CRYOPRESERVATION OF BOAR SPERMATOZOA: THE CURIOUS FUNCTIONS OF SEMINALPLASMA DURING FREEZING OR THAWING

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ABSTRACT

There are various advantages, such as the preservation of genetic resources, an improvement of the reproduction performance in summer, long-distance transportation of semen and the possibility of progeny test, in the cryopreservation technique of boar semen. However, cryopreserved boar spermatozoa have not been routinely available to swine artificial insemination (AI) due to the lower conception and farrowing rates than those of fresh semen. To improve the reproductive performance after AI, many researchers have approached technical modification of temperature program during freezing, freezing extender, thawing solution and AI method (e.g. deep intra uterine insemination). In this study, we focused and analyzed on the role of the seminal plasma of ejaculated semen, and succeed to develop novel freezing and thawing method as following. In the freezing process from semen collection: 1. Ejaculated seminal plasma is immediately removed by centrifugation after semen collection because the fraction contains negative factors, such as bacteria or lipopolysaccharide (LPS). 2. The sperm pellet is resuspended in a semen extender that contains both common antibiotics and polymyxin B (an inactivator of LPS). 3. Hyperosmotic pressure (400 mOsm/ kg) and low concentration glycerol (2%) solution are used for freezing extender (conventional extender; 300 mOsm/ kg, 3% glycerol).

Next step, in thawing process, the frozen straws picked out from the liquid nitrogen tank are melted in 38oC for 1 min, and the content injects into 10% (v/v) seminal plasma-containing thawing solution. When the frozen-thawed spermatozoa treated with seminal plasma were artificially inseminated to sows, the reproductive performance (conception rate; 80.2% and litter size; 10.1 piglets) was comparable to that of liquid semen (73.9%, 10.3 piglets). These results indicated that seminal plasma has positive roles on the AI using cryopreserved boar semen. However, because boar seminal plasma is contaminated with various kinds of bacteria and/or viruses, the development of thawing solution without animal derived materials is required for more sanitary AI.

Cryo-capacitation just after thawing which arises from increasing of intercellular Ca2+ ([Ca2+]i) in sperm, decreases the motility and fertilization ability of frozen-thawed sperm. To suppress this induction, EGTA was added to diluent to chelate the transient increment of the ion. The addition of EGTA significantly suppressed both the increase [Ca2+]i level and the induction of cryo-capacitation.

To yield a high pregnancy rate, not only improvement of sperm function mentioned above but remodeling of the uterine environment after AI is also a key step. We found that cortisol, which is a known immunosuppressive agent, existed in boar seminal plasma and regulated intrauterine environment to enhance implantation in sows. In this symposium, we will also introduce the reproductive performances obtained by AI using the novel diluent.

Keywords**:** Sperm, Seminal Plasma, Cryopreservation, Pig

INTRODUCTION

Insemination in pig farming in Japan is generally accomplished through natural mating. However, this approach requires the breeding of a large number of boars. Furthermore, factors such as danger during mating and decrease in the motility of boar sperm during the transition from summer to fall frequently cause reproductive failure, thus, rendering natural mating as unfavorable. These reproductive challenges have prompted researchers to conduct studies on artificial insemination (AI) using fresh semen. In recent years, fresh semen collected from breeding farms that has passed semen quality testing has been distributed to production farms, and an AI method utilizing this resource has been established. AI conducted using fresh semen ensures the use of only those sperm that have sufficient motility. Therefore, reproductive performance (i.e., pregnancy rates and total productive litter size) following AI has improved compared to natural mating. However, because the shelf life of semen is approximately 7 days, the transport time from semen distribution agencies to production farms is unable to accommodate the sows' sudden estrus. Furthermore, changes in the temperature during transport negatively affect the condition of the sperm, often resulting in 40% diffusion rate. This is significantly different from that reported in other developed countries, where the diffusion rate of these AI methods is more than 80%. The difference can be explained by the unsuitability of Japan's climate to the transportation of fresh semen, along with the limitations in semen supply from various pork brands. The resulting low utilization rate of fresh semen, thus, is the main limiting factor for the improvement of efficient pork production and brood-stock breeding.

AI using frozen–thawed semen or sperm circumvents the above-mentioned flaws involving liquid semen, and sperm can be easily stored in tanks containing liquid nitrogen. Therefore, the AI method can improve the efficiency in pork production by not missing the fertilization windows. Moreover, genetic resources such as superior males and valuable breeds can be preserved semi-permanently and distributed later. Recently, application of the progeny tests routinely performed on beef cattle by using frozen–thawed semen or sperm to the pig breeding is considered. However, for pigs, the low conception rates and low litter sizes from AI (due to low sperm motility rate following freeze–thaw) render its implementation largely impractical (Johnson *et al.* 2000). The objective of the present study was to optimize the freezing and thawing methods of semen or sperm to develop an AI method that has high reproductive performance.

Cooling and Freezing Process; development of the novel freezing extender

Body text: Damage to the sperm plasma membrane and acrosomal membrane occurs when sperm is frozen, and the sperm functionality (fertility) is impaired following thawing (Curry 2000; Aboagla and Terada 2003). This damage is believed to result from intracellular and extracellular ice crystal formation at the time of freezing. Therefore, the addition of glycerol, a cryoprotectant that replaces or adheres to intracellular free water, to freezing extenders is typical. However, glycerol itself has cytotoxic properties to which pigs are more highly sensitive than that for other animal species (Gilmore *et al.* 1998), which prohibits freezing with the glycerol concentration used for cattle (7-10%). Therefore, the development of freezing extenders that compensate the function of glycerol is required.

By raising the osmotic pressure of the freezing extender to achieve extracellular dehydration of intracellular free water, it is possible to prevent ice crystal formation without resorting to the use of glycerol. By using sperm motility, the normal rates of plasma and acrosomal membrane following thawing as indicators, a hypertonic diluted solution with an osmotic pressure of 400 mOsm/kg (normally 300 mOsm/kg) yielded positive results (Okazaki *et al.* 2009a). In addition, by using an osmotic pressure of 400 mOsm/kg, we demonstrated that glycerol concentration could be reduced from 3% (the concentration used in conventional freezing treatments for boar semen) to 2%. Compared to existing freezing extender (300 mOsm/kg, 3% glycerol), our novel freezing extender (400 mOsm/kg, 2% glycerol) showed higher motility rate of sperm after thawing (Fig. 1).

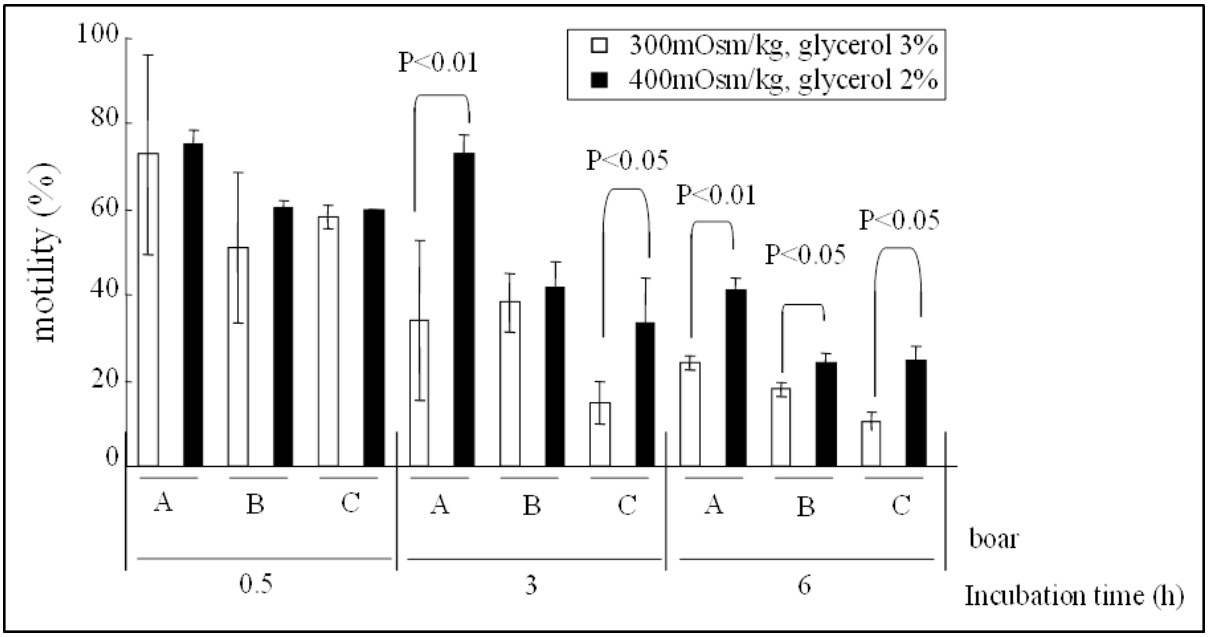

Fig. 1. Comparison of the novel freezing extender (400 mOsm/kg, 2 % glycerol) with the conventional NSF extender (300 mOsm/kg, 3 % glycerol) on post-thawed sperm motility (Okazaki *et al.* 2009a). Data are means and +/- SEM.

Modification of the semen treatment during cooling

The use of the novel freezing extender described above also resulted in low sperm motility following thawing; furthermore, some specimens (boars) could not be cryopreserved. The difference in the freezability of these specimens is a major barrier to the dissemination of this technology. Previous reports have shown that genetic factors influence the freezability of specimens (Thurston *et al.* 2001, 2002). On the other hand, when freezing individual epididymal sperm specimens with low freezability, significantly elevated sperm motility following thawing has been demonstrated in comparison to frozen ejaculated sperm (Okazaki *et al.* 2012a). Therefore, variations in freezability might be due not only to genetic factors but also to the composition of seminal plasma following ejaculation.

Pig semen is typically harvested using the gloved-hand technique, which decreases the sterility of the collection method. Both gram-negative and gram-positive bacteria are inherently present in the collected semen, and a significant negative correlation is often observed between bacterial count and sperm motility. Because the bacteria in seminal plasma are largely gram negative, and the endotoxin lipopolysaccharide (LPS) is also detected in the seminal plasma, it is thought that the endotoxin affects freezability (Okazaki et al., 2009b). Therefore, by using sperm samples, we performed expression and functional analyses of toll-like receptor (TLR) 2, which recognizes endotoxins (lipoproteins and peptidoglycans) of gram-positive bacteria, as well as TLR4, which acts as an LPS receptor. In present study, both receptors were expressed in sperm (Okazaki *et al.* 2009b; Fujita *et al.* 2011). Further, the addition of LPS to the sperm culture medium reduced the sperm motility rate and survival rate in a concentration-dependent manner, as well as induced caspase-3-dependent programmed cell death. This study, thus, showed that for the first time, sperm retains the initial immune response function of recognizing bacteria.

Next, we examined the effect of polymyxin B (PMB), an antibiotic that acts as an inactivating agent for LPS on sperm samples. Hosseinzadeh *et al.* (2003) previously examined the effects of PMB on *Chlamydia trachomatis* in human sperm; however, no other studies have shown the positive effects of PMB on pig sperm. When boar sperm was collected, frozen, and thawed after exposure to a pretreatment solution containing 100 μg/mL PMB, the sperm motility rate and normal cell membrane and normal acrosomal membrane rates were significantly higher than those when using pretreatment solutions that solely contained penicillin G (Okazaki *et al.* 2010; Fig. 2).

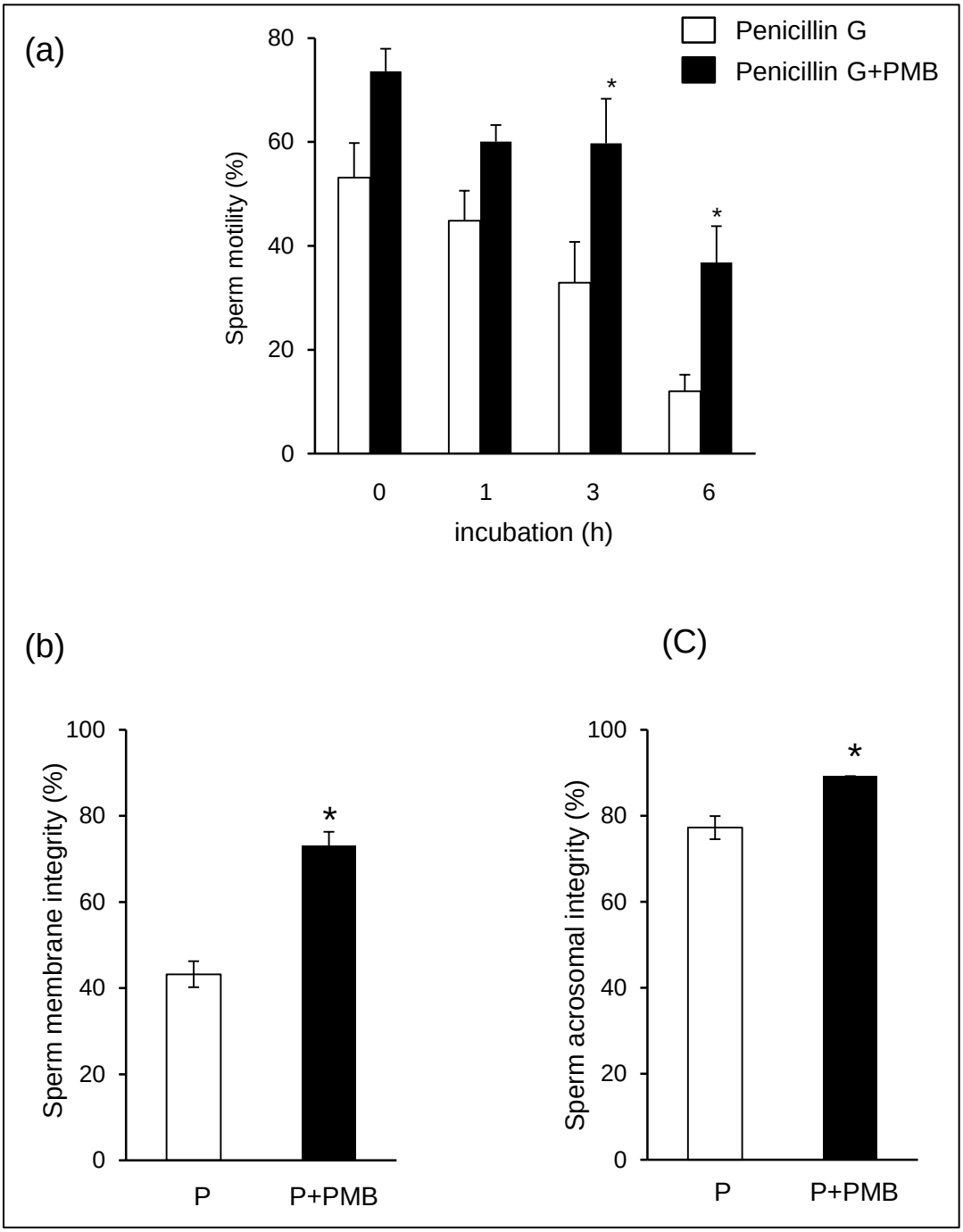

Fig. 2. PMB treatment just after ejaculation of boar sperm improves post-thawed sperm quality

The sperm frozen with penicillin G alone (P) or penicillin G+PMB (P+PMB) was thawed, and then incubated to measure the motility (a) , membrane integrity (b) and acrosomal integrity (c) (Okazaki *et al.* 2010). The membrane integrity and acrosomal integrity were detected by PI-SYBR14 and FITC-PNA staining, respectively. Values are mean+/-SEM. *; The addition of PMB to Penicillin G containing medium increased the motility of sperm as compared with that by Penicillin G alone ($P<0.05$).

Gram-positive bacteria have also been detected in boar semen (Kharenko. 1975). However, currently no antibiotics are present that can inactivate the endotoxins produced by such gram-positive bacteria. In addition, seminal plasma contains elements such as pH fluctuation factors and cholesterol that negatively affect freezing of the sperm (Zeng and Terada 2001; Althouse *et al.* 2000). Therefore, freezing methods in which seminal plasma containing these negative factors is removed immediately following semen collection are inferred to be optimal for the freezing of specimens with low freezability. The removal of seminal plasma immediately after semen collection improved *in vitro* fertility and sperm motility following thawing for specimens with poor freezability (PF). It also did not affect *in vitro* fertility or the rate of sperm motility following thawing for specimens with good freezability (GF). The conditions, thus, facilitate improvement in freezing the majority of the specimens (Okazaki *et al.* 2009c; Okazaki and Shimada 2012; Table 1).

Table 1. Comparative analysis of post-thawed sperm motility between conventional and novel method

	Boar No.																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	average	success rate (>60%)
	Post-thawed sperm motility (%)																										
conventional method	40	41	42	46	46	47	46	50	86	71	26	24	9	39	41	45	25	47	35	33	35	40	1	40	20	39	8
novel method	75	75	71	79	78	81	85	62	60	50	55	82	78	77	70	74	65	65	80	94	89	41	56	60	84	71	88

To examine the effects of removing seminal plasma and PMB combination treatment on post-thaw sperm motility, collected semen was divided into two groups and then frozen by each treatment (Okazaki and Shimada 2012). Conventional method; pre-treatment solution (Modena) with Penicillin G was equally added to collected semen and cooled to 15 ^{0}C over 2h. Novel method; collected semen was immediately centrifuged to remove seminal plasma and then resuspended in pre-treatment solution with Penicillin G + PMB. Freezing program after cooling was similar to both methods. Sperm motility was analyzed by CASA at 1 h incubation after thawing. The proportion of boars (n=25) indicated sperm motility of >60% was 8% (conventional) and 88% (novel) respectively.

Thawing process; the positive effects of seminal plasma on the function of thawed sperm

The conception rate for AI using PF freeze–thawed sperm with seminal plasma removed was exceedingly low (9%) despite the favorable rate of sperm motility following thawing (Okazaki *et al.* 2009c). One of the cited causes of the low conception rate for AI using freeze–thawed semen is cryo-capacitation following thawing (Watson. 2000). This phenomenon has been attributed to the stresses of freezing and thawing on sperm. Additionally, the seminal plasma of mammals inhibits capacitation (Davis and Niwa 1974; Kanwar *et al.* 1979; Vadnais and Roberts 2007). On the basis of the previous studies, it was concluded that the cause of the low conception rates in AI that we performed was two-fold: spontaneous capacitation of freeze–thawed sperm with seminal plasma removed immediately following collection, and the acrosomal damage caused by subsequent acrosome reaction. Therefore, we experimented on adding seminal plasma to the thawing solution, anticipating a positive effect, in which seminal plasma would inhibit a sperm capacitation-like effect following thawing. The results of the experiment showed that adding 10% (v/v) seminal plasma to the thawing solution was sufficient in suppressing concentration-dependent capacitation and acrosomal damage (Okazaki *et al.* 2009c).

On the basis of the described results, the following treatment methods for freeze–thaw of pig semen were devised (Okazaki and Shimada 2012; Fig. 3):

1. Centrifugation immediately following semen collection and removal of the factors in the seminal plasma that adversely affect freezing.

2. Dilution of the pretreatment solution containing PMB and antibiotics that is normally used in sperm pellets (penicillin G, streptomycin, and amikamycin) and cooling at 15℃.

3. Use of freezing extender with hyperosmolarity and low concentration of glycerol (400 mOsm/kg + 2% glycerol).

4. AI by dissolving the cryopreserved semen in a warm water bath and the addition of a 10% (v/v) seminal plasma thawing solution.

Implementation of AI by using these techniques yielded an 80.2% conception rate and single litter size of 10.1 piglets, which are equivalent to artificial insemination by using fresh semen (73.9% conception rate and single litter size of 10.3 piglets, respectively) (Okazaki *et al.* 2011).

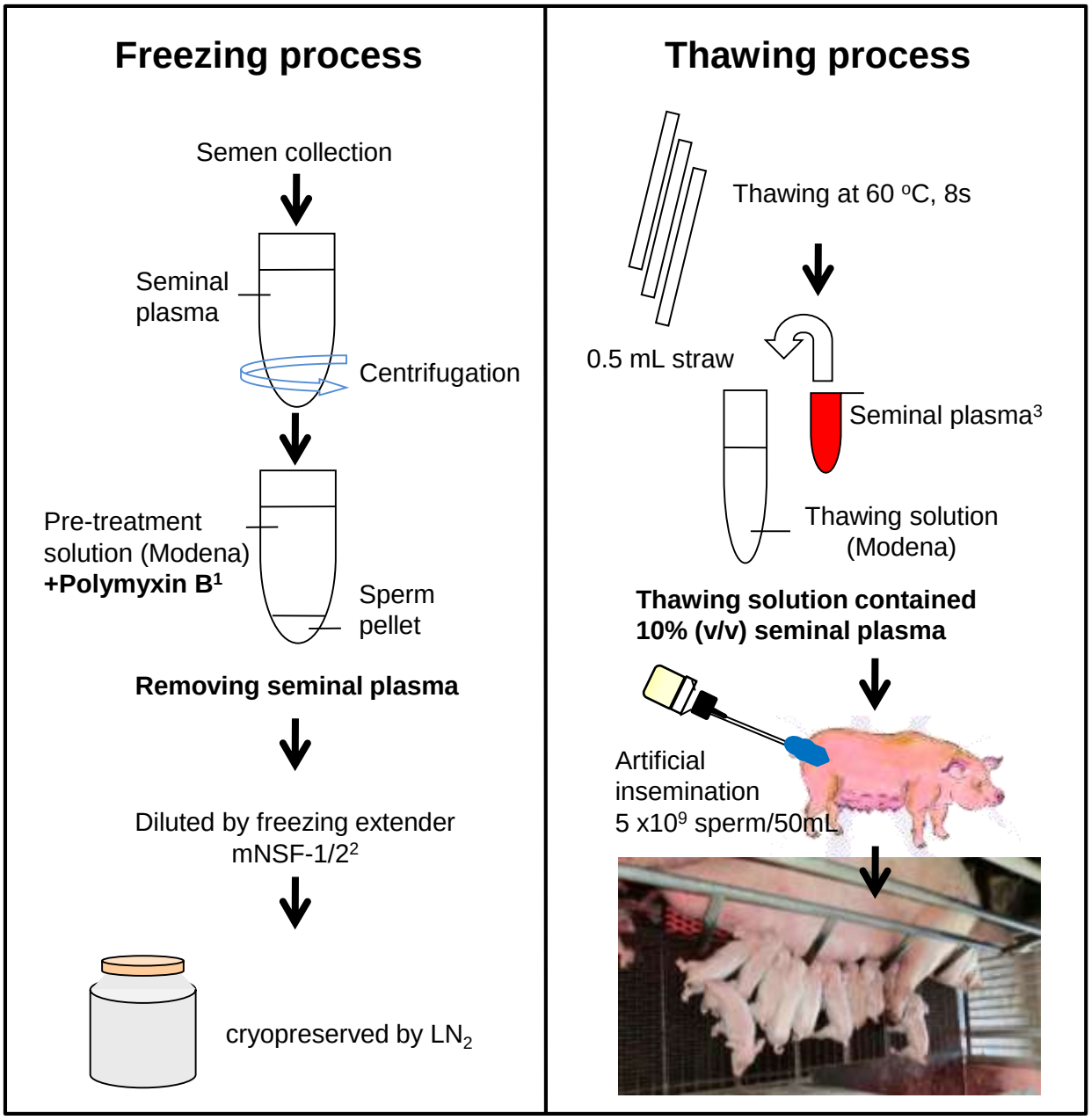

Fig. 3. Overview of novel freezing and thawing method for boar semen (Okazaki and Shimada 2012). [1] 100 □g/mL of PMB is added to the pre-treatment solution. [2] mNSF-1 (modified Niwa and Sasaki freezing extender-1) is NSF-1 with osmotic pressure modified from 300 mOsm/kg to 400 mOsm/kg. mNSF-2 contains 94.5% mNSF-1, 1.5% Orvus Es Paste (OEP) and 4% glycerol (conventional NSF-2 contain 6% glycerol). [3] Seminal plasma, added to the thawing solution, is collected from a boar with high reproductive performance by liquid AI or natural mating.

The necessity for development of chemically defined thawing solution

AI by using the developed thawing solutions containing seminal plasma demonstrated reproductive performance equivalent to that of fresh semen. However, the use of seminal plasma in pig breeding increases economic losses due to the release of viruses such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), and Aujeszky's disease (ADV) (Guérin and Pozzi 2005; Kim *et al.* 2003; Maes *et al.* 2008). Therefore, if the AI is carried out with seminal plasma contaminated with pathogens or viruses, there is concern regarding the spread of disease among non-infected farms and specific-pathogen free (SPF) farms where such viruses do not exist. Further, because the composition of seminal plasma largely fluctuates with the season and specimen (Murase *et al.* 2007), the properties of the thawing solution containing seminal plasma are difficult to stabilize.

Thus, to ensure its stable and safe use at every farm, it is necessary to investigate the positive effects of seminal plasma and to develop a synthetic thawing solution without seminal plasma that consists of chemicals that are functionally equivalent to that of the thawing solution containing seminal plasma.

The addition of EGTA to thawing solution suppresses cryo-capacitation in thawed sperm

Capacitation in ejaculated sperm induces a sudden increase of Ca^{2+} in sperm cells (Arnoult *et al.* 1999; Baldi *et al.* 2000). Increasing intercellular Ca^{2+} ($[Ca^{2+}]_i$) is considered to be the product of the influx of extracellular Ca^{2+} as an initial trigger for ion emission from intracellular Ca^{2+} stores (Treviño *et al.* 2004; Wennemuth *et al.* 2000). On the other hand, in the case of thawed sperm, physical damage to the sperm plasma membrane from freeze–thaw results in a direct intracellular influx that bypasses Ca^{2+} channels. Initially, Fluo-3/AM was used as an indicator of intercellular $Ca2^{+}$ ($[Ca^{2+}]_i$) to determine whether intercellular Ca^{2+} ($[Ca^{2+}]_i$) was increasing in thawed pig sperm as well. Immediately after thawing, strong Ca^{2+} signals were detected in the sperm head and midpiece, and the signal intensity increased in a culture time-dependent manner (Okazaki *et al.* 2012b). In these sperm, phosphorylation of protein tyrosine residue was detected, cryo-capacitation was induced, and the addition of Ca^{2+} to culture medium caused an increase in these signals. However, when 10% (v/v) seminal plasma was added to the culture medium, Ca^{2+}-dependent cryo-capacitation was inhibited. These findings suggest that cryo-capacitation occurring at the time of thawing is regulated by extracellular Ca^{2+}, and the influx of Ca^{2+} is inhibited by the action of the seminal plasma.

To imitate these functions of seminal plasma, we examined the effect of EGTA (*O*,*O*′-bis-(2-aminoethyl)-ethyleneglycol-*N,N,N′,N*′-tetraacetic acid), which is one of the divalent ion chelators, on increasing of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and cryo-capacitation in sperm. The addition of 6 mM EGTA to the thawing solution inhibited cryo-capacitation in the same manner as the addition of seminal plasma, and no increase in Ca^{2+} was observed despite continuous observation following thawing. This effect was maintained in the culture for an extended period. These results demonstrated that the inhibitory action of seminal plasma on cryo-capacitation could be simulated by the addition of EGTA to the thawing solution. The thawing solution containing 6 mM EGTA improved the rate of sperm motility following thawing compared to additive-free solutions.

Cortisol in seminal plasma controls inflammation in the uterus after AI

To confirm whether a sufficient number of fetal implantations has occurred by means of AI utilizing a thawing solution containing EGTA as previously described, the rate of implantation (number of fetuses/ number of corpora lutea) was calculated in uteri recovered from sows 30 days after one course of AI (50×10^8 sperm/50 mL) following superovulation treatment with PMSG-hCG. A low implantation rate (51%) was observed when thawing solutions containing EGTA were used, whereas a thawing solution containing 10% (v/v) seminal plasma yielded an implantation rate of 78% (Fig. 4; Okazaki *et al.* 2012b). The use of thawing solutions containing EGTA but no seminal plasma resulted in the implanted fetus undergoing phagocytosis by the immune cells; thus, high mortality was observed. These results suggest that seminal plasma contains factors that control the intrauterine immune system and promote (or possibly normalize) fetal implantation.

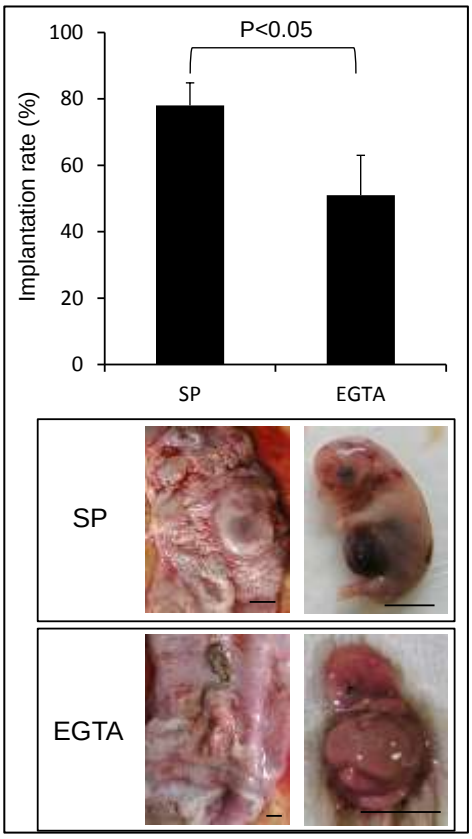

Fig. 4. Implantation rate of the fetuses 30 days after AI with seminal plasma (SP) or without SP but with EGTA. When the sows were artificially inseminated without seminal plasma, the implantation rate of fetuses was low and the number of dead fetuses increased (Okazaki *et al.* 2012b). Scale bar= 10 mm.

Leukocytes that have migrated to the uterus following AI consume residual dead sperm cells in the uterine cavity and play a role in the preparation of the uterine environment (Matthijs *et al.* 2000). On the other hand, Rozenboom *et al.* (1998, 2001) reported that in the presence of seminal plasma, these leukocytes disappeared within 24 h following AI. Furthermore, mouse and human seminal plasma contain a variety of cytokines, chemokines, and anti-inflammatory steroid hormones (Gopichandran *et al.* 2006; Politch *et al.* 2007; Tarter *et al.* 1986). Consequently, it is considered that immunosuppressive factors contained within seminal plasma suppressed cellular immunity, which occurs when sperm cells become antigens following AI. Immunity is also reduced by the time the embryo arrives in the uterus, and leukocyte attack of the embryo is inhibited. However, the immunosuppressive factors within boar semen and the intrauterine environment following AI remain completely unclear, as does their involvement in reproductive performance.

Therefore, we have attempted to identify seminal plasma immunosuppressive factors, focusing on steroid hormones. Enzyme immunoassay showed that these factors have a cortisol concentration of 0.92 ng/mL. We determine the number of leukocytes in the uterine cavity following AI as an index of intrauterine immunosuppression induced by cortisol. Within 6 h following AI, polymorphonuclear leukocytes (PML) had migrated into the uterine cavity; however, different treatment methods (fresh semen, freeze–thawed semen without cortisol, freeze–thawed semen with added cortisol) yielded no significant differences in PML count (Okazaki et al. unpublished data). At 24 and 48 h following AI, although significantly high values were demonstrated for injection of freeze–thawed semen without cortisol, the values for cortisol injected into the uterus decreased to the same levels as those for fresh semen containing seminal plasma. These results suggest that the cortisol in seminal plasma possesses an immunosuppressive action that reduces the migration of neutrophils for intrauterine foreign bodies such as sperm within 24 h of AI.

AI test using chemically-defined thawing solution (EGTA + cortisol)

During their natural estrus, 45 sows were subjected to AI using freeze–thawed semen and a synthetic thawing solution containing EGTA and cortisol, and their reproductive performance was calculated. This procedure yielded a very high conception rate of 84.4% and favorable single litter sizes of 8.7 piglets (4–14) (Okazaki et al. unpublished data). There was a concern for side effects on the fetuses due to the injection of the steroid hormone cortisol, and thus, only extremely small quantities of cortisol were injected into the uterus. No malformations were observed in the resulting offspring, and development was normal (Fig. 5). Therefore, the use of the described thawing solution in AI is considered to impart no effects on safety.

Fig. 5. The piglets produced by AI technique using EGTA + cortisol thawing solution. The malformation of piglet was not observed.

CONCLUSION

We have developing a novel method of AI for pigs using freeze–thawed semen. This method involves the following steps:

1. Immediate removal of seminal plasma following semen collection;

2. Dilution of the sperm using a pre-treatment solution containing 100 μg/mL of PMB;

3. Hypertonic osmotic pressure of 400 mOsm/kg and freezing of sperm by using a freezing extender with a final glycerol concentration of 2%; and

4. Conducting AI with sperm thawed using either thawing solution of 10% (v/v) seminal plasma or thawing solution containing 6 mM EGTA + 5 μg cortisol.

On the basis of the 60-year history of research on the production of freeze–thawed semen or sperm for pig farming, we primarily focused on designing technical improvements. The results of the present study provide us with new techniques that are based on established knowledge and information on the physiological functions of sperm and seminal plasma. Dissemination of this technique involving freeze–thawed semen or sperm to pig farms will facilitate the development of breeding pig stocks and preservation of genetic resources in increasing the productivity, and improving hygiene management (such as safeguarding against disease) in the industry. These benefits in turn will allow sustainable pork production and ultimately satisfy the needs of consumers.

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