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—Full Paper—

Improved Fertility in Gilts and Sows after Artificial Insemination of Frozen-Thawed Boar Semen by Supplementation of Semen Extender with Caffeine and $CaCl_2$

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Abstract. Supplementation of semen extender with caffeine and $CaCl_2$ for artificial insemination (AI) of fresh spermatozoa has been demonstrated to reduce recruitment of uterine polymorphonuclear leukocytes (PMNs) and the activity of phagocytosis. Here, we determined if addition of caffeine and $CaCl_2$ to semen extender improves the fertility of frozen-thawed boar semen. In experiment 1, gilts were cervically inseminated twice with froxzen-thawed boar spermatozoa (25×10^8 cells per dose) suspended in Modena solution (n=7) or modified Beltsville Thawing Solution supplemented with caffeine and $CaCl_2$ (BCC, n=7). The gilts were slaughtered 4 h later, and their oviducts and uterine horns plus the body of the uterus were flushed to recover PMNs and non-phagocytosed spermatozoa. There was no difference in the total number of uterine PMNs between gilts inseminated with Modena solution and those inseminated with BCC (3.8×10^8 *vs.* 1.5×10^8 cells, respectively); however, the total number of uterine spermatozoa was higher when gilts were inseminated with BCC (40.6×10^6 cells) compared with those inseminated with Modena solution (1.4×10^6 cells, $P<0.05$). In experiment 2, gilts and sows were subjected to intrauterine insemination twice with frozen-thawed spermatozoa suspended (25×10^8 sperm per dose) in Modena (n=21) or BCC (n=21). The overall pregnancy and farrowing rates were higher in females inseminated with BCC (71.4 and 61.9%, respectively) compared with those inseminated with Modena solution (38.1 and 28.6%, respectively, $P<0.05$). However, no significant difference in litter size of piglets was observed between treatments (7.2 ± 1.6 piglets for Modena solution *vs.* 8.2 ± 0.9 piglets for BCC solution). In conclusion, we demonstrated that use of BCC solution for frozen-thawed boar semen produced better pregnancy and farrowing rates following AI than Modena solution, probably by reducing the phagocytosis of spermatozoa.

Key words: Artificial insemination, Caffeine and $CaCl_2$, Frozen-thawed boar semen, Polymorphonuclear leukocytes

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During artificial insemination (AI) in sows, a large quantity of semen is deposited into the uterus. However, at the same time, a considerable amount of spermatozoa is rapidly lost from the uterus; only less than 2% of the inseminated spermatozoa are reported to be recovered from the uterus at 4 h after AI [1]. The backflow of semen and phagocytosis of spermatozoa by polymorphonuclear leukocytes (PMNs) appear to be the two main mechanisms responsible for the reduction in the amount of spermatozoa [2]. In gilts, although the influx of PMNs into the uterus can persist for as long as 36 h after insemination, the number of uterine spermatozoa and the clearance time from the uterus are highly dependent on the presence or absence of seminal plasma [3]. It has been shown that seminal plasma inhibits neutrophil chemotaxis *in vitro* [4] and protects spermatozoa from an inflamed uterine environment, resulting in improved fertility [5, 6]. In the case of AI with frozen-thawed boar semen, however, swine seminal plasma is generally removed during the freezing procedure, and the inseminated spermatozoa may be exposed to an inflamed uterine environment just after AI.

Matthijs *et al*. [7] reported that AI with semen diluted with the semen extender Beltsville thawing solution (BTS) supplemented with caffeine and calcium chloride ($CaCl_2$) results in a significantly lower recruitment of leukocytes and a higher number of non-phagocytosed spermatozoa in the uteri of sows at 4 h after AI. Therefore, supplementation of the thawing solution for frozen semen with caffeine and $CaCl_2$ may lead to immune regulation in the uterus and consequently may improve fertility following AI. On the other hand, Funahashi *et al*. [8, 9] have reported that caffeine stimulates both capacitation and spontaneous acrosome reaction in freshly ejaculated and frozen-thawed boar spermatozoa and that spontaneous acrosome-reacted spermatozoa result in reduced survival.

The objective of this experiment was to determine the additive effects of supplementation of semen extender for frozen-thawed boar semen with caffeine and $CaCl_2$ on the immunological environment in the uterus (experiment 1), fertility (experiment 2) and capacitation and spontaneous acrosome reaction in frozen-thawed boar spermatozoa *in vitro* (experiment 3).

Materials and Methods

Animals and detection of estrus

In experiment 1, prepubertal Large White gilts (age, 6–7 months) were treated with PG600® (400 IU of equine chorionic

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gonadotropin + 200 IU of human chorionic gonadotropin; Intervet, Millsboro, DE) to induce estrus. In experiment 2, estrus detection in 38 (age, 8 months or older) Large White gilts and 4 Large White sows (parity, 1–3) was performed twice a day (1030 and 1630 h) by allowing the females to have nose-to-nose contact with a mature boar and by applying back pressure. The occurrence of estrus was defined as exhibition of a standing reflex in the presence of the boar. Estrus detection was continued until the standing reflex subsided.

Preparation of frozen-thawed boar spermatozoa

The percentage of motile spermatozoa was estimated at 38 C using phase-contrast microscopy at × 200 magnification. Morphologically normal spermatozoa were assessed in wet mounts after dilution with 3% NaCl using phase-contrast microscopy at × 200 magnification. Semen samples with a minimum of 80% motile and morphologically normal spermatozoa from 3 Large White boars with proven fertility of fresh semen were used. The semen samples were collected by the gloved-hand technique and processed according to the modified method of Nogami *et al*. [10].

The collected semen was diluted 1:1 with Modena solution and kept overnight at 15 C. After centrifugation at 800 *g* for 10 min, the precipitating spermatozoa were resuspended at a density of 2×10^9 cells/ml in the first dilutor (0.24 M trehalose solution containing 20% egg yolk and 100 mg/l amikacin) and then cooled to 5 C for 2 to 3 h. At 5 C, these semen samples were further diluted 1:1 with the second dilutor (prepared by adding 1.48% OEP and 6% glycerin to the first dilutor). The resuspended and cooled spermatozoa were packed into 0.5-ml straws, frozen in liquid nitrogen gas at 4 cm above the surface of liquid nitrogen and then stored at –196 C until use.

Thawing and post-thaw sperm evaluation

The frozen semen was thawed in a water bath at 38 C for 30 sec prior to use in the experiments. After thawing, the sperm suspensions were extended at 38 C with 10 ml Modena. Sperm evaluation was performed microscopically at 30 min after thawing according to the motility. Only thawed sperm showing more than 35% motility were used for insemination.

Artificial insemination

Modena and modified BTS containing caffeine and $CaCl_2$ (BCC) were used as thawing solutions for the frozen boar semen (Table 1). All gilts and sows were inseminated twice with 25×10^8 sperm in 50 ml of thawing solution at 24 h and from 30 to 38 h after the onset of estrus. In experiment 1, all inseminations were performed by using a standard cervical catheter (Import-Vet S.A., Barcelona, Spain). Frozen semen from 2 Large White boars (boars A and B, Table2) was used to inseminate the gilts in estrus that were equally assigned to different treatments. In experiment 2, a post-cervical catheter (Import-Vet S.A., Barcelona, Spain) with narrow soft inner tubes extending 150 mm beyond the tip of the outer standard catheter was used to penetrate the cervix and lie in the body of the uterus or in the posterior horn. Frozen semen from the 3 Large White boars was used to inseminate the gilts and sows.

Table 1. Composition of thawing solutions

Composition	Modena (mM)	BCC* (mM)
Glucose	152.61	205.37
Sodium citrate	23.46	20.40
Sodium bicarbonate	11.90	14.88
EDTA, disodium salt	6.99	1.68
Tris	46.66	–
Citric acid	15.10	–
Potassium chloride	–	10.06
Caffeine	–	1.15
Calcium chloride dihydrate	–	3.97
Amikacin	100 mg/l	100 mg/l

*The BCC solution was modified BTS solution supplemented with caffeine and $CaCl_2$.

Collection of PMNs and spermatozoa

At 4 h after the second insemination, the gilts were slaughtered to collect the reproductive tracts. The distal ends of the oviducts were clamped to prevent contamination of the oviducts with spermatozoa from the tip of the uterine horn. The reproductive tracts were transported on ice to the laboratory.

The oviducts, right uterine horn and left uterine horn along with the body of the uterus were separated. The vagina and cervix were removed. Each uterine horn was separated into upper one-third (one side of an oviduct) and lower two-thirds portions. Each oviduct was flushed twice with 2 ml cooled PBS. The upper and lower parts of each uterine horn plus the body of the uterus were flushed twice with 5 ml and 10 ml cooled PBS, respectively. The samples were centrifuged at 800 *g* for 10 min, and the supernatant was discarded. The pellets were resuspended in 1 ml cooled PBS, and the numbers of PMNs and spermatozoa that were not phagocytosed by PMNs in each sample were counted with a hemocytometer after dilution with PBS using phase-contrast microscopy at × 200 magnification. Counting of at least 100 cells per sample required dilution of the sample in most cases. The morphological characteristics of the PMNs and spermatozoa were also studied.

Pregnancy diagnosis

Possible return to estrus was determined by exposing females once daily to a mature boar. All pregnant gilts and sows were allowed to proceed to term, and farrowing rates and litter sizes were recorded.

Chlortetracycline fluorescence assessment of spermatozoa

Chlortetracycline fluorescence assessment of spermatozoa was performed by Chlortetracycline (CTC) fluorescence assay to examine the functional status of the spermatozoa, as described previously [8, 9]. Spermatozoa cultured in Modena or BCC solution were assessed at 30 and 90 min after initiation of culture under a phase-contrast microscope equipped with epifluorescence optics. Each cell was first observed at 1000× magnification under ultraviolet (UV) illumination (excitation, from 330 to 380 nm; emission, at 420 nm) to assess their live/dead status; sperm cells showing bright blue staining of the nucleus (bisbenzimide Hoechst 33258-positive

Table 2. Numbers of uterine polymorphonuclear leukocytes (PMNs) and frozen-thawed boar spermatozoa recovered from each region of the oviduct and uterus 4 h after the second cervical insemination

Treatment	Oviducts	Upper uterus	Lower uterus (plus the body of the uterus)	Total
PMNs				
Modena	$7.4 \pm 4.4 \times 10^4$	$9.0 \pm 2.9 \times 10^7$	$29.1 \pm 6.4 \times 10^7$*	$3.8 \pm 0.8 \times 10^8$
BCC	$3.8 \pm 2.3 \times 10^4$	$5.1 \pm 3.7 \times 10^7$	$9.9 \pm 5.6 \times 10^7$	$1.5 \pm 0.9 \times 10^8$
Spermatozoa				
Modena	0	$1.6 \pm 0.6 \times 10^5$*	$1.2 \pm 0.7 \times 10^6$*	$1.4 \pm 0.7 \times 10^6$*
BCC	$2.2 \pm 2.2 \times 10^3$	$75.4 \pm 29.7 \times 10^5$	$33.1 \pm 12.1 \times 10^6$	$40.6 \pm 12.9 \times 10^6$

The data are presented as means ± SEM. AI was performed twice with spermatozoa (25×10^8 cells) suspended in 50 ml Modena (n=7) or BCC (n=7) solution. * Values with different superscripts within the same row are significantly different (P<0.05).

Table 3. Pregnancy and farrowing rates and litter sizes after intrauterine insemination twice with frozen-thawed boar spermatozoa (25×10^8 cells) suspended in 50 ml Modena or BCC solution

Treatment	Boar	Motility (%)	n	Pregnancy rate (%)	Farrowing rate (%)	Litter size
Modena						
	A	41.7 ± 1.1	6	2/6 (33.3)	2/6 (33.3)	6.0 ± 0
	B	44.2 ± 3.3	6	1/6 (16.7)	1/6 (16.7)	13
	C	42.8 ± 1.2	9	5/9 (55.6)	3/9 (33.3)	4.0 ± 2.3
Total			21	8/21 (38.1[a])	6/21 (28.6[a])	7.2 ± 1.6
BCC						
	A	43.0 ± 2.0	5	3/5 (60.0)	3/5 (60.0)	7.3 ± 2.3
	B	42.5 ± 1.1	6	5/6 (83.3)	4/6 (66.7)	8.8 ± 1.3
	C	41.5 ± 1.1	10	7/10 (70.0)	6/10 (60.0)	8.3 ± 1.6
Total			21	15/21 (71.4[b])	13/21 (61.9[b])	8.2 ± 0.9

The data are presented as means ± SEM. [a,b] Values with different superscripts within the same row are significantly different (P<0.05).

cells) were considered to be dead and were not counted. Subsequently, we examined 200 live sperm under blue-violet illumination (excitation, from 400 to 440 nm; emission, at 470 nm) and classified them according to their CTC staining patterns. The following 3 fluorescent staining patterns were identified: F, uniform fluorescence over the whole sperm head; Cap, a fluorescence-free band in the post-acrosomal region; and AR, almost no fluorescence over the sperm head except for a thin band of fluorescence in the equatorial segment.

Statistical analysis

Data were analyzed using the StatView (Abacus Concepts, Berkeley, CA, USA) software to determine treatment-specific differences. To determine the significance of differences between the 2 groups, namely, the BCC and Modena groups, an unpaired Student's *t*-test was used. The pregnancy and farrowing rates were examined using the chi-square test. The percentage data obtained in experiment 3 were subjected to arcsine transformation and then statistical analysis. If a P value of <0.05 was obtained by analysis of variance (ANOVA), Tukey's HSD test was performed. All data were expressed as means ± standard error of the mean (SEM). P<0.05 was considered to be significant.

Results

Numbers of uterine PMNs and non-phagocytosed spermatozoa

The numbers of uterine PMNs and spermatozoa recovered at 4 h after the second insemination are shown in Table 2. Since PMNs are similar in size or rather bigger than the sperm head and the cells contain multiple nuclei, it is very easy to distinguish PMNs from other somatic cells. The total number of uterine PMNs in gilts inseminated with BCC was approximately 2-fold lower than that in gilts inseminated with Modena solution; the number of PMNs in the lower part of the uterus in gilts inseminated with BCC was significantly lower than that in gilts inseminated with Modena solution (P<0.05).

The total number of spermatozoa in gilts inseminated with BCC was significantly higher than that detected in those inseminated with Modena solution (P<0.05). However, since few cells were recovered from the oviducts, no significant differences were observed between gilts inseminated with the BCC and Modena solutions with regard to the number of oviductal spermatozoa.

Fertility

Fertility following AI with frozen-thawed boar spermatozoa is described in Table 3. The overall pregnancy and farrowing rates achieved when spermatozoa were inseminated with BCC were sig-

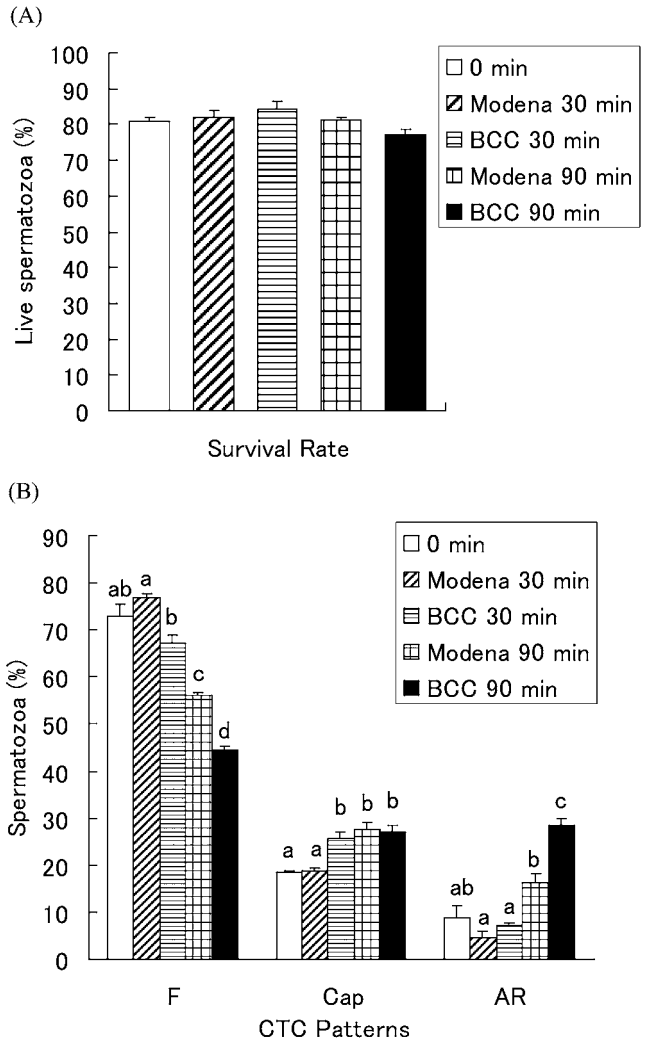

Fig. 1. Viability (A) and functional status (B) of frozen-thawed boar spermatozoa cultured in Modena or BCC solution for 30 and 90 min. The data are presented as means ± SEM. Different letters above the bars within the same CTC pattern denote significant differences (P<0.05).

nificantly higher than those obtained when spermatozoa were inseminated with Modena solution (pregnancy rate of 38.1 *vs.* 71.4% and farrowing rate of 28.6 *vs.* 61.9%, respectively; P<0.05). However, no significant difference was observed between gilts inseminated with BCC and Modena with regard to the mean difference in litter size of piglets (7.2 ± 1.6 *vs.* 8.2 ± 0.9, respectively).

Capacitation and acrosome reaction of spermatozoa in vitro

The viabilities and CTC patterns of the frozen-thawed spermatozoa cultured in the Modena or BCC solution for 30 and 90 min are presented in Fig. 1. The proportion of live spermatozoa did not differ according to the treatment groups and culture times (Fig. 1A). At 30 min after initiation of culture, the proportion of acrosome-intact live cells (F) did not differ between the treatment groups; however, at 90 min after initiation of culture, it was significantly reduced in the BCC group. Similarly, between 0 and 30 min, the proportion of sperm that underwent the acrosome reaction (AR) was not different between the treatment groups, but at 90 min, this proportion was significantly increased in the BCC group.

Discussion

The clearance of spermatozoa from the uterus by PMN recruitment is a necessary process for preparation of the genital tract to receive embryos for implantation. However, if PMN recruitment and phagocytosis of spermatozoa are inhibited or postponed artificially, a larger number of spermatozoa can be placed in oviductal sperm reservoirs. This type of technology may prove beneficial in improving the fertility of frozen-thawed spermatozoa, which are known to have relatively short viability.

In experiment 1, gilts inseminated with BCC solution had a significantly higher total number of frozen-thawed spermatozoa recovered from the uterus and a significantly lower number of PMNs in the lower part of the uterus compared with those inseminated with Modena solution (P<0.05). These results are consistent with a previous study performed using fresh liquid semen after a single AI [7]. In the present study, the number of non-phagocytosed frozen-thawed spermatozoa (10^{6-7} cells) recovered from the uterus was lower than that in the previous study of fresh spermatozoa (10^{7-8} cells) [7]. Seminal plasma-free medium has been shown to induce a post-breeding inflammatory response in gilts in the form of an influx of PMNs into the uterine lumen that persists for more than 36 h [3]. Therefore, most of frozen-thawed spermatozoa seem to be phagocytosed due to the induced uterine inflammation after the first AI. On the other hand, PMN recruitment in sows is elicited after boar seminal factors signal uterine epithelial cells to induce expression of a number of proinflammatory factors, including granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-6 (IL-6) and a key regulator of prostaglandin synthesis, i.e., cyclooxygenase 2 [11]. Caffeine has been shown to increase the intracellular concentration of Cyclic AMP and consequently activate the protein kinase A (PKA) pathway. Cyclic AMP is a very potent immunomodulator that exerts general suppressive effects on the functions (cytokine production and leukocyte chemotaxis) of inflammation *in vitro* and *in vivo* [12, 13]. These results may indicate that influx of PMNs into the uterus in gilts inseminated with BCC solution was suppressed due to inactivation of uterine inflammatory cytokines. Furthermore, incubation of PMNs and boar spermatozoa with caffeine considerably reduced the phagocytosis of spermatozoa *in vitro* [2]. Therefore, most of the recruited PMNs in the uterus of the gilts with BCC solution may not have phagocytosed (or bound) the spermatozoa.

In the second experiment, we evaluated whether uterine immune-regulated AI (UIR-AI) with frozen semen could improve fertility. Frozen-thawed spermatozoa have a limited life span in the female genital tract. The highest rate of fertility post-AI is achieved when inseminations are performed at the optimal time of 4 to 6 h before ovulation [14, 15]. However, it is difficult to directly predict the growth of follicles and the time of ovulation. In case of outside the optimal insemination time, an increase in the

number of frozen-thawed spermatozoa per AI dose improves fertility [16]. The above-mentioned reports and the results of experiment 1 suggest that a greater number of thawed spermatozoa in the uterus of gilts inseminated with BCC solution could produce a sufficient number of functional spermatozoa at the uterotubal junction, resulting in an increase in fertility.

Recently, new applications of AI procedures, such as transcervical deep intrauterine AI (DUI), have demonstrated acceptable fertility in weaned sows using a considerably decreased number of frozen-thawed spermatozoa [17]. However, the success rates of DUI vary among pig farms in which frozen-thawed boar spermatozoa are used for insemination [16]. Therefore, a combination of UIR-AI and DUI may result in improved fertility and reduce the number of frozen-thawed spermatozoa used.

In the present study, a significantly higher proportion of sperm cells had already undergone the acrosome reaction at 90 min, but not 30 min, after thawing when spermatozoa were cultured in BCC rather than Modena solution. These results indicate that exposure of spermatozoa to BCC solution for several hours induced the acrosome reaction and consequently reduced the life span of the spermatozoa. However, this exposure appeared to have no negative effect on spermatozoa fertility because adenosine, which almost certainly present in the female reproductive tract [18], has been shown to induce capacitation but prevent the spontaneous acrosome reaction in frozen-thawed boar spermatozoa [9]. In fact, frozen-thawed boar spermatozoa cultured in BCC solution containing adenosine *in vitro* showed a significant reduction in spontaneous acrosome loss (data not shown). Therefore, spontaneous acrosome reaction of spermatozoa may be prevented by the adenosine in the female reproductive tract.

In conclusion, we demonstrated that the BCC solution for frozen-thawed boar semen produced better pregnancy and farrowing rates following AI than Modena solution, probably by reducing the phagocytosis of spermatozoa.

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References

1. **First NL, Short RE, Peters JB, Stratman FW**. Transport and loss of boar spermatozoa in the reproductive tract of the sow. *J Anim Sci* 1968; 27: 1037–1040.
2. **Woelders H, Matthijs A**. Phagocytosis of boar spermatozoa *in vitro* and *in vivo*. *Reprod Suppl* 2001; 58: 113–127.
3. **Rozeboom KJ, Troedsson MH, Molitor TW, Crabo BG**. The effect of spermatozoa and seminal plasma on leukocyte migration into the uterus of gilts. *J Anim Sci* 1999; 77: 2201–2206.
4. **Rozeboom KJ, Troedsson MH, Rocha GR, Crabo BG**. The chemotactic properties of porcine seminal components toward neutrophils *in vitro*. *J Anim Sci* 2001; 79: 996–1002.
5. **Rozeboom KJ, Troedsson MH, Hodson HH, Shurson GC, Crabo BG**. The importance of seminal plasma on the fertility of subsequent artificial inseminations in swine. *J Anim Sci* 2000; 78: 443–448.
6. **Alghamdi AS, Foster DN, Troedsson MH**. Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils (PMNs) and improves the fertility of fresh semen inseminated into inflamed uteri. *Reproduction* 2004; 127: 593–600.
7. **Matthijs A, Engel B, Woelders H**. Neutrophil recruitment and phagocytosis of boar spermatozoa after artificial insemination of sows, and the effects of inseminate volume, sperm dose and specific additives in the extender. *Reproduction* 2003; 125: 357–367.
8. **Funahashi H, Asano A, Fujiwara T, Nagai T, Niwa K, Fraser LR**. Both fertilization promoting peptide and adenosine stimulate capacitation but inhibit spontaneous acrosome loss in ejaculated boar spermatozoa *in vitro*. *Mol Reprod Dev* 2000; 55: 117–124.
9. **Funahashi H, Nagai T**. Regulation of *in vitro* penetration of frozen-thawed boar spermatozoa by caffeine and adenosine. *Mol Reprod Dev* 2001; 58: 424–431.
10. **Nogami Y, Harada M, Seki T, Hino Y, Tanabe T**. Establishment of practical techniques for boar frozen semen(1). *Bull Okayama Pref Cent Anim Hus Res* 1998; 9: 1–6 (In Japanese).
11. **O'Leary S, Jasper MJ, Warnes GM, Armstrong DT, Robertson SA**. Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. *Reproduction* 2004; 128: 237–247.
12. **Horrigan LA, Kelly JP, Connor TJ**. Caffeine suppresses TNF-alpha production via activation of the cyclic AMP/protein kinase A pathway. *Int Immunopharmacol* 2004; 4: 1409–1417.
13. **Horrigan LA, Kelly JP, Connor TJ**. Immunomodulatory effects of caffeine: friend or foe? *Pharmacol Ther* 2006; 111: 877–892.
14. **Waberski D, Weitze KF, Gleumes T, Schwarz M, Willmen T, Petzoldt R**. Effect of time of insemination relative to ovulation on fertility with liquid and frozen boar semen. *Theriogenology* 1994; 42: 831–840.
15. **Bertani GR, Scheid IR, Fialho FB, Rubin MI, Wentz I, Gonçalves PB**. Effect of the time of artificial insemination with frozen-thawed or fresh semen on embryo viability and early pregnancy rate in gilts. *Theriogenology* 1997; 48: 933–945.
16. **Bolarín A, Roca J, Rodríguez-Martínez H, Hernández M, Vázquez JM, Martínez EA**. Dissimilarities in sows' ovarian status at the insemination time could explain differences in fertility between farms when frozen-thawed semen is used. *Theriogenology* 2006; 65: 669–680.
17. **Roca J, Carvajal G, Lucas X, Vazquez JM, Martinez EA**. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. *Theriogenology* 2003; 60: 77–87.
18. **Cometti B, Dubey RK, Imthurn B, Jackson EK, Rosselli M**. Oviduct cells express the cyclic AMP-adenosine pathway. *Biol Reprod* 2003; 69: 868–875.