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A potential relationship between the acrosome response characteristics of bovine spermatozoa and their *in vitro* fertilizing ability

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Summary

The aim of the work was to study a potential relationship between acrosome response characteristics of bovine spermatozoa and their ability to fertilize oocytes and produce *in vitro* embryos. Sperm of artificial insemination bulls with a high rate ($22.0 \pm 4.1\%$, group A, $n = 7$) or a low rate ($10.3 \pm 4.1\%$, group B, $n = 8$) of embryos were used. For acrosome assessment, motile spermatozoa from a Percoll gradient were incubated with or without heparin and examined by the fix-vital sperm assay (FVSA). The differences between the heparin-treated (H^+) and the non-treated (H^-) spermatozoa were significant ($p < 0.01$) in all bulls at all tested intervals. According to the kinetics of the heparin response, the bulls fell into three categories: fast (FR, $n = 7$), moderate (MR, $n = 5$) or slow (SR, $n = 3$) acrosome responses ($p < 0.01$). Five MR bulls were found in group A in comparison with two MR bulls in group B (57.1 vs 12.5%; $p < 0.05$). Intensity of the acrosome response (response index) was significantly higher in bull group A compared with bull group B (7.0 vs 4.6, $p < 0.01$). A positive correlation was recorded between response index and embryo rate ($r = 0.668$, $p < 0.01$). In conclusion (*a*) the kinetics of spermatozoa response to heparin may be important for *in vitro* fertilization, bulls with a moderate response appearing to be most suitable for embryo production; (*b*) greater spermatozoa response to heparin was related to more effective embryo production.

Keywords: Acrosome, Bulls, Embryos, Heparin, Spermatozoa

Introduction

Fertilization is a complex process that depends on a successful interaction of mature, developmentally competent oocytes with motile, capacitated spermatozoa capable of undergoing an acrosome reaction. Only capacitated spermatozoa can bind to the zona pellucida and penetrate oocytes.

In assisted reproduction, frozen-thawed semen is usually used for *in vitro* fertilization (IVF). A necessary prerequisite for fertilization is to isolate a population of motile spermatozoa with an intact plasmalemma and acrosome. The most common methods used are separation on Percoll or bovine serum albumin (BSA) gradients (Parrish *et al.*, 1995; Suzuki *et al.*, 2003), by the swim up technique (Parrish *et al.*, 1985, 1988; Januskauskas *et al.*, 2000*a*) or PureSperm medium (Mendes *et al.*, 2003).

The freezing-thawing and separating process may cause structural and functional changes in the acrosome and may increase the ability of spermatozoa to undergo capacitation and the acrosome reaction (Pérez *et al.*, 1996). Due to premature capacitation, the time necessary for the acrosome reaction may be reduced or sperm viability and fertility may be decreased (Watson, 1996, 2000, Cormier & Bailey, 2003).

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Several methods are currently available for the evaluation of sperm viability and acrosome integrity, with the vital fluorescent staining methods being preferred (Cross & Watson, 1994, Garner & Johnson, 1995, Brito *et al.*, 2003). The simple and prompt fix-vital sperm assay (FVSA) described by de Leeuw *et al.* (1991) is suitable for a simultaneous assessment of viability and acrosomal status of glutaraldehyde-fixed spermatozoa stained with Hoechst 33258.

Although some authors have studied the acrosomal status of mammalian sperm, they usually evaluated fresh ejaculated semen (Way *et al.*, 1995), fresh or frozen-thawed and washed spermatozoa treated with heparin or calcium ionophore in relation to field fertility of bulls (Whitfield & Parkinson, 1995, Januskauskas *et al.*, 2000*a*, *b*; Pereira *et al.*, 2000). Little is known about the acrosomal status of motile bovine spermatozoa, separated from frozen-thawed semen and treated with heparin for several hours, that participate in the IVF process. Similar information is needed for the prediction of fertilizing ability of individual bulls involved in IVF programmes.

The aim of the present study was to study a potential relationship between acrosome characteristics of bovine spermatozoa during heparin-induced capacitation and their ability to fertilize oocytes and produce *in vitro* embryos.

Materials and methods

Animals

Fifteen 2-year-old bulls of the Czech pied breed with good outcomes in artificial insemination (from 57.3% to 67.3% non-return rate) but variable results in the *in vitro* system (from 8.0% to 28.9% embryo rates) were used in the experiments. Based on the efficiency of IVF, the bulls were assigned to one of two groups: A ($n = 7$) or B ($n = 8$) with a high and a low embryo rates, respectively ($22.0 \pm 4.1\%$ and $10.3 \pm 4.1\%$).

Motile sperm

Separation and incubation

From each bull, two insemination doses from two batches were thawed in a water bath at 37 °C for 60 s. The thawed semen was pooled and motile spermatozoa were separated using Percoll (Sigma Chemical, Prague, Czech Republic) gradient (Parrish *et al.*, 1995). The spermatozoa were washed by centrifugation at 200 *g* for 10 min and the pellet was resuspended in IVF-TALP medium. The sperm concentration was adjusted to give 25×10^6 spermatozoa/ml in 500 µl medium. To a 250 µl aliquot, 10 µg/ml heparin was added. Both the heparin-treated (H^+) and the non-treated (H^-) sperm suspensions were incubated at room temperature for 6 h.

Fixation and staining

From the heparin-treated and the non-treated sperm suspensions, 20 µl samples were taken at 1 h intervals. They were fixed with 20 µl of 2.5% glutaraldehyde and stained with 20 µl of Hoechst 33258 bisbenzimide at a final concentration of 20 µg/ml. Wet slides were prepared by placing a 5 µl drop of stained spermatozoa under a coverslip.

Evaluation

From each bull, 800 spermatozoa from the heparin-treated (H^+) and 800 spermatozoa from the non-treated (H^-) populations (two slides from each sample) were evaluated at each interval, using a phase-contrast fluorescence microscope (Jenamed) with a 410 nm excitation filter, at a magnification of ×400. In the total of spermatozoa evaluated, the percentages of acrosome-intact (AI), acrosome-reacted (AR) and acrosome-denuded (AD) spermatozoa were recorded.

Oocyte fertilization and embryo production

Prior to sperm assessment, embryos from each bull were prepared by the standard method (Machatkova *et al.*, 2004). A total of 3549 oocytes, an average of 236 oocytes per bull, were fertilized.

Cumulus–oocyte complexes (COCs) obtained by slicing of the cortex of ovaries of slaughtered cows were used. They were matured in 500 µl of TCM-199 medium (Earle's salt), with the addition of 0.20 mM sodium pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin (Sigma Chemical, Prague, Czech Republic), 5% ECS (estrus cow serum), and gonadotropins (P.G. 600 15 U/ml, Intervet, Boxmeer, The Netherlands) in microplate wells (Nunclon Intermed, Roskilde, Denmark) for 24 h. Motile spermatozoa were separated by the procedure described above. Fertilization was carried out in modified Tyrode's medium (IVF-TALP) containing 1×10^6/ml spermatozoa and 10 µg/ml heparin. Twenty-four hours after fertilization, cumulus cells were removed and presumptive zygotes were transferred to a Buffalo rat liver cell line monolayer (ATCC, Rockville, MD) and cultured at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

The embryo rate was expressed as percentages of embryos that reached the blastocyst stage from fertilized oocytes on day 8 after fertilization.

Statistical analysis

The data were analysed by the ANOVA procedure, chi-square test, non-parametric exact test and Pearson correlation test using Version 6.1 for Windows software

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Table 1 Bull categories according to the kinetics of the acrosome response of their spermatozoa to heparin before and after 6 h of incubation

Spermatozoal treatment	Acrosomal status	Incubation (h)	FR bulls ($n = 7$)	MR bulls ($n = 5$)	SR bulls ($n = 3$)
		0	80.3 ± 3.4^{a}	84.3 ± 5.9^{a}	85.0 ± 4.0^{a}
H^-	Acrosome-intact (AI)	6	60.7 ± 8.0^{a}	56.3 ± 7.8^{ab}	72.3 ± 4.4^{ac}
H^+		6	0.1 ± 0.3^{a}	3.7 ± 2.9^{b}	22.8 ± 3.4^{c}
		0	18.2 ± 3.2^{a}	14.6 ± 6.6^{a}	14.8 ± 6.0^{a}
H^-	Acrosome-reacted (AR)	6	35.5 ± 8.0^{a}	39.2 ± 7.0^{a}	26.1 ± 4.2^{a}
H^+		6	96.4 ± 0.9^{a}	92.5 ± 2.0^{b}	75.0 ± 2.8^{c}
		0	1.5 ± 0.8^{a}	1.1 ± 1.3^{a}	0.2 ± 0.2^{a}
H^-	Acrosome-denuded (AD)	6	3.8 ± 0.9^{a}	4.5 ± 1.1^{a}	1.6 ± 0.5^{b}
H^+		6	3.5 ± 0.9^{a}	3.8 ± 1.6^{a}	2.2 ± 1.0^{a}

Values are the percentage of spermatozoa (mean ± SD).
Within the same row, values with different superscripts differ significantly ($^{a\text{-}b, a\text{-}c, b\text{-}c}$ $p < 0.01$; $^{ab\text{-}ac}$ $p < 0.05$).

(SPSS, Inc.). The results were expressed as mean ± SD values.

Results

Acrosome response of spermatozoa to heparin

After separation, highly viable and acrosome-intact spermatozoa populations (82.3 ± 5.5% viable and 82.6 ± 4.7% acrosome-intact) were recovered from the semen of all test bulls. During 6 h of incubation, the proportions of AI and AR spermatozoa changed dramatically in the presence of heparin in comparison with slow gradual changes in the percentages of AI and AR spermatozoa in the absence of heparin. The differences between the H^+ and H^- spermatozoa populations were significant ($p < 0.01$) in all bulls at all intervals tested.

Kinetics of the acrosome response

Using the changes in acrosomal status in H^+ spermatozoa populations, it was possible to categorize the individual animals as bulls with a fast (FR, $n = 7$), moderate (MR, $n = 5$) or slow (SR, $n = 3$) response to heparin. The differences ($p < 0.01$) found among the categories following 6 h of incubation with heparin are shown in Table 1.

The kinetics of the acrosome response of spermatozoa in the three categories of bulls is shown in Figs. 1 to 3. Each category was characterized by a different curve showing changes in the proportions of AR spermatozoa in H^+ and H^- populations during 6 h of incubation. Significant differences in the percentages of AR spermatozoa between these categories appeared as early as after 1 h and continued for the whole incubation.

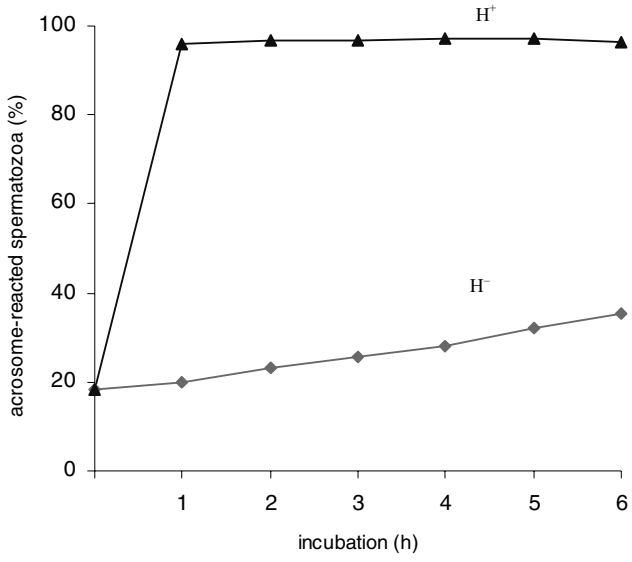

Figure 1 Kinetics of acrosomal changes of spermatozoa in bulls with a fast response (FR) to heparin.

When the kinetics of the acrosome response of spermatozoa was related to embryo production in the bulls, it was found that five of seven in group A in comparison with two of eight in group B were MR bulls (57.1 vs 12.5%; $p < 0.05$), as shown in Fig. 4.

Intensity of the acrosome response

To express the ability of spermatozoa to respond to heparin treatment, the response index was established. This was a ratio of the percentage of AR spermatozoa after 6 h of incubation with heparin to that before incubation (0 h). The heparin response index was significantly higher in group A bulls compared with group B bulls ($p < 0.01$) and a positive correlation was

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Table 2 Comparison of the response index of spermatozoa to heparin and embryo production

Group of bulls	Acrosome-reacted spermatozoa (%) Incubation (h) 0	6	Response index*	Embryo development* (%)
A ($n=7$)	13.1 ± 3.4	91.8 ± 7.7	7.0 ± 2.0^{a}	22.0 ± 4.1^{a}
B ($n=8$)	19.5 ± 4.2	90.1 ± 9.7	4.6 ± 1.1^{b}	10.3 ± 1.8^{b}

Values are the mean ± SD.
Within the same column, values with different superscripts differ significantly ($^{a\text{-}b}$ $p < 0.01$).
*Pearson's correlation coefficient, $r = 0.668$.

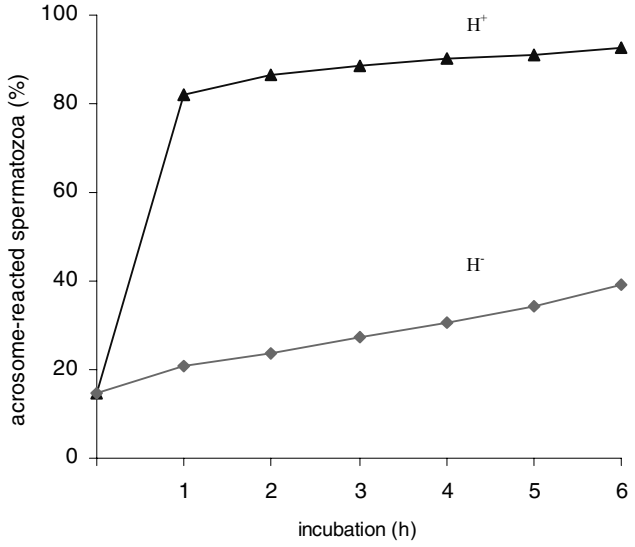

Figure 2 Kinetics of acrosomal changes of spermatozoa in bulls with a medium response (MR) to heparin.

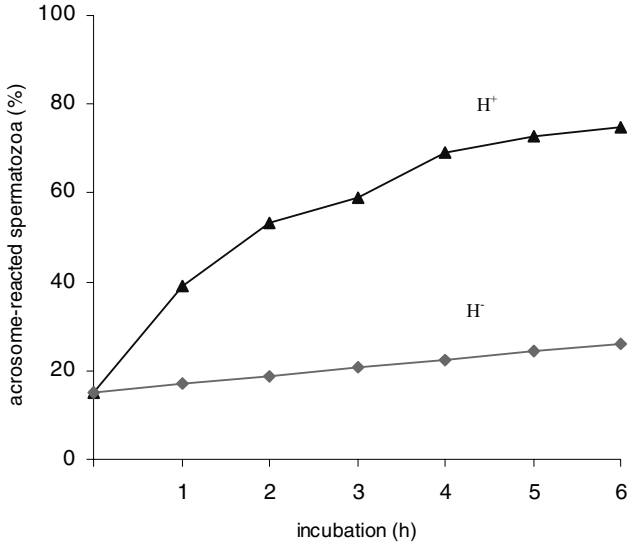

Figure 3 Kinetics of acrosomal changes of spermatozoa in bulls with a slow response (SR) to heparin.

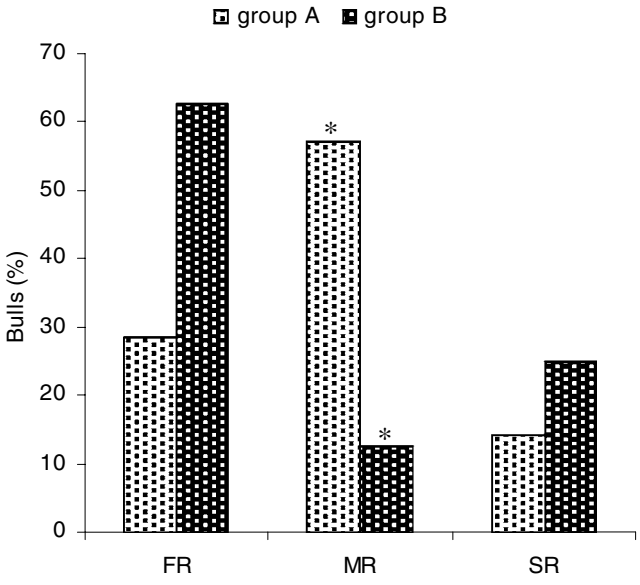

Figure 4 Distribution of the bulls according to the kinetics of the acrosome response in group A bulls with a high rate ($22.0 \pm 4.1\%$) and group B bulls with a low rate (10.3 ± 1.8) of embryos. FR, bulls with a fast response; MR, bulls with a moderate response; SR, bulls with a slow response ($^{*}p < 0.05$).

found between the heparin response index and embryo development (Table 2).

Discussion

The methods of oocyte fertilization and embryo production have become fully effective in breeding programmes in cattle. Although great advances in methods which permit repetitive yields of experimental embryos have recently been achieved in this field, the proportion of embryos derived from high-performance parents is still highly variable. This is also due to the use of standard conditions of fertilization for all individual

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donors without regard to the functional status of their gametes.

One of the means available that may assist in increasing the yield of embryos from bulls with high genetic merits is to test spermatozoa *in vitro* in order to predict their fertilizing ability. The results of such tests may serve to modify conditions of fertilization in individual bulls, to minimize differences amongst them and to enhance the probability that transferable embryos of a required genotype will be produced.

Currently, the decisive criterion for the selection of bulls for an IVF programme is their *in vivo* fertility. Although a positive correlation has been reported between *in vivo* and *in vitro* bull fertility (Eid & Parrish, 1995; Ward *et al.*, 2001; Hillery *et al.*, 1990; Marquant-Le Guienne *et al.*, 1990; Lansbergen *et al.*, 1997), some authors failed to confirm this relationship (Aurich & Hahn, 1993; Schneider *et al.*, 1996). In bulls with the same non-return rate, the fertilizing ability of individual animals in the IVF system varies greatly, with the rate of cleaved zygotes ranging from 25% to 82% and embryo yields from 6% to 45% (Yang *et al.*, 1995). Under standard fertilization conditions, bulls differ markedly in their ability to provide *in vitro* embryos (Katska & Smorag, 1996), including those bulls proved to be highly fertile when used *in vivo* (Palma & Brem, 1994).

With this background knowledge, we based our study on a group of bulls with a good non-return rate but varying in *in vitro* embryo production. We have tested the response of their spermatozoa to a capacitation agent to find out whether there is a relationship between the characteristics of sperm response and the efficiency of IVF. By separation of motile spermatozoa with intact acrosomes, we obtained from all bulls involved, as did Somfai *et al.* (2002) and Suzuki *et al.* (2003), healthy and comparable populations that were further treated by heparin. Our assumption was that capacitation of spermatozoa would be stimulated by heparin as compared with heparin-free conditions (Parrish *et al.*, 1985, 1988; Kitiyanant *et al.*, 2002) and that the behaviour of spermatozoa would be different in each bull in relation to its individual qualities (Sumantri *et al.*, 1996).

Our results confirmed a considerable variability amongst the bulls tested in the response rate of spermatozoa to heparin during incubation. They also demonstrated that the kinetics of sperm response was related to the efficiency of IVF, because most of the bulls with a higher production of embryos had spermatozoa with a moderate response to heparin, whereas the bulls with a low embryo production had spermatozoa with either a fast or slow response. Takahashi *et al.* (1992) drew a similar conclusion from the positive correlation found between the normal reaction of non-separated human spermatozoa incubated in tubal fluid and the outcomes achieved in the IVF system. They regarded a good timing of both capacitation and the acrosome reaction as an important factor affecting the fertilization outcome.

When we compared the bulls with a high embryo production with those with a low production in terms of the intensity of capacitation changes of their spermatozoa, we found a positive correlation between the intensity of sperm response to heparin and the percentage of embryos produced. This is in agreement with the previous finding that the capacitation process is markedly influenced by the presence of specific surface antigens that facilitate binding of heparin to a spermatozoon (Bellin *et al.*, 1996). When spermatozoa have a higher affinity to heparin, they also have a better ability to undergo the acrosome reaction and to bind to the zona pellucida; this correlates with a higher fertilizing ability in both *in vivo* and *in vitro* conditions (Watanabe *et al.*, 1997). A possible relationship between heparin-induced capacitation in a population of separated spermatozoa and the efficiency of IVF or non-return rates of the bulls involved has also been described (Blottner *et al.*, 1990; Whitfield & Parkinson, 1992; Kawaguchi 1992). On the other hand, the evaluation of a separated spermatozoa population may not be related to bull fertility *in vivo* because the abnormal sperm were removed during separation on a Percoll gradient.

It can be concluded that the two criteria established in this study, i.e. the kinetics and intensity of acrosomal response, may improve the prognosis of bovine spermatozoa fertility as concerns *in vitro* fertilization. These findings will be useful not only in the prediction of bulls' fertilizing abilities in the *in vitro* system, but also for modification of fertilization conditions in high-performance sires employed in the production of embryos with high genetic merits.

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