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Relationship between acrosome integrity changes and *in vitro* fertilising ability of bovine spermatozoa

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ABSTRACT: This study was designed to investigate the characteristics of acrosomal changes during capacitation of bovine spermatozoa in relationship to *in vitro* fertility of individual bulls. Motile spermatozoa were separated from frozen-thawed semen by a swim-up procedure and capacitated in IVF-TALP medium with or without heparin. The spermatozoa were evaluated in terms of acrosomal changes at 0, 3, 4, 5, 6 and 8 h of capacitation. Proportions of acrosome-reacted spermatozoa at 5 h and 0 h of capacitation were used for calculation of the heparin response index. Variations in the heparin response index were found among individual bulls. Based on the mean response index value of all bulls, they fell into three categories: bulls with greater, intermediate and no response to heparin (GRH, IRH and NRH, respectively). Differences in the heparin response index between the bull categories were significant ($P < 0.05$). Higher D7 and D8 embryo development rates were found in the IRH vs. NRH bulls ($P < 0.05$). In conclusion, this study shows that the spermatozoa of bulls with a greater or intermediate response to heparin appear to be most suitable for *in vitro* embryo production compared with spermatozoa of bulls with no response to heparin.

Keywords: bull; spermatozoa; acrosome; embryos; IVF

A necessary prerequisite for efficient *in vitro* embryo production is a sufficient number of motile acrosome-intact sperm capable of fertilising the oocyte.

Different separation methods are used for the preparation of a population of motile spermatozoa from frozen-thawed bull semen, with swim-up being one of the most commonly used techniques for sperm isolation. This process enables the selection of a population of viable spermatozoa with intact acrosomes (Alomar et al. 2006).

At the same time, a high proportion of motile spermatozoa must be able to undergo the acrosomal reaction at the appropriate time to ensure efficient fertilisation of the oocyte. In order to induce the acrosome reaction, culture media for *in vitro* fertilisation are supplemented with different capacitating agents, preferentially with heparin, which has a capacitating effect on bovine spermatozoa (Van Soom and de Kruif 1996; Pereira et al. 2000; Mendes et al. 2003). Sumantri et al. (1996) demonstrated that under uniform capacitation conditions, the proportion of sperm capacitated at the same time is highly variable depending on the bull breed.

A number of studies have focused on the evaluation of acrosome status (Kitiyanant et al. 2002; Puente et al. 2011; Almadaly 2012), sperm capacitation and variations among different bull breeds (Demyda-Peyras et al. 2012). There are also numerous studies concerned with the high variability in embryo production under *in vitro* conditions, using spermatozoa from different bulls, but only a few studies have investigated a potential relationship between the *in vitro*-induced acrosome reaction and *in vivo* fertility of bulls (Whitfield and

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Parkinson 1995; Januskauskas et al. 2000; Birck et al. 2010; Kumar et al. 2014). Moreover, studies examining the relationship between the acrosome reaction of sperm from individual bulls and the fertility potential of these bulls under *in vitro* conditions are missing. For practical use of bulls for *in vitro* production of embryos, methods to assess a bull's fertilising ability under *in vitro* conditions are needed.

The purpose of the present study was to evaluate the acrosomal integrity of motile sperm, separated from the whole sperm population of individual bulls, and to characterise events occurring during capacitation in relationship to their *in vitro* fertilising ability.

MATERIAL AND METHODS

Tested bulls

Insemination doses from two-year-old bulls of the Czech Pied breed (n = 9) from one artificial insemination station, with non-return rates from 60.2% to 66.4%, were used in the experiments.

Separation of motile spermatozoa

After the thawing of an insemination dose, spermatozoa were separated using a modified swim-up method. Sperm were carefully deposited under 1 ml equilibrated (38.8 °C in 5% CO_2) modified Tyrode's medium for sperm isolation (SP-TALP) in the bottom of the tube and incubated at 38.8 °C in 5% CO_2 for one hour. After incubation, 120 µl of the bottom part of the medium were removed and the upper part with motile sperm was centrifuged twice at 200 G (1000 rev/min) for 10 min. The pellet was diluted with the fertilising IVF-TALP medium to give the final concentration of 25 × 10^6 sperm per ml of medium.

Treatment of spermatozoa and capacitation

Spermatozoa were capacitated either in a medium with 10 µg/ml heparin (H^+) or in a control medium without heparin (H^-) according to a protocol described previously (Reckova et al. 2008). The heparin-treated (H^+) and non-treated (H^-) spermatozoa were evaluated at 0, 3, 4, 5, 6 and 8 h of capacitation (hc).

Acrosome assessment

The spermatozoa samples were fixed in an ethanol-acetone solution for 10 min and then stored at 4 °C until examination. Just before the examination, sperm were stained with PSA-FITC. From each bull, at each interval, 2 × 400 spermatozoa from the heparin-treated (H^+) and 2 × 400 spermatozoa from the heparin non-treated (H^-) populations (two slides from each sample) were evaluated, using a phase-contrast fluorescence microscope with a 530 nm excitation filter. The percentages of acrosome-intact spermatozoa (AR^+ spermatozoa with intensely fluorescent acrosomal cap), acrosome-reacted spermatozoa ($AR^\pm$ spermatozoa with disrupted, patch-like fluorescent acrosomal cap indicating vesiculation and breakdown of acrosomal membrane) and acrosome-denuded spermatozoa (AR^- spermatozoa displaying a fluorescent band at the equatorial segment or a loss of the acrosomal cap) were recorded. Only $AR^\pm$ spermatozoa able to penetrate and fertilise oocytes were included in this study.

The fertilising ability of spermatozoa

Oocyte maturation and fertilisation. Oocyte maturation and fertilisation were performed as described previously by Machatkova et al. (2006). Briefly, oocytes were matured in 500 µl of TCM 199 medium with the addition of 0.2mM sodium pyruvate, 50 IU/ml penicillin, 50 µg/ml streptomycin, 5% ECS (oestrus cow serum) and gonadotropins (PG 600 15 UI/ml; Intervet, Boxmeer, The Netherlands) in culture dishes for 24 h. They were inseminated with bull spermatozoa isolated using the swim-up method, using SP-TALP medium. Fertilisation was carried out in IVF-TALP medium containing 1 × 10^6/ml spermatozoa and 10 µg/ml heparin. Cumulus cells were removed from oocytes by vortexing either at 6 h or 18 h after insemination (hi).

Penetration and fertilisation assessment. The presumptive zygotes were fixed in 2.5% glutaraldehyde and stained with bisbenzimide Hoechst 33258. The oocytes were examined under a fluorescence microscope at the wave length of 450 nm. An oocyte was considered to be penetrated when the female pronucleus formation and decondensing sperm head were present at 6 hi, and to be fertilized when the female and male pronuclei, syngamy or the first mitotic division were present at 18 hi. On average, 166

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and 160 oocytes were inseminated by spermatozoa of each bull and evaluated in terms of penetration and fertilisation, respectively, in three replicates.

Embryo development assessment. Embryos from each bull were prepared using a standard protocol described previously by Machatkova et al. (2008). Cumulus cells were removed from presumptive zygotes at 24 hi by vortexing. They were transferred to a Buffalo rat liver cell line monolayer and cultured in B2 medium with 10% ECS for eight days at 38.8 °C in a humidified atmosphere of 5% CO_2 in air. Embryo development was expressed as percentages of embryos that reached the morula or early blastocyst stages on Day 7 (D7) and the advanced or expanded blastocyst stages on Day 8 (D8) from all presumptive zygotes. On average, 381 oocytes were inseminated by spermatozoa of each bull and evaluated in terms of embryo development in three replicates.

Statistical analysis

The data were analysed by the Student's *t*-test using Statistica 8.0 statistical software (StatSoft).

RESULTS

Categorisation of tested bulls

The percentage of $AR^{\pm}$ spermatozoa capacitated in the presence or absence of heparin differed among the tested bulls. To characterise the level of spermatozoa reaction to heparin in individual bulls, a response index was calculated for each bull using the following formula: ratio of $AR^{\pm}$ spermatozoa from the total number of H^+ spermatozoa capacitated for 5 h to proportion of $AR^{\pm}$ spermatozoa from the total number of H^- spermatozoa at 0 hc (before capacitation). The values of the heparin response index ranged from 4.48 to 1.05 in the tested bulls. On the basis of mean ± S.E.M. (2.11 ± 0.76) of the response index values in all bulls, three categories were made: bulls with greater (2.11 plus 0.76; index > 2.87; two bulls), intermediate (index from 2.87 to 1.35; five bulls) and no response (2.11 minus 0.76, index < 1.35; two bulls) to heparin. Differences in the values of the mean response index were significant among the bull categories ($P < 0.05$; Table 1).

Acrosome changes in spermatozoa of bull categories

The kinetics of acrosome changes in spermatozoa was specific to GRH, IRH and NRH bulls.

Bulls with a greater response to heparin. The mean $AR^{\pm}$ spermatozoa rate increased from 20.4% to 81.9% during 8 h-capacitation with heparin. In H^+ spermatozoa, the highest increase in $AR^{\pm}$ spermatozoa rate was found from 0 hc to 3 hc and from 3 hc to 4 hc. On the other hand, in H^- spermatozoa, the highest increase in $AR^{\pm}$ spermatozoa rate was observed from 4 hc to 5 hc (Figure 1A).

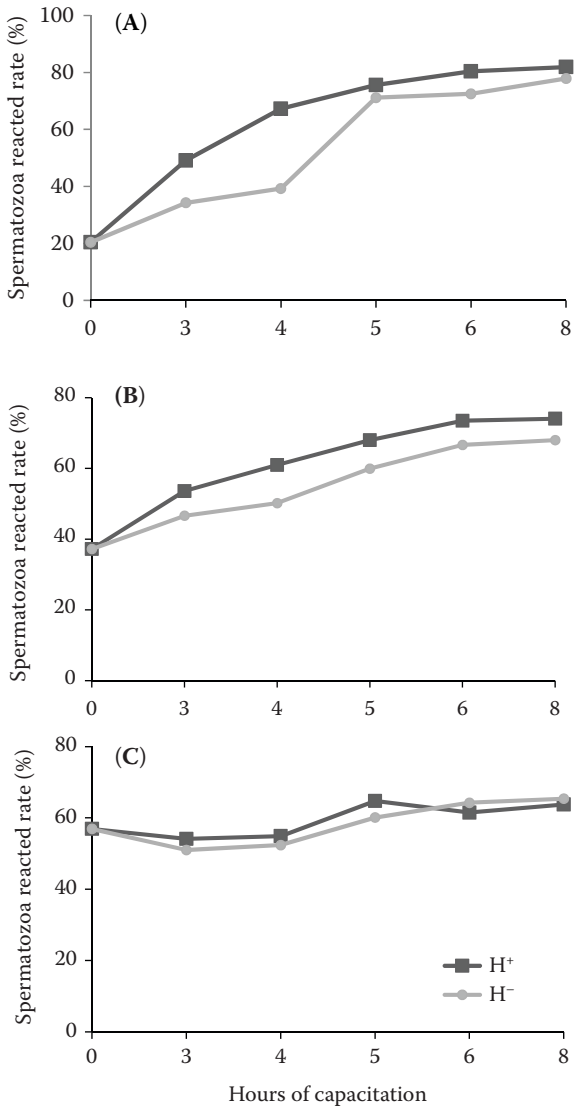

Figure 1. Kinetics of acrosome changes in bulls with greater (**A**), intermediate (**B**) and no response to heparin (**C**): Spermatozoa were capacitated with (H^+) or without (H^-) heparin and assessed at 0, 3, 4, 5, 6 and 8 h

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Table 1. Response of spermatozoa of tested bulls to heparin

		Spermatozoa response to heparin								
		greater (GRH)		intermediate (IRH)					none (NRH)	
		bulls								
		1	2	3	4	5	6	7	8	9
Spermatozoa treatment	hours	spermatozoa reacted rate (%)								
H^-	0	17.3	23.5	41.0	27.0	44.8	45.3	27.8	71.0	42.8
H^+	5	77.3	74.0	80.0	52.5	83.5	79.5	44.5	84.5	45
Heparin response index*		4.48	3.15	1.95	1.94	1.87	1.76	1.60	1.19	1.05
Mean response index ± S.E.M. of all bulls		2.11 ± 0.76								
Mean response index of bull categories		3.82^a				1.82^b			1.12^c	

H^+ spermatozoa capacitated for 5 h to proportion of $AR^{\pm}$ spermatozoa from the total number of H^- spermatozoa at 0 hc (before capacitation)

Bulls with greater heparin response > 2.87 (mean response index of all bulls plus S.E.M. value of all bulls)

Bulls with intermediate heparin response < 2.87 > 1.35 (mean response index of all bulls plus and minus S.E.M. value of all bulls)

Bulls with no heparin response < 1.35 (mean response index of all bulls minus S.E.M. value of all bulls)

*heparin response index = ratio of proportion of $AR^{\pm}$ spermatozoa from the total number of

$^{a,b,c}P < 0.05$

Bulls with an intermediate response to heparin. The mean $AR^{\pm}$ spermatozoa rate increased from 37.2 % to 74.1 % during 8 h-capacitation with heparin. In H^+ spermatozoa, the highest increase in $AR^{\pm}$ spermatozoa rate was found from 0 hc to 3 hc. In H^- spermatozoa, the highest increase in $AR^{\pm}$ spermatozoa rate was observed both from 0 hc to 3 hc and from 4 hc to 5 hc (Figure 1B).

Bulls with no response to heparin. The mean $AR^{\pm}$ spermatozoa rate increased from 56.9 % to 63.8 % during 8 h-capacitation with heparin. In H^+ spermatozoa, the highest increase in $AR^{\pm}$ spermatozoa rate was found from 4 hc to 5 hc. In H^- spermatozoa, the highest increase in $AR^{\pm}$ spermatozoa rate was observed from 4 hc to 5 hc and from 5 hc to 6 hc (Figure 1C).

Fertilising ability of spermatozoa in bull categories

Oocyte penetration. Differences in the penetration rate of oocytes at 6 hi were found among the bull categories. A significantly higher penetration rate was reached in IRH bulls in comparison with NRH bulls and a higher penetration rate was found in IRH bulls compared with GRH bulls (Figure 2).

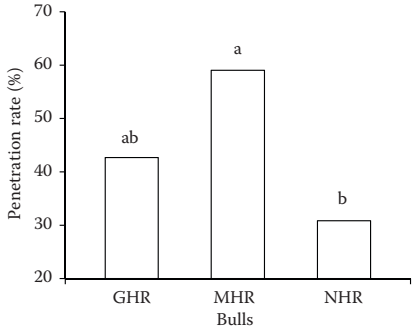

Figure 2. Oocyte penetration in bulls with different responses to heparin: A total of 335, 832 and 333 oocytes were inseminated by spermatozoa of GRH, IRH and NRH bulls, respectively. Mean percentage of penetrated oocytes for each bull category was assessed at 6 h after insemination. Values with different superscripts differ significantly (a–b, $P < 0.05$)

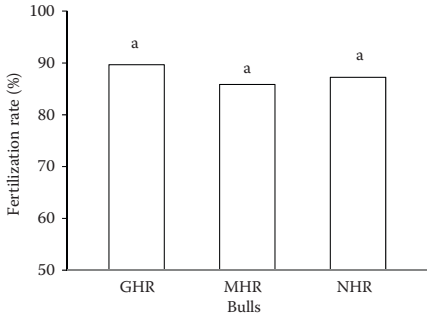

Figure 3. Oocyte fertilization in bulls with different responses to heparin: A total of 318, 805 and 321 oocytes were inseminated by spermatozoa of GRH, IRH and NRH bulls, respectively. Mean percentage of fertilized oocytes for each bull category was assessed at 18 h after insemination. Values with the same superscripts are not significantly different (a–b, $P < 0.05$)

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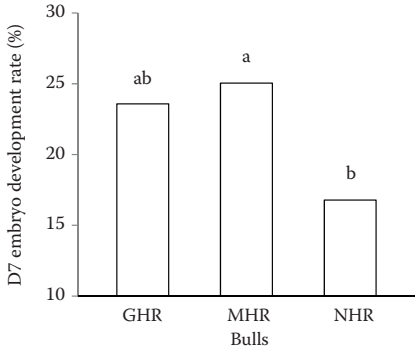

Figure 4. D7 embryo development in bulls with different responses to heparin: Presumptive zygotes were cultured in B2 medium on a cell monolayer. A total of 819, 1841 and 769 presumptive zygotes derived from GRH, IRH and NRH bulls, respectively, were assessed at Day 7. Mean percentages of D7 embryos that were between the morula and early blastocyst stages were calculated from all presumptive zygotes. Values with different superscripts differ significantly (a–b, $P < 0.05$)

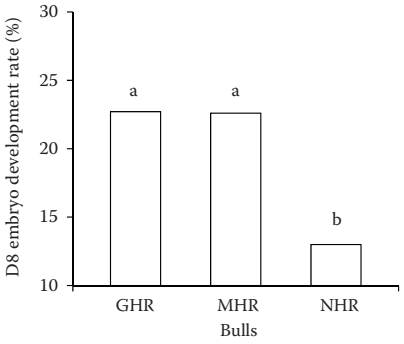

Figure 5. D8 embryo development in bulls with different responses to heparin: Presumptive zygotes were cultured in B2 medium on a cell monolayer. The total of 819, 1841 and 769 presumptive zygotes derived from GRH, IRH and NRH bulls respectively, were assessed at Day 8. Mean percentages of D8 embryos that were between the advanced and expanded blastocyst stages were calculated from all presumptive zygotes. Values with different superscripts differ significantly ((a–b, $P < 0.05$)

Oocyte fertilisation. In contrast to penetration, the fertilisation rate of oocytes at 18 hi did not differ among the bull categories. No significant differences in the fertilisation rate were found among GRH, IRH and NRH bulls (Figure 3).

D7 embryo development. Differences in embryo development rate at Day 7 were found among the bull categories. A significantly higher D7 embryo rate was reached in IRH bulls compared with NRH bulls and a higher D7 embryo rate was found in IRH bulls in comparison with GRH bulls (Figure 4).

D8 embryo development. Similarly to embryo development at Day 7, differences in embryo development at Day 8 were observed among the bull categories. Significantly higher D8 embryo rates were obtained in GRH and IRH bulls than in NRH bulls (Figure 5).

DISCUSSION

Analyses of semen reflect the sperm-producing ability of bulls and their spermatogenesis status. However, this information cannot be used as a predictor of fertilising ability of their spermatozoa (Mukhopadhyay et al. 2008). It is necessary to find methods which would improve the prediction of field fertility and *in vitro* embryo production in high value bulls because a positive correlation between *in vivo* and *in vitro* fertilising capacity of spermatozoa was found by Ward et al. (2003). However, even if high-field fertility bulls are used for embryo production, their spermatozoa may not efficiently fertilise oocytes under *in vitro* conditions (Ward et al. 2001; Ward et al. 2002). Therefore, it is necessary to test the fertilising ability of spermatozoa before embryo production. In the present study, we focused our investigations on the character of acrosome changes in bovine spermatozoa during their capacitation related to their *in vitro* fertiliding ability.

In order to induce the acrosome reaction, culture media for *in vitro* oocyte fertilisation are supplemented with different agents, in cattle preferentially with heparin, which stimulates capacitation of bovine spermatozoa (Parrish et al. 1988; Pereira et al. 2000; Mendes et al. 2003; Parrish 2014). Authors who have described the behaviour of spermatozoa during capacitation and acrosome reaction onset reported that the first changes in acrosome morphology appear after 2 h, independently of capacitation conditions (Bilaspuri and Babbar 2007). We observed initial morphological changes in acrosomes earlier, during the motile spermatozoa separation process. We assume that this difference is due to the use of a modified swim-up procedure in our study instead of separation on a Percoll gradient. In our protocol spermatozoa are capacitated during the process of separation for 1h at 38.8 °C.

Bilaspuri and Babbar (2007) reported that sperm capacitation reached its maximum after 5 h of incu-

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bation. On the other hand, Giritharan et al. (2005) described a maximum increase in the acrosome-reacted spermatozoa rate already after 4 h. In our study, sperm capacitation reached its maximum after 5 h, but only in bulls with no response to heparin, whereas in bulls with greater and intermediate response to heparin, the percentage of acrosome-reacted sperm further increased between 5 and 6 h and reached its maximum after 6 h of capacitation, similarly as reported in Molnarova et al. (2006).

Dhanju et al. (2006) demonstrated that in individual bulls the proportion of acrosome-reacted spermatozoa after 6 h ranged from 62% to 87%. In our tested bulls, the rate of acrosome-reacted spermatozoa after 5 hours was in the range of 44.5% to 84.5%. In bull categories, the increase of acrosome-reacted spermatozoa rate after 5 h was 55.3%, 30.8% and 7.9% for those with greater, intermediate and no response to heparin, respectively.

A relationship between the bull breed and the kinetics of acrosome changes in bovine spermatozoa was described by Sumantri et al. (1996) and Demyda-Peyras et al. (2012). In addition to that, our study confirms that individual bulls within a breed exhibit characteristic kinetics of acrosome changes. In our experiments, Czech Pied breed bulls of the same age which were kept at one insemination station were used. The motile spermatozoa of the bulls were separated and capacitated by heparin under standard conditions. Based on our findings, we assume that the level of spermatozoa response to heparin is different, not only among breeds but also among individual sires, and is most likely genetically determined.

A positive correlation (r = 0.26) between the percentage of acrosome-reacted spermatozoa after 4 h of capacitation and embryo cleavage rate was found by Giritharan et al. (2005). In our study, the bulls with greater and intermediate response to heparin showed the best results in percentages of penetrated and fertilised oocytes and D7 and D8 embryo rates. However, the present study failed to find a correlation between these parameters, most likely because of the rather low numbers of bulls in each group.

CONCLUSION

In this study, differences were observed in the acrosomal changes during *in vitro* capacitation of bovine spermatozoa. On the basis of the acrosome changes of spermatozoa in individual sires, three bull categories were characterised as those with greater, intermediate and no response to heparin. The differences in the mean response index were significant among the three groups. A relationship between the mean response index and *in vitro* embryo production was found for the different bull categories. It can be concluded that the heparin response index can be used for *in vitro* fertility prediction in bulls and that those with a greater or intermediate response appear to be more suitable for *in vitro* embryo production than bulls with no response to heparin.

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