Effect of acrosome reaction progress in frozen-thawed boar spermatozoa on the efficiency of *in vitro* oocyte fertilization

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ABSTRACT: A good functional status of cryopreserved boar spermatozoa is very important for successful fertilization of porcine oocytes and *in vitro* embryo production. The purpose of the study was to evaluate the changes in functional status of boar spermatozoa separated from frozen-thawed semen and capacitated *in vitro* by caffeine. The effect of acrosome reaction development in spermatozoa on the efficiency of oocyte fertilization has been studied in boars A, B and C. Motile spermatozoa were separated by Percoll gradient, untreated (control) or treated with both 1mM and 2mM caffeine, and capacitated or co-cultured with matured oocytes. The motility, viability, chromatin and acrosome integrity, and fertilizing ability of spermatozoa were assessed. The separation significantly increased ($P < 0.05$) the percentage of viable spermatozoa in all tested boars and percentages of motile and acrosome intact spermatozoa in boars B and C. The capacitation significantly decreased ($P < 0.05$) the percentages of viable and motile spermatozoa, but after capacitation, the motility and viability were significantly higher ($P < 0.05$) for the caffeine-treated spermatozoa than for the untreated controls. A fall in the proportion of acrosome-intact spermatozoa was different for each caffeine concentration and each boar, but in all boars, acrosome reaction progress was faster and, similarly, monospermy and the total efficiency of fertilization were significantly higher ($P < 0.01$) for the spermatozoa treated with 1mM caffeine than for those treated with 2mM caffeine. It can be concluded that there is a potential relationship between the acrosome reaction progress in frozen-thawed boar spermatozoa and the efficiency of fertilization of porcine oocytes. A faster AR induced in spermatozoa by appropriate caffeine treatment resulted in a higher monospermy rate and total efficiency of fertilization. Thus, it is important to test sires before their semen is used for *in vitro* embryo production. The faster AR induced by 1mM caffeine was more effective in terms of monospermy and total efficiency of fertilization.

Keywords: pig; cryopreserved sperm; assessment; capacitation

In recent years a great effort has been made to improve the efficiency of porcine reproduction biotechnology. Although many improvements have been achieved in *in vitro* maturation (IVM) and fertilization (IVF) of oocytes, today's methods for porcine embryo production are still not efficient enough (Nagai et al., 2006).

The results of *in vitro* fertilization of pig oocytes using fresh semen greatly vary in the same boar. Large differences in fertilizing ability of spermatozoa among individual boars or their ejaculates exist not only in fresh but also in frozen semen (Sirard at al., 1993; Wang et al., 1995; Long et al., 1999; Selles et al., 2003; Alminana et al., 2005). This

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variability influences the yield of both fertilized and polyspermic oocytes (Xu et al., 1996b).

To eliminate this problem, cryopreserved semen from boars with proven *in vitro* fertility should be used and a high number of insemination doses with a standard fertilizing ability of spermatozoa should be obtained from their ejaculates (Gil et al., 2008). It has been documented that, in addition to the quality of oocytes (Marchal et al., 2001), the quality of spermatozoa and conditions of fertilization can be a reason for polyspermic fertilization (Sirard et al., 1993; Abeydeera, 2001). A good functional status of frozen-thawed boar spermatozoa is a prerequisite for successful *in vitro* fertilization. Only the maintenance of viability, motility, and acrosome and chromatin integrity after thawing can guarantee the ability of spermatozoa to capacitate, undergo an acrosome reaction (AR) and penetrate oocytes. For that reason, in pigs, the fertilization medium usually contains caffeine, a phosphodiesterase inhibitor known to induce capacitation (Wang et al., 1991; Fraser, 2008), to accelerate the acrosome reaction (Funahashi et al., 2000) and to stimulate penetration of spermatozoa into oocytes (Wang et al., 1991). Nevertheless, it is widely accepted that a caffeine-induced AR may be responsible for polyspermic fertilization of porcine oocytes. It has been described that one of the reasons for high polyspermy is a high proportion of spermatozoa at the early stage of a spontaneous acrosome reaction, at which point the spermatozoa can tightly bind to the zona pellucida (ZP) and penetrate oocytes in large numbers (Funahashi et al., 2000; Funahashi and Nagai, 2001).

Although more effective methods for *in vitro* fertilization have recently been developed, a high incidence of polyspermy and a low incidence of normospermy in porcine oocytes still remain major problems (Funahashi, 2003).

Several assays for evaluation of the functional status of boar spermatozoa are available at present (Johnson et al., 2007). However, their value for predicting sperm fertilizing ability varies and, moreover, little of this information concerns cryopreserved boar spermatozoa (Gadea et al., 2001). Therefore, more methods for evaluation of the fertilizing ability of frozen-thawed boar spermatozoa are needed.

The aim of this study was to characterize changes in motility, viability, and chromatin and acrosome integrity of spermatozoa separated from frozen-thawed boar semen and capacitated *in vitro* by different levels of caffeine. Our attention was focused on the effect of acrosome reaction progress in spermatozoa on the efficiency of *in vitro* fertilization of oocytes.

MATERIAL AND METHODS

Semen collection. Three mature, 2- to 5-year-old boars of Pietrain (A) and Large White (B and C) breeds with proven fertility were used. Sperm-rich fractions of ejaculates were collected by the gloved-hand technique into plastic bags, placed in a thermo box and transported within 1 h to the laboratory and immediately assessed for spermatozoa motility and acrosome integrity as described below. Only ejaculates with at least 70% motility and 90% acrosome integrity of the spermatozoa were used for cryopreservation.

Semen cryopreservation. Semen was gently mixed with isothermal BTS medium (Beltsville thawing solution, Minitübe, Tiefenbach, Germany) at a 1 : 1 ratio (v/v) and kept in darkness at laboratory temperature for 30 min. Subsequently, it was stored at 17 °C for 20 h, and equilibrated by a previously described method (Martecikova et al., 2008a,b) and frozen according to the method of Westendorf et al. (1975) modified by Thurston et al. (1999) and Carvajal et al. (2004). Shortly, after cooling from 17 °C to 5 °C in a cooling extender (11% egg yolk (v/v), 0.12M lactose, 0.11M trehalose and 100 µg/ml kanamycin sulfate), the semen was equilibrated for 1 h and was then resuspended in a freezing extender (0.22M lactose, 22.8% egg yolk (v/v), 1,3 Equex STM, Minitube, Tiefenbach, Germany and 7.5% glycerol (v/v) to the final concentration of 0.5×10^9 spermatozoa per ml. The straws (IMV, French straws, Cassou, France) were placed in liquid nitrogen vapour at 1 cm above the nitrogen level for 20 min, then plunged into liquid nitrogen and stored until use.

Spermatozoa separation and capacitation. Semen thawing was carried out by holding straws at room temperature for 15 s and plunging them into a 37 °C water bath for 30 s. The semen was equilibrated at 38 °C for 30 min and spermatozoa were separated on a 40/60% Percoll gradient by centrifugation at 550 g for 5 min at 38 °C, after that the pellet of motile spermatozoa was removed and washed twice in BTS medium by centrifugation at 550 g for 2.5 min. The spermatozoa were resuspended (10×10^6 spermatozoa/ml) in mTBM (modified Tris buffered medium) contain-

ing 113.1mM NaCl, 3mM KCl, 10mM $CaCl_2 \cdot H_2O$, 20mM Tris, 11mM glucose, 5mM sodium pyruvate and 0.4% BSA), as described by Abeydeera and Day (1997), supplemented with 1mM or 2mM caffeine (Sigma Chemicals Co., Prague, Czech Republic) or only in medium without caffeine. Both the caffeine-treated and untreated (control) spermatozoa, in 200 ml aliquots, were capacitated in a humidified atmosphere of 5% CO_2 at 39 °C for three hours.

Spermatozoa assessment. Motility, viability and acrosome integrity were assessed 30 min after thawing, after separation and caffeine treatment (0 h) and during capacitation at 1-, 2- and 3-hour intervals. Chromatin integrity was evaluated 30 min after thawing, after separation and caffeine treatment (0 h) and after 3-hour capacitation.

Motility was evaluated subjectively in a 15 ml drop on a warm slide (38 °C), using a phase contrast microscope at a magnification of × 400. At least 2 × 200 spermatozoa per replica were checked in each boar. For viability assessment, wet mounts were prepared by mixing spermatozoa with 2.5% aqueous glutaraldehyde solution (GAH, v/v) at a ratio of 1 : 1 and adding bisbenzimide Hoechst 33258 (20 µg/ml in citrate buffer containing 0.154M sodium chloride and 0.015M trisodium citrate at pH 5.5). The mounts were immediately evaluated by epifluorescence at a × 400 magnification. Two populations of spermatozoa were distinquished: live Hoechst negative spermatozoa and dead Hoechst positive spermatozoa. At least 2 × 200 spermatozoa per replica were checked in each boar.

For chromatin integrity evaluation, the sperm chromatin structure assay (SCSA) was used (Evenson and Wixon, 2006). At least 3 × 5000 spermatozoa were checked per replica in each boar.

For acrosome status evaluation, smeared slide glasses were air-dried, fixed in 3.9% formalin solution (v/v) and stained by the Farrelly method for 15 s with both 5% (v/v) aniline blue and 0.5% (v/v) crystal violet. Acrosome-intact, acrosome-reacted and acrosome-denuded spermatozoa were evaluated using phase contrast at a magnification of × 1000. At least 2 × 200 spermatozoa were checked per replica in each boar. Only spermatozoa with an unchanged acrosome were classified as acrosome-intact. When the acrosome membrane was disrupted in apical, medial or equatorial parts, the spermatozoa were categorised as acrosome non-intact. When the acrosome was missing, the spermatozoa were considered acrosome-denuded.

A fall in the proportion of acrosome-intact spermatozoa was regarded as progress of the acrosome reaction in this study.

Oocyte maturation and fertilization. Cumulus oocyte complexes (COCs) were collected by slicing the ovarian cortex. The ovaries were obtained from sows slaughtered between the middle luteal and the early follicular phase of the oestrous cycle (Machatkova et al., 2008). Only COCs with dark, evenly granulated ooplasm and at least two compact layers of cumulus cells were selected for maturation. These were matured in TCM-199 medium with Earle's salts supplemented with 0.20mM sodium pyruvate, 0.57mM cysteamin, 50 IU/ml penicillin, 50 µg/ml streptomycin (Sigma Chemicals Co., Prague, Czech Republic), 10% fetal calf serum and gonadotropins (P.G.600 15 IU/ml; Intervet, Boxmeer, Holland) in an atmosphere of 5% CO_2 at 39 °C for 46 h. Matured oocytes were denuded of cumulus cells, placed in mTBM supplemented with 1mM or 2mM caffeine and co-cultured with 1mM and 2mM caffeine-treated spematozoa, respectively, for 3 h at 39 °C in an atmosphere with 5% CO_2. Presumptive zygotes were cultured in PZM-3 medium (Yoshioka et al., 2002) for 15 h.

Fertilization assessment. The presumptive zygotes were fixed in 2.5% GAH (v/v), stained with Hoechst 33258 solution in citrated buffer (20 µg/ml) at room temperature for 10 min and rinsed three times in Dulbecco-PBS. They were examined by epifluorescence at a magnification of × 400 on wet mounts prepared in 5 µl glycerin buffer (50% glycerol (v/v), 0.02M CH_3COOH and 0.05M Na_2HPO_4).

Oocytes were regarded as penetrated when they had at least one swollen sperm head or a male pronucleus in the ooplasm. Those with more than one swollen sperm head or male pronucleus were considered polyspermic. Only the oocytes with one male and one female pronucleus and two polar bodies were classified as monospermic.

The proportions of penetrated from co-cultured oocytes and monospermic or polyspermic from the penetrated oocytes were assessed. The total efficiency of fertilization (%) was calculated by the formula:

$$\frac{\text{monospermic oocytes } (n)}{\text{co-cultured oocytes } (n)} \times 100$$

Statistical analysis. The experiments were carried out in three replicas. The data were statistically analyzed by the Student's *t*-test or Chi-squared test and the results were expressed as mean ± SD values.

RESULTS

Motility

Motility after thawing, separation and caffeine treatment of spermatozoa in the tested boars are shown in Table 1.

In all boars, the percentage of motile spermatozoa increased after separation, as compared with that after thawing, but the increase was significant ($P < 0.05$) only in boars B and C. In all boars, the percentage of motile spermatozoa decreased significantly ($P < 0.05$) after 3 h-capacitation, as compared with that after separation. The percentage of motile spermatozoa was significantly higher ($P < 0.05$) after 3 h-capacitation with caffeine in comparison with the untreated control. No significant differences in motility were observed between the spermatozoa treated with 1mM and those with 2mM caffeine.

Viability

Viability after thawing, separation and caffeine treatment of spermatozoa in the tested boars are documented in Table 2.

The percentage of viable spermatozoa increased after separation in comparison with that after thawing and the increase was significant ($P < 0.05$) in all boars. In all boars, 3 h-capacitation resulted in a significantly decreased viability, as compared with that after separation. As with motility, the viability of spermatozoa was also significantly higher ($P < 0.05$) after 3 h-capacitation with caffeine, as compared with the untreated control. No significant differences in viability were found between the 1mM caffeine-treated and 2mM caffeine-treated spermatozoa.

Chromatin integrity

In contrast to statistically significant changes in motility and viability, the differences in chromatin integrity among thawed, separated and capacitated spermatozoa of the tested boars were not significant (Table 3).

Acrosome integrity and acrosome reaction progress

Acrosome integrity of spermatozoa after thawing, separation and caffeine treatment in the tested boars is shown in Table 4.

In all boars, the percentage of acrosome intact-spermatozoa increased after separation compared to that after thawing, but the increase was significant ($P < 0.05$) only in boars B and C. The per-

Table 1. Motility of spermatozoa after thawing, separation and caffeine treatment

Boar	Motile spermatozoa (%, mean ± SD)					
	after thawing	after separation	after caffeine treatment			
			mM	1 h	2 h	3 h
A	57.5 ± 2.5^{a}	60.8 ± 3.4^{a}	0 (control)	59.2 ± 3.4^{a1}	50.0 ± 2.9^{b1}	40.8 ± 3.4^{c1}
			1	61.7 ± 2.4^{a1}	50.8 ± 3.4^{b1}	46.7 ± 2.4^{b2}
			2	60.0 ± 2.9^{a1}	50.8 ± 1.9^{b1}	45.8 ± 4.5^{b2}
B	35.8 ± 3.4^{a}	48.3 ± 2.4^{b}	0 (control)	45.8 ± 1.9^{b1}	40.8 ± 1.9^{b1}	35.8 ± 3.4^{ac1}
			1	49.2 ± 1.9^{b1}	47.8 ± 2.4^{b2}	41.7 ± 2.4^{c2}
			2	47.5 ± 2.5^{b1}	48.3 ± 2.4^{b2}	40.0 ± 2.9^{ac2}
C	41.7 ± 2.4^{a}	49.2 ± 1.9^{b}	0 (control)	48.3 ± 2.4^{b1}	35.8 ± 4.5^{ac1}	28.3 ± 2.4^{d1}
			1	49.2 ± 1.9^{b1}	38.3 ± 2.4^{ac1}	35.0 ± 2.9^{c2}
			2	48.3 ± 2.4^{b1}	35.1 ± 2.9^{c1}	33.3 ± 2.4^{c2}

Values with different superscripts in the same row (letters) or column (numerals) are significantly different for the same boar at least at $P < 0.05$. Different letters in the rows or numerals in the columns express the effect of separation and capacitation or caffeine concentration

Table 2. Viability of spermatozoa after thawing, separation and caffeine treatment

Boar	Viable spermatozoa (%, mean ± SD)					
	after thawing	after separation	after caffeine treatment			
			mM	1 h	2 h	3 h
A	58.8 ± 2.7^{a}	68.9 ± 4.6^{b}	0 (control)	64.9 ± 2.9^{b1}	58.9 ± 1.5^{ac1}	44.4 ± 3.6^{d1}
			1	67.4 ± 4.1^{b2}	62.5 ± 1.6^{c2}	53.5 ± 4.9^{ad2}
			2	67.3 ± 3.6^{b2}	61.5 ± 2.1^{c1}	53.4 ± 5.3^{ad2}
B	48.6 ± 3.7^{a}	53.7 ± 1.8^{b}	0 (control)	51.1 ± 1.4^{b1}	43.7 ± 1.5^{c1}	40.2 ± 2.8^{c1}
			1	53.5 ± 2.4^{b1}	49.2 ± 1.8^{ad2}	47.2 ± 4.8^{ad2}
			2	52.5 ± 1.6^{b1}	52.6 ± 3.0^{b2}	49.0 ± 2.8^{ad2}
C	44.8 ± 2.4^{a}	50.9 ± 2.4^{b}	0 (control)	51.2 ± 2.8^{b1}	41.6 ± 2.4^{ac1}	32.3 ± 3.8^{d1}
			1	51.6 ± 4.2^{b1}	43.2 ± 3.3^{ac2}	39.0 ± 2.8^{c2}
			2	51.6 ± 2.4^{b1}	39.0 ± 4.1^{c1}	39.4 ± 2.8^{c2}

Values with different superscripts in the same row (letters) or column (numerals) are significantly different for the same boar at least at $P < 0.05$. Different letters in the rows or numerals in the columns express the effect of separation and capacitation or caffeine concentration

centage of acrosome intact-spermatozoa decreased after 3 h-capacitation in comparison with that after separation, the decrease was significant ($P < 0.05$) in boars A and C.

In all boars, the percentage of acrosome-intact spermatozoa was lower, or significantly lower ($P < 0.05$), for the 1mM compared to the 2mM caffeine-treated spermatozoa or the untreated control during the whole capacitation. However, the AR dynamics were different for each boar, as shown by the fall in the percentage of acrosome-intact spermatozoa occurring at different intervals, namely, at 1–2 h for boar A, at 2 h for boar B, and between 2 and 3 h for boar C (Figures 1, 2 and 3).

Fertilizing ability

The results of fertilization for oocytes co-cultured with the untreated or caffeine-treated spermatozoa of the tested boars are presented in Table 5.

The rate of penetration was significantly higher ($P < 0.05$) for the 2mM caffeine-treated than for the 1mM caffeine-treated spermatozoa only in boar A; in boars B and C the difference in the rate of penetration was not significant. On the other hand, in all boars, the rate of monospermy and the total efficiency of fertilization were significantly higher ($P < 0.05$) for oocytes co-cultured with the spermatozoa treated with 1mM caffeine

Table 3. Chromatin integrity of spermatozoa after thawing, separation and caffeine treatment

Boar	DNA intact-spermatozoa (%, mean ± SD)			
	after thawing	after separation	after caffeine treatment	
			mM	3 h
A	98.9 ± 0.2^{a}	98.7 ± 0.5^{a}	0 (control)	97.6 ± 1.3^{a1}
			1	98.4 ± 0.3^{a1}
			2	98.5 ± 0.3^{a1}
B	99.2 ± 0.1^{a}	98.0 ± 0.3^{b}	0 (control)	97.8 ± 0.5^{b1}
			1	97.9 ± 0.7^{ab1}
			2	97.6 ± 0.7^{ab1}
C	99.1 ± 0.0^{a}	97.0 ± 1.2^{ab}	0 (control)	97.1 ± 0.7^{b1}
			1	97.2 ± 0.6^{b1}
			2	96.9 ± 0.4^{b1}

Values with different superscripts in the same row (letters) or column (numerals) are significantly different for the same boar at least at $P < 0.05$. Different letters in the rows or numerals in the columns express the effect of separation and capacitation or caffeine concentration. Only values for spermatozoa with a non-detectable DNA fragmentation index were used in the table

Table 4. Acrosome integrity of spermatozoa after thawing, separation and caffeine treatment

Boar	Acrosome intact-spermatozoa (%, mean ± SD)					
	after thawing	after separation	after caffeine treatment			
			mM	1 h	2 h	3 h
A	53.8 ± 2.8^{a}	55.8 ± 3.9^{a}	0 (control)	51.0 ± 2.9^{b1}	48.5 ± 2.8^{b1}	45.8 ± 3.0^{c1}
			1	44.0 ± 5.3^{b2}	31.4 ± 2.2^{c2}	30.9 ± 1.0^{c2}
			2	50.5 ± 1.0^{b1}	38.6 ± 4.2^{c1}	33.3 ± 1.9^{c2}
B	26.2 ± 4.3^{a}	48.9 ± 4.8^{b}	0 (control)	48.0 ± 4.8^{b1}	44.1 ± 6.6^{b1}	40.6 ± 4.4^{b1}
			1	43.0 ± 3.4^{b2}	32.0 ± 3.6^{c2}	30.2 ± 3.2^{d2}
			2	44.9 ± 2.7^{b2}	40.5 ± 2.6^{c1}	31.1 ± 2.4^{d2}
C	42.6 ± 3.8^{a}	51.0 ± 3.2^{b}	0 (control)	45.3 ± 3.5^{c1}	39.9 ± 2.2^{d1}	40.2 ± 1.6^{d1}
			1	31.8 ± 4.6^{c2}	29.5 ± 2.6^{c2}	18.9 ± 5.4^{d2}
			2	33.3 ± 2.1^{c2}	33.1 ± 1.6^{c3}	31.0 ± 2.0^{c3}

Values with different superscripts in the same row (letters) or column (numerals) are significantly different for the same boar at least at $P < 0.05$. Different letters in the rows or numerals in the columns express the effect of separation and capacitation or caffeine concentration

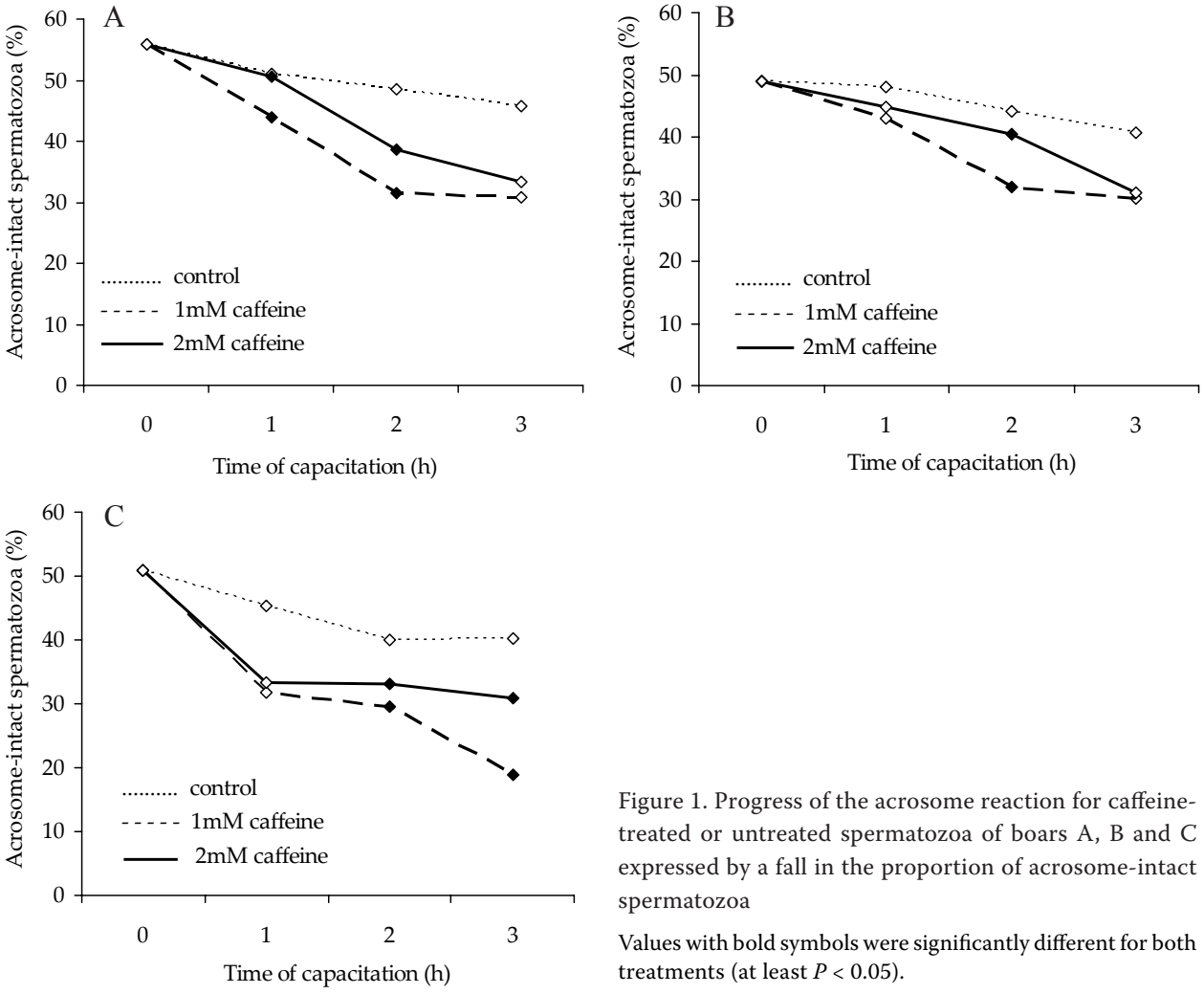

Figure 1. Progress of the acrosome reaction for caffeine-treated or untreated spermatozoa of boars A, B and C expressed by a fall in the proportion of acrosome-intact spermatozoa

Values with bold symbols were significantly different for both treatments (at least $P < 0.05$).

Table 5. Efficiency of fertilization of oocytes co-cultured with untreated or caffeine-treated spermatozoa

Boar	Spermatozoa treatment by caffeine (mM)	Oocytes co-cultured with spermatozoa (*n*)	Oocytes (%, mean ± SD)			Total efficiency of fertilization (%)
			penetrated	monospermic/ penetrated	polyspermic/ penetrated	
A	0 (control)	83	8.4 ± 0.9[1]	87.5 ± 12.5[1]	12.5 ± 12.5[1]	7.3 ± 0.3[1]
	1	82	51.1 ± 1.1[2]	67.0 ± 4.9[2]	33.0 ± 5.0[2]	34.4 ± 1.8[2]
	2	83	67.4 ± 0.7[3]	32.3 ± 12.0[3]	67.7 ± 11.5[3]	21.9 ± 8.0[3]
B	0 (control)	84	5.0 ± 0.8[1]	100.0 ± 0.0[1]	0.0 ± 0.0[1]	5.0 ± 0.8[1]
	1	83	48.7 ± 9.2[2]	74.3 ± 7.3[2]	25.7 ± 5.7[2]	34.7 ± 3.1[2]
	2	82	62.9 ± 6.1[2]	34.4 ± 0.5[3]	65.6 ± 1.1[3]	20.6 ± 1.7[3]
C	0 (control)	80	26.3 ± 3.8[1]	57.0 ± 1.4[1]	19.5 ± 2.8[1▲]	15.0 ± 2.5[1]
	1	83	89.5 ± 2.5[2]	30.5 ± 5.8[2]	69.5 ± 7.0[2]	26.0 ± 4.4[2]
	2	83	89.2 ± 0.1[2]	8.0 ± 1.9[3]	92.0 ± 1.9[3]	7.1 ± 1.7[3]

Values with different superscripts in the same column were significantly different for the same boar ($P < 0.05$). Total efficiency of fertilization (%): monospermic oocytes from those co-cultured with spermatozoa
▲23.5% asynchronic oocytes/penetrated in boar C were not included in the table

in comparison with those co-cultured with spermatozoa treated with 2mM caffeine or the untreated control.

The effect of caffeine on the total efficiency of fertilization was confirmed when the result of each of the three spermatozoa treatments was related to the total number of oocytes co-cultured with spermatozoa regardless of the boar's individuality (Table 6).

DISCUSSION

The assessment of *in vitro* fertility in boars still presents a great problem. The prerequisite for effective fertilization of oocytes is a good functional status of spermatozoa which must retain their ability to undergo the acrosome reaction. Therefore, we evaluated the basic functional parameters of cryopreserved boar spermatozoa, i.e., motility, viability, chromatin and acrosome integrity and we focused on the monitoring of changes associated with separation and capacitation of the spermatozoa used for oocyte fertilization.

While the chromatin integrity of spermatozoa did not change significantly during these procedures, the motility, viability and acrosome integrity of frozen-thawed boar semen was positively influenced by separation on a Percoll gradient. The effect of sperm separation was different in each boar. These results are in agreement with those of Suzuki and Nagai (2003).

In our study, caffeine was used to stimulate capacitation and AR in spermatozoa. We found a positive effect of caffeine on spermatozoa not only in terms of AR induction, as observed by De Jonge et al. (1991) and Funahashi et al. (2000), but also in terms of viability and motility as described by Garbers et al. (1973). In our experiments this was manifested as a lower proportion of acrosome-in-

Table 6. Efficiency of fertilization based on the total number of oocytes co-cultured with the untreated, 1mM- and 2mM-caffeine-treated spermatozoa independently of individuality of boar

Spermatozoa treatment by caffeine (mM)	Oocytes co-cultured with spermatozoa (*n*)	Oocytes (%, mean ± SD)		Total efficiency of fertilization (%)
		penetrated	monospermic/penetrated	
0 (control)	247	13.5 ± 9.1[1]	81.5 ± 1.8[1]	7.7 ± 1.7[1]
1	248	63.1 ± 21.3[2*]	56.4 ± 20.3[2]	31.7 ± 16.5[2]
2	248	73.1 ± 13.1[3**]	24.4 ± 13.4[3]	16.5 ± 9.0[3]

Values with different superscripts in the same column were significantly different ($P < 0.01$; $^{*,**}P < 0.05$). Total efficiency of fertilization (%): monospermic ocytes from those co-cultured with spermatozoa

tact spermatozoa and higher proportions of motile and viable spermatozoa in the caffeine-treated than in the untreated spermatozoa in all tested boars.

The acrosome reaction is an important prerequisite for the fertilizing capacity of spermatozoa (Birck et al., 2009) and the ability of spermatozoa to undergo the AR affects the male fertilizing potential under both *in vivo* and *in vitro* conditions. Studies on the induction of capacitation and AR with the aim of evaluating the fertilizing ability of boars have been reported (Funahashi and Nagai, 2001). In our experiments we investigated a potential relationship between AR progress in cryopreserved boar spermatozoa and their ability to penetrate and fertilize matured porcine oocytes.

Although the AR progress was different in each boar, at certain capacitation intervals, it was faster for 1mM than for 2mM caffeine-treated spermatozoa in all of them. Similarly, monospermy and the total efficiency of fertilization were more effective in oocytes co-cultured with 1mM caffeine-treated spermatozoa, even though penetration was moderately decreased. At this caffeine level, AR progress was accelerated at the beginning of capacitation in boars A and B, and resulted in a slight decrease in penetration but an evident increase in monospermy. In boar C, in contrast, AR was accelerated at the end of the capacitation and therefore penetration was unchanged and only a slight increase in monospermy was observed.

Funahashi and Nagai (2001) have reported that, in pigs, high polyspermy results from a high proportion of spermatozoa at an early stage of spontaneous AR. In another study Funahashi (2003) has found that polyspermy could be reduced by a decrease in the proportion of spermatozoa with an incompletely reacted acrosome which can tightly bind to ZP and mask free receptors. We suggest that, in our experiments, modification in the caffeine concentration resulted in faster AR progress, a decrease in the proportion of spermatozoa at an early AR stage and an increase in monospermy and the total efficiency of fertilization.

It is known that the rate of penetration and polyspermy in porcine IVF systems may also be influenced by boar selection (Vazquez at al., 1993; Wang et al., 1995; Xu et al., 1996a,b) and not only by the concentration of a capacitation agent. We suggest that additional factors can play a role in the fertilizing ability of boar spermatozoa, and one of these may be the individual zona pellucida binding ability of spermatozoa.

It can be concluded that there is a potential relationship between the progress of acrosome reaction in boar cryopreserved spermatozoa and the efficiency of *in vitro* fertilization of porcine oocytes. The AR progress can be modified by caffeine treatment, and better results are achieved with a caffeine concentration that induces a faster decrease in the proportion of acrosome-intact spermatozoa. Because the ability of spermatozoa to undergo the AR is specific for each boar, it is very important to test the spermatozoa of sires for the caffeine response before they are used for *in vitro* embryo production.

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