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MONITORING OF CHROMATIN INTEGRITY CHANGES IN THE POPULATION OF MOTILE BOVINE SPERM CAPACITATED IN VITRO

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Abstract

REČKOVÁ, Z., MACHATKOVÁ, M., RYBÁŘ, R., MÁCHAL, L.: *Monitoring of chromatin integrity changes in the population of motile bovine sperm capacitated in vitro*. Acta univ. agric. et silvic. Mendel. Brun., 2007, LV, No. 2, pp. 65–70

The objective of our study was to standardize a method for chromatin integrity assessment in a separated population of bovine sperm and monitor the changes occurring during sperm capacitation stimulated with heparin. Frozen sperm of 11 young bulls of the Czech pied breed with a defined fertility in both in vitro system (from 12.9% to 25.8% embryos) and in insemination (from 60.2% to 66.4% pregnancy) was used in our experiments.

Bovine spermatozoa were isolated by Percoll gradient centrifugation from frozen-thawed semen using Tyrode's medium (SP-Talp) and resuspended in a fertilization medium (IVF-Talp). The spermatozoa were incubated at laboratory temperature at a concentration 25×10^6 per cm^3 for 6 h either in IVF-Talp medium with heparin (H^+) or without heparin (H^-). Samples were obtained immediately after sperm thawing (PS), following motile spermatozoa separation (P0), and their three (P3) and six hour (P6) incubation. The samples were examined by flow cytometry. Two measurements were carried out in each of the samples so that a total of 10 thousand spermatozoa were analysed. Proportion of spermatozoa with undetectable DNA fragmentation index (non-DFI sperm) i.e. spermatozoa with undamaged chromatin structure were determined using SCSA-soft software.

Chromatin integrity changes of spermatozoa before and after separation and capacitation differed markedly in individual bulls. Separation of motile spermatozoa increased significantly the mean proportion of non-DFI sperm in tested bulls (from 94.2 to 96.4%, $P \leq 0.01$). While in most of the bulls the mean proportion of non-DFI sperm remained nearly constant during incubation (H^-) (mean, P0 – 96.4%, P3 – 95.6%, P6 – 95.5%), it gradually decreased during capacitation (H^+) (mean, P0 – 96.4%, P3 – 95.2%, P6 – 94.2%). The differences were statistically significant (P0 vs. $P3H^+$, P0 vs. $P6H^+$, $P \leq 0.05$). Significant difference ($P \leq 0.05$) in the mean proportion on non-DFI sperm was also found between capacitated ($P6H^+$) and incubated ($P6H^-$) spermatozoa.

The results of our study suggest the following outcomes. Separation of motile spermatozoa by Percoll gradient increased the proportion of spermatozoa with undamaged chromatin structure. Sperm incubation induced gentle damage of chromatin integrity which was potentiated by heparin in capacitated spermatozoa. The proportion of spermatozoa with undamaged chromatin structure remained relatively high in the course of capacitation, therefore we can assume it to be high enough for a potential oocyte fertilization.

bulls, spermatozoa, chromatin integrity, flow cytometry

A sufficient number of viable and motile spermatozoa with intact acrosome and undamaged chromatin structure are needed to be able to penetrate and fertilize oocytes in in vitro conditions.

Different separation techniques can be used to obtain populations of viable and motile spermatozoa from frozen-thawed bull sperm. The most commonly used separation method for frozen-thawed sperm separation is the Percoll gradient centrifugation (Galli et al., 2003). Major advantage of motile sperm separation by Percoll gradient compared to other methods is a relatively high yield of spermatozoa (Parrish et al., 1995). Sperm separation also increases the proportion of viable spermatozoa with intact acrosome (Alomar et al., 2006), however, their chromatin structure can be disrupted by the separation process. Sperm of some bulls are highly sensitive to such a treatment, therefore more careful methods are needed for separation, as are the swim-up method or centrifugation on Sephadex column. Further, it was confirmed that increased levels of reactive oxygen groups can occur during centrifugation with a subsequent risk of sperm chromatin damage (Aitken and Clarkson, 1988; Zalata et al., 1995).

Successful oocyte fertilization necessitates a certain portion of spermatozoa to undergo an acrosomal reaction in a corresponding time. Acrosome reaction of spermatozoa can be stimulated by supplementation of the fertilization media with different capacitation agents, most frequently heparin (Pereira et al., 2000; Mendes et al., 2003). Both separation and capacitation of spermatozoa can induce specific damage of the chromatin structure (Silva and Gadella, 2006).

Boe-Hansen et al. (2003) suppose that lower fertility of some bulls under in vitro conditions could be due to chromatin integrity changes during preparation of spermatozoa for oocyte fertilization or at their capacitation. Fatehi et al (2006) found that bovine sperm with damaged chromatin are able of fertilization, however, their embryos show impaired development which is in correlation with the level of sperm chromatin damage.

Assessment of sperm DNA integrity and other qualitative and quantitative characteristics as are viability, acrosome intactness and mitochondrial activity can be done by flow cytometry (Graham et al., 1990; Watson et al., 2002; Nagy et al., 2004; Gillan et al., 2005). Sperm Chromatin Structure Assay described by Evenson et al. (2002) is a suitable method for the assessment of bovine sperm chromatin integrity.

Monitoring of chromatin changes in the population of spermatozoa capacitated under standard conditions could result in more precise prediction of the fertilization ability in individual bulls. Therefore it is of importance to find out whether chromatin integrity changes occur during separation and capacitation of spermatozoa and to elucidate impact of those changes on efficiency of in vitro fertilization in individual bulls.

The objective of our study was to standardize of the method of chromatin integrity assessment in the population of motile spermatozoa, and monitoring of changes occurring during their separation and capacitation.

MATERIALS AND METHODS

Tested bulls

Frozen sperm from AI bulls (n = 11) of Czech pied breed with a defined in vitro (from 12.9 % to 25.8 % of embryos) and in vivo fertility (from 60.2 % to 66.4 % pregnancy) was used in our experiment.

Separation of motile sperms and their incubation

After thawing of the insemination doses in water bath at 37 °C for 60 s, bull sperm was layered on Percoll gradient. After a 30 min centrifugation (700G) supernatant was removed and the pellet containing motile spermatozoa was washed twice in an isolation medium SP-Talp (200G) for 10 min. The pellet was resuspended in IVF-Talp medium, and after determination of sperm concentration it was diluted with the fertilization medium to the concentration of 25×10^6 spermatozoa per cm^3. The spermatozoa were incubated at laboratory temperature in IVF-Talp medium with heparin and without heparin (10 µg/cm^3) for 6 h.

Sperm sample preparation

Immediately after thawing (PS), separation (P0), three-hour (P3) incubation, and six-hour (P6) incubation, the samples were collected from both untreated (H^-) and heparin treated (H^+) sperm. The obtained samples were diluted with TNE buffer (0.15M NaCl, 0.01M Tris HCl, 1 mM disodium EDTA, pH 7.4) to the concentration of 2×10^6 spermatozoa in cm^3, frozen and stored at –80°C until further use.

Sperm sample examination

Prior to examination, sperm samples were thawed in a water bath at 37 °C and placed on ice at the temperature ±4 °C. The method described by Evenson et al. (2002) was used for sample examination. Two measurements were accomplished in each of the samples, with a total of 10 thousand spermatozoa analysed.

Statistical analysis

The proportion of spermatozoa with undetectable DNA fragmentation index (non-DFI sperm) i.e. spermatozoa with undamaged chromatin structure was

obtained by SCSA-soft software. The obtained values were analysed statistically by Wilcoxon test in the programme Statistica.

RESULTS

The separation of motile spermatozoa increased significantly ($P < 0.01$) mean proportion (mean ± S.M.E.) of non-DFI sperm (from 94.2% ± 4.17 to 96.4% ± 1.46) in the tested bulls. The proportion of non-DFI sperm was lower in semen after thawing than the proportion of non-DFI sperm after separation in individual bulls. Before separation, the higher variability in proportions of non-DFI sperm among the bulls was found than that in proportions of non-DFI sperm after separation (Table I).

I: *Effect of separation on mean proportion of non-DFI sperm*

	PS (%)	P0 (%)
Minimum	79.4	92.5
Maximum	99.3	99.1

The lesser decrease of the mean proportion (mean ± S.M.E.) of non-DFI sperm occurred during incubation of H^- sperm, however, the changes were not significant (P0 – 96.4 ± 1.46, P3 – 95.6 ± 2.54, P6 – 95.5 ± 2.61). The bulls remained relatively a homogeneous group with prolonged incubation time (Table II).

II: *Effect of incubation on mean proportion of non-DFI sperm in H^- sperm*

	P0 (%)	P3 (%)	P6 (%)
Minimum	92.5	88.6	85.6
Maximum	99.1	99.1	99.2

The decrease of the non-DFI sperm proportion (mean ± S.M.E.) was higher in capacitated H^+ sperm (P0 – 96.4 ± 1.46, P3 – 95.2 ± 2.38, P6 – 94.2 ± 4.06) compared to incubated H^- sperm. Significant differences were found between P0 and P3, and between P0 and P6 sperm ($P \leq 0.05$) in capacitated H^+ sperm. During sperm capacitation, the variability between bulls has increased like as the variability before separation (Table III). The significant difference ($P6H^-$ vs. $P6H^+$, $P \leq 0.05$) was found after a 6-hour incubation of H^- sperm and capacitation of H^+ sperm.

III: *Effect of capacitation on mean proportion of non-DFI sperm in H^+ sperm*

	P0 (%)	P3 (%)	P6 (%)
Minimum	92.5	86.6	81.4
Maximum	99.1	99.0	99.1

DISCUSSION

The fertility of bulls used in *in vitro* system varies markedly in individual sires (Galli et al., 2003; Katska-Ksiazkiewicz et al., 2005). Fertilization rate of oocytes does not correspond in some of the bulls with good results in insemination. Data found in the literature have not sufficiently clarified the differences in fertilization efficiency in individual bulls; therefore we decided to monitor the changes of chromatin integrity of sperm that were prepared for in vitro fertilization.

The effect of sperm chromatin integrity on their fertilizing ability has so far been studied predominantly in human medicine when seeking after the causes of infertility in men. Several authors that tried to find answer to the question of chromatin integrity effect on fertilizing ability of human spermatozoa came to the conclusion that a negative correlation between damaged chromatin integrity and fertility exists (Larson et al., 2000; Zini et al., 2001; Virro et al., 2004; Lewis and Aitken, 2005; Evenson and Wixon, 2006).

Some of the authors tried to find relationship between chromatin integrity of bovine sperm and fertility of bulls, however their results are ambiguous. Madrid-Bury et al. (2005) showed high correlation between chromatin stability of frozen bovine sperm and a non-return rate. In contrast, Hallap et al. (2005) did not confirm correlation between chromatin stability of sperm and fertility of bulls. Tendencies to find relationship between chromatin integrity of bovine sperm, their morphology and fertilizing ability also appeared but have not been confirmed. On the other hand, Katska-Ksiazkiewicz et al. (2005) found that bovine sperm with damaged chromatin can be morphologically normal and penetrate the oocytes.

The effect of different separation methods on sperm chromatin integrity has been investigated by many authors. Chohan et al. (2004) showed that the use of a gradient centrifugation has a positive effect on chromatin quality of 90% of human frozen sperm. Hallap et al. (2005) demonstrated that the swim-up method increased significantly the percentage of bovine spermatozoa with a stable chromatin structure compared to thawed sperm. Under our experimental conditions, separation of motile bovine sperm using Percoll gradient also increased significantly the proportion of spermatozoa with undamaged chromatin structure. The positive effect of separation on the proportion of spermatozoa with undamaged chromatin structure differed in individual bulls; however separation decreased variability among the bulls. Increasing level of sperm DNA damage in boar's semen incubated for 72 h has been demonstrated by Boe-Hansen et al. (2005). Negative effect of exogenous hydrogen peroxide on sperm chromatin in the incubated sperm of bulls has

been recorded by Krzyzosiak et al. (2000). However, no data has been found in the literature so far, on chromatin integrity changes in separated and capacitated bovine spermatozoa. In bovine spermatozoa separated in our laboratory, incubation induced a certain level of chromatin integrity damage which was potentiated by heparin in capacitated spermatozoa.

In conclusion we have demonstrated that sperm separation using Percoll gradient has a positive effect on the proportion of spermatozoa with undamaged chromatin structure. Incubation of spermatozoa induced a gentle damage of sperm chromatin integrity which was potentiated with heparin. During sperm preparation, chromatin structure differed significantly among bulls, but after a 6-hour sperm capacitation the rate of spermatozoa with undamaged chromatin structure was relatively high for a potential oocyte fertilization.

SOUHRN

Monitorování změn integrity chromatinu v populaci motilních bovinních spermií kapacitovaných v podmínkách in vitro

Cílem práce bylo standardizovat metodu hodnocení integrity chromatinu u separované populace bovinních spermií a monitorovat změny, ke kterým dochází v průběhu jejich kapacitace stimulované heparinem.

V experimentu bylo použito zmrazené sperma 11 mladých býků českého strakatého plemene s definovanou plodností v in vitro systému (od 12,9 % do 25,8 % embryí) i v inseminaci (od 60,2 % do 66,4 % březosti). Bovinní sperma bylo separováno pomocí Tyrodova média (SP-Talp) na gradientu Percollu a resuspendováno ve fertilizačním médiu (IVF-Talp). Spermie byly inkubovány při laboratorní teplotě v koncentraci 25×10^6 v cm^3 po dobu šesti hodin jak v médiu doplněném o heparin (H^+), tak i bez heparinu (H^-). Vzorky byly odebrány ihned po rozmrazení spermatu (PS), po separaci spermií (P0), jejich, tříhodinové (P3) a šestihodinové (P6) inkubaci. Získané vzorky byly vyšetřeny pomocí průtokové cytometrie, metodou popsanou Evensonem et al., (2002). U každého vzorku bylo celkem hodnoceno 10 tisíc spermií ve dvou měřeních. Podíl spermií s nedetekovatelným DNA fragmentačním indexem (non-DFI sperm), tj. spermií s nepoškozenou strukturou chromatinu byl stanoven pomocí SCSA-soft softwaru. Získané hodnoty byly statisticky zpracovány v programu STATISTICA pomocí Wilcoxnova testu.

Změny integrity chromatinu spermií v průběhu jejich separace a kapacitace byly u sledovaných býků značně individuální. Separace motilních spermií signifikantně zvýšila průměrný podíl non-DFI spermií u všech sledovaných býků, průměrně od 94,2 % do 96,4 % ($P \leq 0,01$). Zatímco u většiny býků zůstal průměrný podíl non-DFI spermií během jejich inkubace (H^-) téměř konstantní (průměr, P0 – 96,4 %, P3 – 95,6 %, P6 – 95,5 %), tak v průběhu kapacitace (H^+) se průměrný podíl non-DFI spermií postupně snižoval (průměr, P0 – 96,4 %, P3 – 95,2 %, P6 – 94,2 %). Zjištěné rozdíly byly statisticky průkazné (P0 vs. P3H^+a P0 vs. P6H^+, $P \leq 0,05$). Statisticky významný rozdíl ($P \leq 0,05$) v průměrném podílu non-DFI spermií byl také nalezen mezi kapacitovanými (P6H^+) a inkubovanými spermiemi (P6H^-).

Z výsledků naší studie je možné vyvodit následující závěry. Separace motilních spermií na gradientu Percollu zvýšila podíl spermií s neporušenou strukturou chromatinu. Inkubace spermií vyvolala mírné poškození integrity chromatinu spermií, které bylo u kapacitovaných spermií potencováno účinkem heparinu. V průběhu procesu kapacitace zůstal podíl spermií s neporušenou strukturou chromatinu relativně vysoký a lze tedy předpokládat, že byl dostatečný pro potenciální fertilizaci oocytů.

býci, spermie, integrita chromatinu, průtoková cytometrie

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