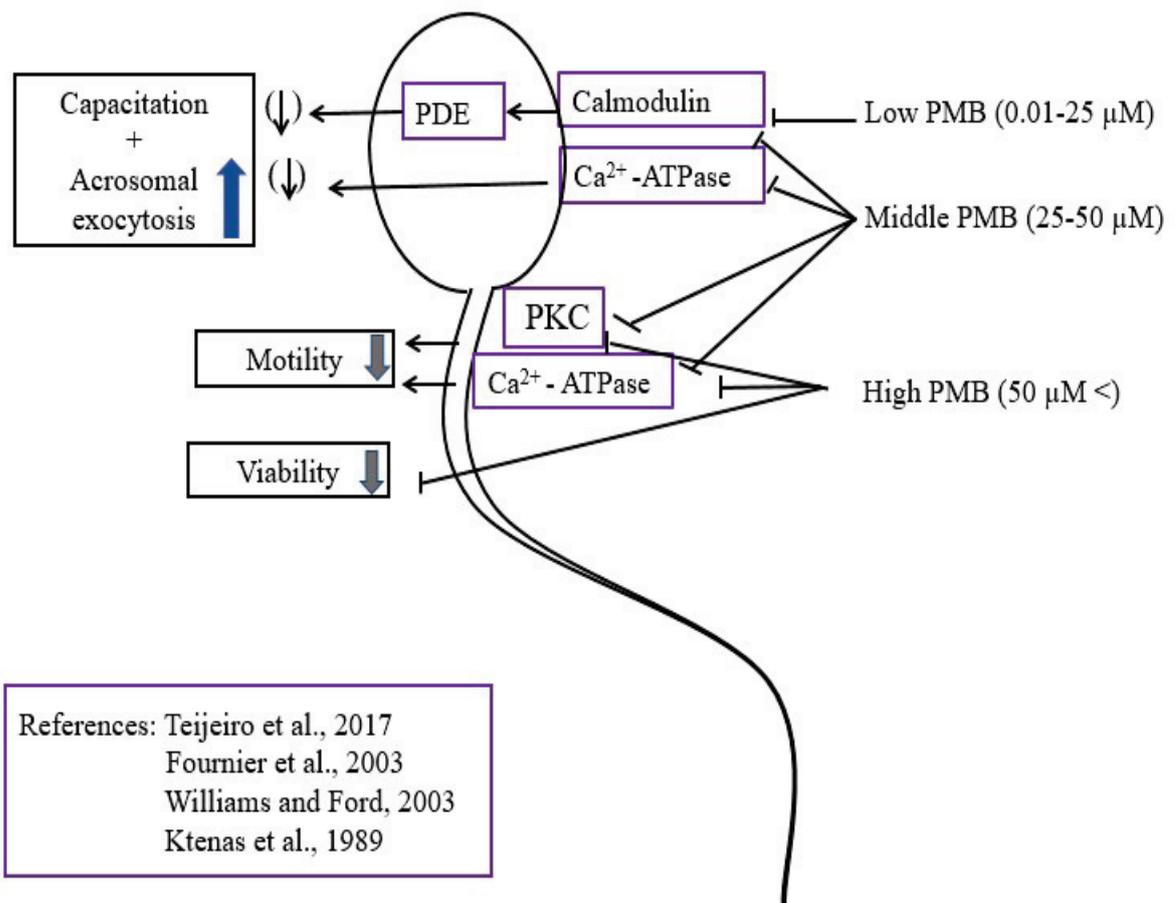
This study sought a possible direct effect of PMB on boar spermatozoa freed from seminal plasma and extender and hence from LPS because PMB is known to act as inhibitor of a variety of signalling molecules in somatic cells. The results showed that PMB decreased motility and viability at μ M levels but increased acrosomal exocytosis at lower concentrations. Thus the study suggests that PMB has direct effects on boar spermatozoa. This should be taken into consideration when it is added to boar semen extender. In our previous study, boar spermatozoa collected during summer season, when "summer infertility" takes place, were found to be more sensitive to calcium ionophore A23187, suggesting that boar spermatozoa have a higher inducibility of acrosomal exocytosis in summer (Murase et al., 2007). It may be important to point out that if PMB is added to semen diluted in summer, inducibility of spermatozoa would be further increased. On the contrary, it is possible that PMB could increase acrosomal exocytosis of spermatozoa that may have decreased their

ability to undergo acrosomal exocytosis for some reason, for example, by sub-fertility. It is still unknown whether PMB similarly enhances acrosomal exocytosis when it is added to extended semen during storage and whether the increased inducibility of acrosomal exocytosis is related to fertility after AI. Further experiments are address these issues.

Conclusion

The present study showed that PMB has direct effect on acrosomal exocytosis in boar spermatozoa and pointed out the importance to take into consideration the direct effect of PMB on acrosomal exocytosis in boar spermatozoa, when it is used for AI in pigs. It was hypothesized from this study 1) that in boar ejaculated spermatozoa, nanomolar concentrations of PMB may inhibit calmodulin which in turn would accumulate cAMP to enhance acrosomal exocytosis and 2) that higher concentrations of PMB may inhibit Ca^{2+} -ATPase or PKC resulting in decreased motility and enhanced acrosomal exocytosis, and 3) the intermediate concentrations of PMB may inhibit both calmodulin, Ca^{2+} -ATPase and PKC. Further studies are required to test the hypothesis of the mechanisms by which PMB acts on acrosomal exocytosis in spermatozoa and to reveal the effect of PMB added to diluted semen during storage on acrosomal exocytosis.

Hypotheses



Section II

Polymyxin B Added during Liquid Storage at 17°C Increases Progressive Motility and Acrosomal Exocytosis in Boar Spermatozoa

Introduction

The addition of antibiotics to semen extenders is a common feature, usually aimed to avoid bacteria growth in extended semen (Lopez Rodriguez et al., 2017) which are harmful to the quality of semen. Endotoxins produced by bacteria interfere with spermatozoa survival time in semen and cause sperm agglutination and reduced motility (Diemer et al., 1996). However, in somatic cells, it has been shown that PMB is an inhibitor of signaling molecules including calmodulin (Hegemann et al., 1991), protein kinase C (Mazzei et al., 1982; Wise et al., 1982), Ca²⁺-ATPase and K⁺-channel (Harding et al., 1994). Thus PMB has direct actions on cells other than neutralizing effect on LPS of bacteria.

Okazaki et al. (2010) demonstrated that PMB added to extended and stored boar semen maintained sperm motility higher than without its addition. However, the influence of PMB added to stored semen on other sperm characteristics and acrosomal exocytosis is as yet unclear. Because PMB has an inhibitory effect on signalling molecules known in somatic cells (see above), it was inferred that PMB might affect signal transduction pathways regulating sperm fertilizing functions, including acrosomal exocytosis. The objective of this section was to investigate the effects of PMB added during storage on sperm characteristics and acrosomal exocytosis induced by calcium and A23187.

Materials and Methods

Storage of boar semen extender with PMB:

Boar semen was purchased from Fuji Nojo Service, boar AI center in Japan. The sperm rich fraction collected by the gloved-hand technique from 3 Landrace boars (collected at the end of August) were diluted 6-fold with BTS (Johnson et al., 1988) with different concentrations of PMB and preserved at 17°C. Ejaculates of acceptable quality (i.e. total motility > 95%, Vigour grade 4 and Agglutination score 0) were used. Semen was diluted (constant dilution at 6-fold) at a temperature of ~29 °C. PMB was dissolved in H₂O at 100X of final concentrations. For use, the stock was allowed to thaw at room temperature and added 100-fold to BTS for final concentrations of 0 (H₂O added as vehicle control), 25, 50 and 75 µM. Then semen was added to give a constant sperm concentration of 1.2×10^8 cells/ml, and kept at 17°C for 48 hrs. The preserved semen was agitated once daily.

Assessment of motility in BTS

After 2 days of storage, 0.5 ml of sperm from each treatment (0, 25, 50 and 75 µM PMB) was heated in water bath at 38.5 °C for 15 min, and examined at 20× magnification under a phase contrast microscope at 38.5°C. Subjective assessment of motile spermatozoa (% Total motility) and progressively motile spermatozoa (% Progressive motility) was carried out.

Washing of spermatozoa

Boar spermatozoa were washed as described in Chapter 2, Section I.

Assessment of sperm characteristics after incubation

To determine the effect of PMB after 48 hr storage, boar spermatozoa were subjected to visual assessment of percentages of total motility, progressive motility, sperm vigor grade and agglutination score. The washed spermatozoa were pre-incubated for 10 min and then incubated without A23187 at 37°C in air for 30 min. After incubation sperm motility were examined as described in previous chapter. Sperm vigor of flagellar beating on a grade of 0 to 4 (sperm vigor grade) were subjectively evaluated. Criteria of Vigour grade and Agglutination score were classified briefly in Chapter 3, Section I.

Induction of acrosomal exocytosis by calcium ionophore A23187

After washing, PMB treated spermatozoa were pre-incubated in the saline medium containing 3 mM CaCl₂ for 10 min and then stimulated with or without (control) 0.3 μM of A23187 at 37°C in air for 30 min. At various intervals of stimulation (5, 10, 15 and 30 min) subsamples were taken and fixed by mixing with an equal volume of 2% glutaraldehyde/0.165 M cacodylate buffer (pH 7.3). Spermatozoa were then examined under a phase contrast microscope (400x), and the acrosomal exocytosis was examined (using 3 replicates for each experiment).

After incubation, sperm motility and viability were examined in order to examine if any degenerative effect of PMB was present on spermatozoa. Motility was examined as above and sperm viability was measured by staining with propidium iodide (PI) according to Harrison and Vickers, 1990.

Statistical analysis

All data are expressed as the mean \pm SEM. % Total motility, % Progressive motility, % Viability, Sperm vigor grade and Agglutination score were analysed by One-way ANOVA followed by the Tukey's multiple comparison. Percentage of acrosomal exocytosis was analysed by two-way ANOVA (time after stimulation \times PMB concentrations) followed by Bonferroni multiple comparison test. Values of $P < 0.05$ were considered to be statistically significant.

All analyses were carried out using a statistical software program (GraphPad Prism Version 6.0; GraphPad Software, San Diego, CA, USA)

Results

Effect of PMB on motility and viability:

Addition of PMB (0, 25, 50 and 75 μM) did not significantly affect % Total motility but significantly increased % Progressive motility of stored spermatozoa with BTS containing and the increase was significant at 75 μM PMB compared to control ($P < 0.05$, Table 1).

One-way ANOVA revealed a significant effect of PMB concentrations on % Total motility and % Viability of washed and incubated spermatozoa without A23187 but did not reveal significant effect of % Progressive motility, Sperm vigor grade and Agglutination score (Table 2).

Table 2 shows % Total motility, % Progressive motility, % Viability, Sperm vigor grade and Agglutination score in spermatozoa stored with different concentrations of PMB, washed and incubated for 30 min. The % Total motility and % Viability decreased significant by at 25 μM PMB, compared to 0 μM but did not significantly differ at 50 μM and 75 μM PMB ($P < 0.05$) compared to 0 μM % Progressive motility, Agglutination score, and Sperm vigor grade did not differ significantly among any concentrations of PMB examined (One-way ANOVA).

Effect of PMB on %Acrosomal Exocytosis:

In spermatozoa, stored in the presence of PMB and subsequently washed and incubated without A23187, there was no significant main effect of incubation time and PMB nor interaction (2-way ANOVA, $P > 0.05$, Fig. 1, A).

When boar spermatozoa were stimulated with Ca^{2+} /A23187, the Two-way ANOVA revealed that there was a significant interaction of % Acrosomal exocytosis between incubation time and PMB ($P < 0.0001$). Storage with 50-75 μM PMB resulted in significant enhancement of acrosomal exocytosis induced by Ca^{2+} /A23187 at 15 min and 30 min of stimulation (Fig.1 B; 2-way ANOVA, Bonferroni multiple comparison test) while were no significant differences among the different concentration of PMB at 5 and 10 min ($P > 0.05$).

Table 1. Effect of PMB on motility (%) in BTS stored for 48 h:

PMB (μM)	% Total Motility	% Progressive motility
0	90.0 \pm 3.5	26.7 \pm 4.7 ^a
5	88.3 \pm 3.5	33.3 \pm 4.7 ^{ab}
50	88.3 \pm 3.5	36.7 \pm 4.7 ^{ab}
75	91.7 \pm 3.5	43.3 \pm 4.7 ^b

a, b: Different superscripts represents significant differences (One-way ANOVA, $P < 0.05$)

Values are mean \pm SEM from 3 replicates.

Table 2. The effect of different concentrations of PMB (0-75 μ M) during storage for 48 h on % Total motility, % Progressive motility, % Viability, Sperm vigor grade and Agglutination score in spermatozoa, washed and incubated for 30 min.

PMB (μM)	% Total Motility	% Progressive motility	% Viability	Agglutination Score	Sperm Vigor
0	61.7 \pm 2.6 ^a	20.0 \pm 4.7 ^a	64.0 \pm 2.0 ^a	2.0 \pm 0.4 ^a	2.3 \pm 0.5 ^a
25	50.0 \pm 2.6 ^b	20.0 \pm 4.7 ^a	55.7 \pm 2.0 ^b	1.7 \pm 0.4 ^a	2.3 \pm 0.5 ^a
50	61.7 \pm 2.6 ^a	26.7 \pm 4.7 ^a	69.0 \pm 2.0 ^a	1.3 \pm 0.4 ^a	2.7 \pm 0.5 ^a
75	65.0 \pm 2.6 ^a	25.0 \pm 4.7 ^a	68.7 \pm 2.0 ^a	1.7 \pm 0.4 ^a	2.7 \pm 0.5 ^a

a, b: Different superscripts represents significant differences (One-way ANOVA, P < 0.05)

Values are mean \pm SEM from 3 replicates.

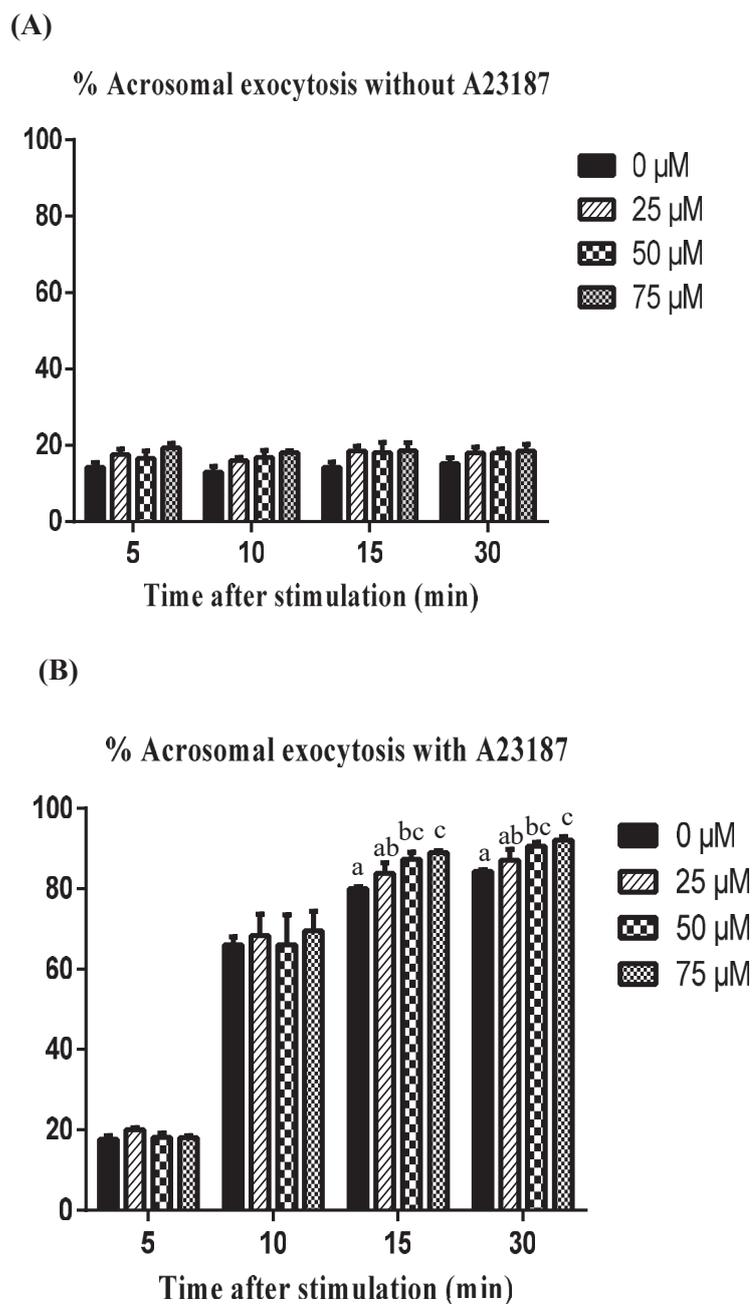


Figure 1. Effect of PMB on boar sperm acrosomal exocytosis induced by calcium and A23187. Acrosomal exocytosis after incubation in the absence (A) or presence (B) of 0.3 μ M A23187 for 5, 10, 15 and 30 min of incubation following preincubation for 10 min with different concentrations of PMB. Interaction between incubation time and concentrations of PMB was significant (Two-way ANOVA, $P < 0.0001$). a-c: Significant differences ($P < 0.05$). Values are mean \pm SEM from 3 replicates.

Discussion

Effect of PMB on motility with BTS:

For AI in pigs, preservation of liquid boar semen at 16–20°C is still a common practice (Bryła and Trzcí, 2015; Huo et al., 2002). To meet the different needs of the pig industry, several extenders have been developed to preserve semen in liquid form for short and long-term storage. In this study, short-term (BTS) were used to test whether storage of semen from 3 mature fertile boars at 17°C for 48 h would affect sperm characteristics after addition of PMB. However, sperm motility is an important indicator for the evaluation of sperm quality while sperm motility in a medium is an indicator of an active metabolism and the integrity of membranes (Johnson et al., 2000). In this study, when PMB added to extended semen during storage, the percentages of total motility in BTS did not differ significantly compared to control. Okazaki et al., (2010) has already shown that addition of PMB to boar semen extender maintains motility longer than without PMB.

In present study, PMB did not significantly decrease the sperm motility and viability immediately after the end of incubation without A23178. Although the addition of 50 to 75 µM PMB in BTS did not affect both sperm motility and viability in washed spermatozoa as compared with that of sperm stored without PMB, a lower concentration (25 µM) significantly affected both parameters after 30 min of incubation. Adding PMB as an antibiotic neutralizes bacterial LPS and diminish the noxious effects of LPS observed by Okazaky al., 2010. One recent study showed that after addition of PMB in bull sperm extender, both beneficial (with 100 µg/ml, 66.3% viability), and toxic effects (with 1000 µg/ml, 42.9% viability) were observed (Rashedi et al., 2016).

The results shown by Okazaki et al. (2010), in which sperm motility was maintained higher with PMB and penicillin G during storage than penicillin G alone. In previous study, (Chapter 3, section I) I observed that after incubation with PMB (washed spermatozoa were directly exposed to PMB), the percentages of sperm motility and viability were decreased by 75-100 μ M. But in this study, when sperm were stored with 0-75 μ M in BTS and then washed and incubated, the % motility and viability did not differ by 50-75 μ M levels of PMB and even increased progressive motility (Table 1). This discrepancy might be due to binding of PMB to LPS present in the extended semen at least to some extent and thus reduced availability of remaining PMB molecules in spermatozoa.

Effect of PMB on% Acrosomal exocytosis:

In Chapter 3; section I, a possible effect of PMB on boar spermatozoa that freed from seminal plasma and extender was investigated. In this section I was observed whether the addition of PMB in diluted boar semen during storage could affect acrosomal exocytosis induced by calcium and calcium ionophore A23187 after 30 min stimulation. When spermatozoa stored with PMB (0-75 μ M) and stimulated with Ca^{2+} /A23187, the acrosomal exocytosis significantly increased with 50-75 μ M PMB at 15 and 30 min of incubation. Similarly to total motility, it was considered that less PMB may have been available to spermatozoa due to binding of PMB to LPS present to some extent in extended semen. This may be reason why 75 μ M PMB added to extended semen enhanced exocytosis without decreasing motility and viability.

These results suggest that boar ejaculate can be stored with PMB by diluting ejaculates with BTS supplemented with PMB, which gives increase in progressive motility and

enhancement of acrosomal exocytosis triggered by Ca^{2+} /A23187 without affecting the sperm motility and viability.

Chapter 4

Summary

Artificial insemination has been increasingly employed for pig breeding. Summer infertility has been known globally for a long time in pig reproduction. It has been reported that boar spermatozoa collected during summer show hypersensitivity to calcium and the calcium ionophore A23187 (Ca^{2+} /A23187) to undergo the acrosome reaction, which was related to a tendency of low magnesium (Mg^{2+}) concentration present in seminal plasma and lowered farrowing rate after AI. It is also likely to take place that, at high ambient temperature during summer bacteria are more easily proliferate. In an attempt to recover lowered fertility during summer, this study sought a possibility to add additives, magnesium ions (Mg^{2+}) and antibacterial agent polymyxin B sulphate (PMB), to extended and stored semen to alleviate hypersensitivity of boar spermatozoa and to examine the effect of PMB on acrosomal exocytosis. Acrosomal exocytosis is an essential step to fertilization, information on the direct effect is of significance to employ Mg^{2+} and PMB for boar semen storage. This study was conducted to test the hypothesis that magnesium and PMB acts on sperm functions and to reveal the effect of Mg^{2+} and PMB added to extended semen during storage on standard sperm characteristics and acrosomal exocytosis.

Ca^{2+} influx is required for the mammalian sperm acrosomal exocytosis while Mg^{2+} acts as a physiological antagonist of calcium ions (Ca^{2+}). Section 1 of the Chapter 2 explored the role of Mg^{2+} in exocytosis of the boar sperm acrosome stimulated by Ca^{2+} /A23187. Ejaculated boar spermatozoa were stored in a chilled state, washed and incubated for stimulation with 3 mM Ca^{2+} and 0.3 μM of the calcium ionophore A23187 (Ca^{2+} /A23187) in the presence of different concentrations of MgSO_4 or MgCl_2 . When spermatozoa were treated with Ca^{2+} /A23187 and 0–3 mM MgSO_4 or MgCl_2 for 10 and 15 min, % acrosomal exocytosis was significantly lower with 0.1 mM of both MgSO_4 and MgCl_2 than the other concentrations at both intervals. When spermatozoa were stimulated with Ca^{2+} /A23187 in the presence of 0–0.1 mM MgSO_4 for up to 60 min, a clear dose-dependent inhibition was observed with the

maximal inhibition at 0.1 mM. When spermatozoa were similarly treated with higher concentrations of MgSO₄ (0.1–3 mM), % acrosomal exocytosis was the lowest again at 0.1 mM. These results suggest that micromolar levels of Mg²⁺ in the presence of millimolar levels of Ca²⁺ may probably be able to inhibit acrosomal exocytosis triggered by Ca²⁺/A23187 in boar spermatozoa.

Section 2 of the Chapter 2 explored the effect of Mg²⁺ added during storage of boar spermatozoa. The addition of MgSO₄ (0–8 mM) did not influence motility or progressive motility after storage at 17°C. When spermatozoa stored with added MgSO₄ were washed and stimulated for acrosomal exocytosis with Ca²⁺/A23187, it was shown that the % acrosomal exocytosis was significantly lower at 10 and 15 min of stimulation with 4 mM MgSO₄ than the other concentrations. When motility and viability were checked immediately after incubation, no difference in motility and viability among different concentration of MgSO₄ was observed. Thus it was confirmed that acrosomal exocytosis was not due to degenerative changes by cell death. The findings might become useful information to develop a novel method to reduce the excess induction of acrosomal exocytosis taking place in boar spermatozoa collected in summer, characteristic to summer infertility.

PMB has been reported to be effective in boar semen storage by binding to the lipopolysaccharide complex to neutralize its endotoxic activity. However, PMB has also been shown to directly modify signal transduction pathways in somatic cells. Section 1 of the Chapter 3 examined the direct effect of PMB on boar spermatozoa. In spermatozoa stored, washed and incubated with various concentrations of PMB (0–100 µM), total motility and viability were not significantly different between 0 µM and 50 µM PMB but gradually declined at 75 and 100 µM. However, progressive motility was significantly reduced at the concentration of 25 µM of PMB. Vigor grade was affected at 75 µM PMB, but agglutination scores were not affected. In separate experiments, spermatozoa

similarly stored and washed were preincubated in the presence of 0–50 μM PMB, concentrations that did not affect total motility and viability, followed by incubation for stimulation of acrosomal exocytosis with $\text{Ca}^{2+}/\text{A23187}$. Preincubation with 0.01–0.5 μM and 1–50 μM resulted in significant enhancement of acrosomal exocytosis induced by A23187 at 10 and 15 min, respectively. Preincubation with PMB did not affect acrosomal exocytosis without A23187. These results show that PMB can affect boar sperm motility and viability directly while acrosomal exocytosis triggered by $\text{Ca}^{2+}/\text{A23187}$ can be enhanced by PMB.

Section 2 of the Chapter 3 examined the changes in sperm motility and acrosomal exocytosis induced by $\text{Ca}^{2+}/\text{A23187}$ in spermatozoa extended and stored with different concentrations (0–75 μM) of PMB. Total motility was not significantly different among different concentrations of PMB added but progressive motility was significantly increased with the increasing concentrations of PMB. Stored spermatozoa were then similarly washed, incubated to stimulate acrosomal exocytosis with $\text{Ca}^{2+}/\text{A23187}$ for 5, 10, 15 and 30 min and, at the end of incubation, analyzed for total motility, progressive motility, vigor grade, viability and agglutination. Acrosomal exocytosis triggered by $\text{Ca}^{2+}/\text{A23187}$ was significantly enhanced by 50 and 75 μM of PMB both at 15 min and 30 min of stimulation. Total motility and viability significantly declined at 25 μM but did not significantly differ with 50 μM and 75 μM PMB compared to 0 μM . There were no significant differences in progressive motility, sperm vigor grade and agglutination score among any concentrations of PMB examined. These results suggest that boar ejaculate can be stored with PMB, which gives increase in progressive motility and enhancement of acrosomal exocytosis triggered by $\text{Ca}^{2+}/\text{A23187}$ without affecting total motility and viability.

In conclusion, magnesium can be added to extended boar semen during storage without influencing motility, and the addition slows the occurrence of acrosomal exocytosis, which might possibly alleviate excess induction of acrosomal exocytosis in spermatozoa collected in summer, potentially leading to increase in fertility. The addition of PMB may possibly improve boar sperm fertility by increasing progressive motility, which is essential for sperm migration up to the fertilization site, and the ability to undergo acrosomal exocytosis, which is essential for spermatozoa to penetrate the oocyte. However, it should be taken into consideration when PMB is used for spermatozoa collected during summer because the added PMB might further enhance the excess occurrence of acrosomal exocytosis.

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