Molecular morphology and function of bull spermatozoa linked to histones and associated with fertility

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Abstract

Sub-par fertility in bulls is influenced by alterations in sperm chromatin, and it might not be solved with increased sperm concentration in artificial insemination. Appropriate histone retention during sperm chromatin condensation plays critical roles in male fertility. The objective of this study was to determine failures of sperm chromatin condensation associated with abnormal persistence or accessibility of histones by aniline blue (ANBL) test, expression levels, and cellular localizations of one variant and two core histones (H3.3, H2B, and H4 respectively) in the spermatozoa of low-fertility (LF) vs high-fertility (HF) bulls. The expression levels and cellular localizations of histones in spermatozoa were studied using immunoblotting, immunocytochemistry, and staining methods. The bioinformatics focused on the sequence identity and evolutionary distance of these proteins among three mammalian species: bovine, mouse, and human. We demonstrated that ANBL staining was different within the LF (1.73 (0.55, 0.19)) and HF (0.67 (0.17, 0.06)) groups ($P < 0.0001$), which was also negatively correlated with *in vivo* bull fertility ($r = -0.90$, $P < 0.0001$). Although these histones were consistently detectable and specifically localized in bull sperm cells, they were not different between the two groups. Except H2B variants, H3.3 and H4 showed 100% identity and were evolutionarily conserved in bulls, mice and humans. The H2B variants were more conserved between bulls and humans, than in mice. In conclusion, we showed that H2B, H3.3, and H4 were detectable in bull spermatozoa and that sperm chromatin condensation status, changed by histone retention, is related to bull fertility.

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Introduction

Fertility is one of the most important factors influencing mammalian reproduction and development. Bull fertility is defined as the ability of the sperm to fertilize and activate the egg, to sustain embryonic development, and is usually evaluated by determining sperm characteristics such as motility and morphology (Correa *et al.* 1997). Although there are several approaches to evaluate capacitation status and membrane integrity using microscopy and/or flow cytometry in sperm cells, these techniques may still not be sufficient to predict overall male fertility (Januskauskas *et al.* 1999, Puglisi *et al.* 2012). One of the reasons for the failure of the aforementioned studies is that they primarily focused on the traits that are necessary for spermatozoa to reach and fertilize the oocyte, while ignoring other traits that are essential for egg activation and to support early embryonic development (Silva & Gadella 2006). In addition to these traits, sperm chromatin integrity is also essential for proper

sperm physiology and early embryonic development. For example, studies have revealed that sperm chromatin damage reduces fertility in bulls (García-Macías *et al.* 2007) and in human males (Agarwal & Said 2003). More precisely, abnormal levels of specific nucleoproteins such as protamines (Simon *et al.* 2011) and H4 acetylation (Sonnack *et al.* 2002) in spermatozoa have been found to be associated with human subfertility. The molecular alterations leading to sub-par fertility have also been demonstrated to be important in cattle industry because they are not compensated for by increasing the amounts of spermatozoa used in artificial insemination (AI; Blaschek *et al.* 2011).

The chromatin in mature spermatozoa is composed of DNA wrapped around the nucleoproteins, mainly protamines, but also some histones (D'Occhio *et al.* 2007). Following spermatogenesis, histone–protamine transition takes place during spermiogenesis where histones from early spermatids are subsequently and

partially substituted by transition proteins and, finally, they are almost completely replaced by protamines at later stages (Hales *et al.* 2011). Although the histone-associated sperm DNA is <15% in humans (Gatewood *et al.* 1990), it is suggested that this histone retention provides less condensed DNA regions where paternal genes can be more accessible to transcription factors right after fertilization (Rajender *et al.* 2011). The four types of core (canonical) histones involved in DNA packaging are H2A, H2B, H3, and H4 nucleoproteins, and they form a tightly coiled packed structure along with protamines, called the toroidal model (Dadoue 1995). Although proper histone retention is believed to be vital for the genes that will be activated before and/or after fertilization, an excessive ratio of histone retention occasionally indicates sperm chromatin immaturity and this ultimately causes sperm dysfunctions. In general, the evaluation of excessive histone retention, i.e. sperm chromatin immaturity, can be carried out using several cost-effective methods such as the chromomycin A3, toluidine blue, and aniline blue (ANBL) staining methods (Agarwal & Said 2003). Since histones are rich in lysine, the ANBL dye attaches to nucleohistones and generates a blue color in the sperm head (Franken *et al.* 1999). Post-translational modifications (PTMs) such as methylation, acetylation, and phosphorylation of histones can epigenetically influence the accessibility of the male genome to maternal transcription factors during embryonic development (Miller *et al.* 2010).

Methylated histones are mainly found in heterochromatin regions where the genes are occasionally silenced (Fischle *et al.* 2003), whereas acetylation of histones is strongly associated with the euchromatin architecture, reducing histone–DNA links and facilitating both replacement of protamines and activation of transcription (Struhl 1998) during spermatogenesis (Dadoue 2003). The phosphorylation of serine residues in histones is also commonly associated with gene activation (Prigent & Dimitrov 2003). Interestingly, H2B variants have been shown to be highly expressed during DNA damage and infertility in humans (Zini *et al.* 2008). In addition to H2B, H3 and its replacement variant H3.3 have been demonstrated to be associated with gene activation (Mito *et al.* 2005). Specific PTMs in H3 are localized in the regions of sperm genome that are active during spermatogenesis and early embryonic development (Brykczynska *et al.* 2010, Steilmann *et al.* 2011). Lastly, H4 is associated with the success of chromatin remodeling during spermatogenesis (Kleiman *et al.* 2008) and male inheritance of chromosomal architecture during zygote development (van der Heijden *et al.* 2006).

However, the extent of cellular and molecular associations between expression levels and PTMs of histones that influence bull fertility is not known. Furthermore, the molecular mechanisms of those events that regulate male fertility remain a mystery. Therefore, there is a need for research to elucidate the interconnectedness between

histones in spermatozoa and male fertility at the molecular and cellular levels. The objective of this study was to determine sperm chromatin status, expression levels, and cellular localizations of histones in the sperm cells of high-fertility (HF) vs low-fertility (LF) bulls by taking advantage of bioinformatics. Specifically, we studied the expression levels and cellular localizations of H3.3, H2B, and H4 in the sperm cells of bulls with varying fertility.

Materials and methods

Determination of bull fertility

The prediction of bull fertility scores was done according to the Alta Advantage Program (Alta Genetics, Inc., Watertown, WI, USA), which is periodically updated by information from partnering herds (Peddinti *et al.* 2008). All factors influencing fertility performance of the sires such as the environmental and herd management were adjusted using reliable threshold models by Zwald *et al.* (2004a, 2004b). Fertility estimation with the related parameters was done using the Probit F90 Software developed by Chang *et al.* (2004) (Department of Dairy Science, University of Wisconsin, Madison, WI, USA). The database calculates the fertility of each sire based on the average conception of more than 300 breeding outcomes with the percent deviation of the conception rates. According to this calculation, the s.d. of the population was used as the criterion to classify the fertility of bulls, where HF and LF bulls had a 2 s.d. difference from the mean. Therefore, the HF bulls were 4 s.d. different from their LF counterparts, leading to extreme outliers for the given population.

Isolation of spermatozoa

The frozen semen samples with their fertility scores were provided by Alta Genetic, Inc. (Table 1). In this study, sixteen

Table 1 Fertility phenotypes of the sixteen bulls. Bulls 1–8 are from the LF group and bulls 9–16 are from the HF group. The bulls are individually represented with their fertility scores and the number of breeding. Fertility score was calculated using the Probit F90 Software, and it is expressed as the percent deviation of each conception rate from the average conception rate of all the bulls.

Bull number	Fertility score	Number of breeding
Low-fertility bulls		
1	–12.3	595
2	–8.1	1552
3	–7.2	719
4	–6.4	668
5	–6.3	750
6	–5.9	781
7	–5.6	702
8	–4.2	783
High-fertility bulls		
9	3.3	1024
10	4.2	651
11	4.5	578
12	4.8	300
13	4.8	300
14	4.8	518
15	5.0	326
16	6.2	743

bulls were divided into two groups based on their fertility scores. Semen straws were thawed at 37 °C for 30 s, and spermatozoa were first washed with 2 ml of Percoll in PBS (D'Amours *et al.* 2010) and then washed twice with 5 ml of PBS (pH 7.4) containing a protease inhibitor cocktail (cOmplete, EDTA-free Protease Inhibitor Cocktail, Roche) at 800 g for 5 min. Later, only sperm pellets were stored at –80 °C for immunoblotting; otherwise, they were used immediately following isolation in the ANBL staining and immunocytochemistry experiments.

ANBL test

Smears of the washed sperm were prepared on glass slides and air-dried. Then, the smears were fixed with 3% of glutaraldehyde (PBS, pH 7.4) for 30 min at room temperature (RT). Following fixation, the smears were washed with PBS twice for 5 min each. The smears were then stained in 5% of ANBL in 4% acetic acid (pH 3.5) for 7 min, washed once more with PBS (pH 7.4), and allowed to air-dry. Later, 200 spermatozoa were evaluated per slide (in duplicate) under the 1000× oil immersion objective. Unstained spermatozoa were considered normal and dark blue as sperm cells with failures of chromatin condensation (immature sperm cells) related to excessive retention (Dadoune *et al.* 1988, Dadoune 2003) or accessibility (de Lamirande *et al.* 2012) of sperm histones.

Extraction of sperm nucleoproteins

The nucleoproteins were extracted according to the method of de Yebra & Oliva (1993). In order to lyse the cells, sperm pellets with 5×10^7 cells were washed twice with 400 µl of 1 mM phenylmethylsulfonyl fluoride (PMSF) in ddH₂O. Then, 100 µl of 20 mM EDTA, 1 mM PMSF, and 100 mM Tris (pH 8.0) were added to the pellets followed by the addition of 100 µl of 6 M guanidine hydrochloride, 575 mM dithiothreitol (DTT), and 200 µl of 552 mM sodium iodoacetate. The samples were protected from light and incubated at 20 °C for 30 min. To the samples, 1000 µl of ethanol pre-equilibrated at –20 °C were added, and each sample was then incubated at –20 °C for 1 min and centrifuged for 15 min twice. The pellet was incubated with 1000 µl of 0.5 M HCl at 37 °C for 15 min and then centrifuged at RT for 10 min. The supernatant was held, and 300 µl of 100% trichloroacetic acid (TCA) at 4 °C were added. Following 5 min of incubation at 4 °C, the samples were centrifuged at 4 °C for 10 min. The protein pellet was washed twice with 500 µl of 1% 2-mercaptoethanol in acetone, dried, dissolved in 100 µl of 5.5 M urea, 20% 2-mercaptoethanol, and 5% acetic acid, and stored at –80 °C.

Western blotting

Protein concentration was measured using the Micro BCA Protein Assay Kit (cat #23235, Pierce, Rockford, IL, USA). Sperm nucleoproteins were separated using acetic acid–urea PAGE. The 13.3×8.7×0.1 cm gel was composed by 7.5% stacking/15% separating (0.9 M acetic acid, 2.5 M urea, 2% ammonium persulfate (APS), and 0.9% tetramethylethylenediamine (TEMED)). To each sample, one-half its volume of

loading buffer (5.5 M urea, 20% 2-mercaptoethanol, 5% acetic acid, 50% glycerol, and 0.2% methylene blue) was added. The electrophoresis was performed in 0.9 M acetic acid buffer at 130 V for 80 min. The proteins were then transferred on to a PVDF membrane (Immobilon-P) using a semidry electroblotter (HEP-1, OWL) and 2.5% acetic acid buffer. Subsequently, the membrane was incubated in TBS/1% casein (cat #1610782, Bio-Rad) at RT for 1 h. The blocked membrane was then incubated with a rabbit primary antibody solution (H2B, H3.3, or H4; Abcam Ab1790, Ab97968 and Ab10158 respectively, at 4 °C overnight; Abcam, Cambridge, MA, USA). Thereafter, the membrane was washed thrice with TBS and 0.1% Tween 20 at RT for 10 min each. Incubation with the secondary antibody solution (anti-rabbit IgG conjugated with HRP, Santa Cruz #sc-2313) diluted 1/50 000 in TBS–0.1% Tween 20 was performed at RT for 1 h. Following three washes with TBS+0.1% Tween 20, the proteins were detected using a chemiluminescent HRP substrate (Immobilon Western, cat #P36599), and the expression signals were determined on a ChemiDoc XRS System (Bio-Rad; cat #170-8070) with the Quantity One Software (Bio-Rad).

Immunocytochemistry

To locate and measure the expression of specific histones in the spermatozoa, we carried out immunocytochemistry experiments adapted from the protocols described by Li *et al.* (2008) with modifications. The spermatozoa were washed with PBS containing a protease inhibitor (cOmplete, Roche) and 10 mM EDTA. After washing, the cells were incubated with 20 mM CHAPS for 20 min at RT and washed once with PBS only. In order to decondense sperm chromatin, the samples were incubated with 10 mM DTT and 1 mg/ml of heparin at RT for 30 min (Motoishi *et al.* 1996). The spermatozoa were posteriorly treated and attached to poly-L-lysine-coated coverslips. The coverslip smears were fixed with 4% paraformaldehyde in PBS for 10 min at 4 °C and permeabilized with 0.2% Triton X-100 and 0.1% BSA in PBS for 15 min at RT. Next, the smears were subjected to a series of dehydration steps using 50, 70, 95, and 100% ethanol respectively. Subsequently, fixation was done using 100% methanol for 20 min at –20 °C. Following one washing with a washing buffer (WB: PBS containing 0.1% Triton X-100), the samples were blocked with 1% BSA in the WB for 1 h at RT.

Rabbit polyclonal antibodies against H2B (1:250), H3.3 (1:250), and H4 (1:250) (Abcam) were utilized at 4 °C overnight. Following two rounds of washes in the WB at RT for 15 min, the coverslips were incubated with a secondary antibody solution (1:250; donkey anti-rabbit, DyLight 488, Abcam) with 2.5 µg/ml of DAPI at RT for 1 h. Following a final wash in PBS with 2% Triton X-100 at RT for 15 min, the coverslips were sealed to the slides using a drop of an antifade mounting medium (VECTashield, H-1000). The slides were examined under a confocal fluorescence microscope (Zeiss LSM 510) with 1000× fold magnification using immersion oil. The images were analyzed using the LSM 510 Image Software and 100 spermatozoa, from two slides containing 50 sperm cells each, obtained from the LF and HF bulls were classified based on low, medium, and high histone signals. The area of decondensed sperm head (µm²) was also measured.

Bioinformatics

We compared the sequences of H2B, H3.3, and H4 among the bovine, human, and mouse species using computational tools. The protein sequences of interest were first extensively searched and aligned using UniProt for the bovine, human, and mouse species (www.uniprot.org, UniProt (2012)). Since these mammalian H2B proteins have numerous clusters and variants in the UniProt database, we prealigned these H2B protein clusters of interest that are 90% identical among the mammals prior to the analysis. The multiple alignments were done using the Clustal X method (<http://www.clustal.org>), while phylogenetic trees were created using the neighbor joining method (Saitou & Nei 1987) for each H2B, H3.3, and H4 protein in the UniProt database. (Supplementary Tables 1 and 2 and Supplementary Figs 1 and 2, see section on supplementary data given at the end of this article).

Statistical analysis

We used the statistical package SYSTAT 12 (SYSTAT Software, Inc., Chicago, IL, USA) to analyze the data. The results are expressed as means (S.D. and S.E.M.). In order to verify differences between the LF and HF groups, Student's *t*-test was used ($P < 0.05$). Pearson's correlation was used to establish the relationship between histone retention (ANBL test) or histone expression (western blotting) and bull fertility ($P < 0.05$).

Results

ANBL test

The ANBL test was utilized to assess sperm chromatin condensation by histone retention in the LF and HF bulls. The spermatozoa that stained blue represented the deficient condensation of chromatin (nuclear immaturity) with excessive persistent histones and the unstained spermatozoa were those with normal sperm chromatin (Fig. 1). There was a significant difference in positive cells between the LF (1.73 (0.55, 0.19)) and HF (0.67 (0.17, 0.06)) groups ($P < 0.0001$). A linear regression and Pearson's correlation were used to analyze the relationship between the percentage of positive

Figure 2 Linear regression analysis between aniline blue (ANBL) test results and fertility scores of the low- and high-fertility bulls. A negative and significant Pearson's correlation was found between the ANBL test results and fertility scores of the low- and high-fertility bulls ($r = -0.90$, $P < 0.001$).

spermatozoa and fertility index for each bull (Fig. 2). These parameters were negatively correlated to each other ($r = -0.90$, $r^2 = 0.82$; $P < 0.0001$).

Western blotting

We used an immunoblotting approach to evaluate the expression of H2B, H3.3, and H4 in the sperm cells of bulls with varying fertility. Our results showed that the histones were consistently detectable in all the samples; however, we did not find any significant differences in histone levels in the sperm cells of the LF bulls compared with those of the HF bulls (Fig. 3). In addition, no correlation was found between the fertility index and western blotting results of H2B ($P = 0.98$), H3.3 ($P = 0.84$), and H4 ($P = 0.73$).

Immunocytochemistry

We determined the localization of histones in the spermatozoa of the bulls with the lowest and highest

Figure 1 Bull sperm stained with aniline blue. The unstained sperm on the left has normal chromatin and the dark blue sperm on the right has abnormal chromatin condensation associated with retained and accessible histones.

Figure 3 Western blot (a) and relative quantity of H2B (b), H3.3 (c), and H4 (d) in the low-fertility (LF; black dots) and high-fertility (HF; gray dots) groups. The relative quantity was obtained by calculating the ratio of the band intensity of each bull to that of bull 1. The middle lines are medians, the edges of boxes are the 25th and 75th percentiles, and the whiskers are the minimum and maximum values. There were significant differences between the groups.

fertility using immunofluorescence microscopy. The signals of histone proteins were clearly detectable in the sperm head (Fig. 4 B, C and D). These were present in the post-equatorial, equatorial and, predominantly, in the pre-equatorial region of the sperm head. We also observed histone fluorescence in the midpiece, probably from histones not associated with sperm chromatin. The mean values of total fluorescence intensity from histone signals and area of decondensed sperm head (μm^2) of 100 spermatozoa counted from each of the LF and HF bulls were 2.65×10^6 (1.70; 0.17), 2.60×10^6 (1.38; 0.38), $77.56 \mu\text{m}^2$ (13.80; 1.38) and $79.43 \mu\text{m}^2$ (26.43; 2.64), respectively. The counted sperm cells were divided into three classes (low, medium, and high levels of histone signals) with a range that was calculated by dividing the difference between maximum and minimum total fluorescence intensity values by 3. The percentage, average of total fluorescence intensity, and area of decondensed sperm head of these spermatozoa with low, medium, and high degrees of histone retention are summarized in Table 2. Our immunocytochemistry results confirmed that the largest sperm population (74.5%, average from the two bulls) has a low level of histone retention. In addition, we found a positive correlation between the area of decondensed sperm head and the signal intensities of histones ($r=0.64$; $P<0.0001$).

Bioinformatics

Using UniProt, we identified a total of 21 bovine H2B protein clusters, 12 of which were unique to the bovine species, while the remaining belonged to other mammalian members such as mice and humans. In total, 17 of H2B, 3 of H3.3, and 3 of H4 proteins that belonged to bulls, mice and humans were used for

further bioinformatics analysis. In detail, four mouse, seven human, and six bovine H2B protein sequences were selected for multiple alignments and phylogenetic trees. Bovine H2B variants F2Z4E8 and A5D7N2 were 100% identical to their human counterparts Q99877 (H2B1N) and Q16778 (H2B2E) based on the pairwise alignment; indeed, they were closely related to each other by having the common ancestral sequence based on our phylogenetic trees. Therefore, since bovine F2Z4F9, F2Z4E8, and A5D7N2 were 100% identical to human H2B type 1K, H2B type 1N, and H2B type 2E, they should be considered as bovine H2B1K, H2B1N, and H2B2E. This is consistent with the results obtained from our pairwise alignments and phylogenetic trees. Besides, histone H2B type 1 was 100% conserved among the bovine (P62808), human (P62807), and mouse (Q6ZWY9) species, and they evolved from the common ancestral protein sequence (Supplementary Table 1 and Supplementary Fig. 1). On the other hand, bovine A5D7N2 showed 98.41% identity with human Q8N257 and mouse Q8CGP0 histone H2B type 3B, which were 100% conserved to each other. Besides, this H2B variant was evolutionarily more distant than its human and mouse counterparts. Only one protein variant of H3.3 and H4 per species was identified using the UniProt database, which was 100% identical and evolutionarily conserved among bulls, mice and humans based on multiple sequence alignments and phylogenetic trees (Supplementary Table 2 and Supplementary Fig. 2).

Discussion

Histones are thought to be present in sperm chromatin in a nonrandom distribution. Studies have demonstrated

Figure 4 Immunolocalization of histones in bovine sperm cells. (A1, B1, C1 and D1) DNA stained with DAPI (blue) in a bull sperm cell; (A2, B2, C2 and D2) bright-field images; (A3, B3, C3 and D3) histones stained with DyLight 488-conjugated secondary antibody (green) in a bull sperm cell. (A3) negative control sperm cell incubated with only the secondary antibody; (B3) sperm cell with a low level of histone fluorescence; (C3) sperm cell with a medium level of histone fluorescence; and (D3) sperm cell with a high level of histone fluorescence.

that histone-rich regions of the paternal genome contain genes related to embryonic development (Hammoud *et al.* 2009). Therefore, paternal histones can play a significant role in the evaluation of male fertility and consequently during the early development of embryos. Our hypothesis was that bull fertility was associated with histone retention in sperm cells. In order to test this hypothesis, we carried out a study evaluating sperm chromatin condensation by staining the retained and accessible histones with ANBL and determining the expression of specific histones (H2B, H3.3, and H4) in the sperm cells of bulls with varying fertility. Additionally, a computational biology procedure was performed using an online database to show the extent of conservation of the histone proteins among mammals. The ANBL test was used to stain sperm histones to determine the abnormal reduction of sperm chromatin condensation or immaturity (Hammadeh *et al.* 1996, Chioccarelli *et al.* 2010, Sati & Huszar 2013).

Based on the ANBL test results, we conclude that overall abnormal chromatin condensation associated with abnormal retention or accessibility of sperm histones is related to bull fertility. The percentage of spermatozoa stained by ANBL was higher in the LF group than in the HF group ($P < 0.0001$). The majority of studies using the ANBL test have been conducted in humans, suggesting its predictive value for male fertility (Auger *et al.* 1990, Hammadeh *et al.* 2001). Relatively few studies have examined the association of the ANBL test and bull fertility (Vieytes *et al.* 2008). Among the species, the percentage of ANBL-positive sperm cells has been found to be variable, such as 1% in mice (Shokri *et al.* 2013), 10% in cats (Hingst *et al.* 1995), 10% in rabbits (Steger *et al.* 2005), and 50% in roosters (Santiago-Moreno *et al.* 2009). In bulls, the percentage of ANBL-stained sperm cells was 1–10%, which was associated with sperm chromatin instability and DNA damage (Khalifa *et al.* 2008, Mukhopadhyay *et al.* 2011).

Table 2 Immunocytochemical analysis of sperm histones of high- and low-fertility bulls. Percentage (%), total fluorescence intensity, and area of decondensed sperm head (μm^2) in sperm cells with low, medium, and high levels of histone signals. The results are expressed as means (S.D. and S.E.M.).

Parameters	Low-fertility bulls	High-fertility bulls
Spermatozoa with low level of histone signals		
Percentage (%)	71	78
Total fluorescence intensity	1.77×10^6 (0.76, 0.09)	1.97×10^6 (0.47, 0.05)
Area of decondensed sperm head (μm^2)	58.70 (8.38, 0.99)	58.66 (7.06, 0.80)
Spermatozoa with medium level of histone signals		
Percentage (%)	24	17
Total fluorescence intensity	4.26×10^6 (0.77, 0.16)	4.27×10^6 (0.63, 0.15)
Area of decondensed sperm head (μm^2)	67.05 (9.30, 1.90)	54.25 (6.98, 1.69)
Spermatozoa with a high level of histone signals		
Percentage (%)	5	5
Total fluorescence intensity	7.37×10^6 (1.03, 0.46)	6.75×10^6 (0.57, 0.25)
Area of decondensed sperm head (μm^2)	65.87 (9.53, 4.26)	63.53 (8.45, 3.78)

Our results with a limited significant difference between the two groups could also be an indicator of inefficient spermatogenesis in LF bulls.

Our evaluation based on immunoblotting did not show any statistical association between the specific histones (H2B, H3.3, and H4) and bull fertility. However, we found different sperm populations with regard to the intensity of histone signals (low, medium, and high) (Fig. 4 and Table 2). We also observed a positive correlation between the area of decondensed sperm head and the signal intensities of histones ($P < 0.0001$). Considering that protamines are essential for the compaction of sperm chromatin, insufficient replacement of histones by protamines can reduce the level of chromatin condensation (Carrell *et al.* 2007). Higher levels of histone retention have been shown to be associated with low chromatin condensation, which is believed to influence the size of the sperm head following the decondensation step (Kazerooni *et al.* 2009). Additionally, a study in bulls has demonstrated that DNA damage is related to sperm head abnormalities such as sperm shape and sperm head size (Enciso *et al.* 2011). In our study, the majority of spermatozoa displayed a low level of histone retention (71% in the LF bulls and 78% in the HF bulls), confirming the results obtained from the ANBL test. Scattering of the immunofluorescence signals from histones displayed a punctuated variation in the sperm head (Fig. 4); this pattern was also similar to that reported in previous experiments in swines (Flores *et al.* 2011) and humans (Zhang *et al.* 2006). Additionally, one of the few immunocytochemical studies conducted on bull sperm chromatin has demonstrated that histones are also located in the perinuclear theca (Tovich & Oko 2003). This result is consistent with our findings, where we observed that the histone signals were mainly present in the pre-equatorial region of the sperm head. Histone proteins can also be present in the cytoplasm or be associated with the mitochondria in animal cells (Johnson *et al.* 2007, Kutsyi 2009, Choi *et al.* 2011, Cascone *et al.* 2012); these findings can justify the histone signals observed in the sperm midpiece in our study.

Based on our immunoblotting results, we report that there was no difference in the expressions of sperm histones (H2B, H3.3, and H4) between the LF and HF bulls (Fig. 3), although spermatozoa with increased histone retention (positive to ANBL) were abundant in the LF bulls. Since the percentage of spermatozoa stained by the ANBL dye was $< 2\%$ in both the groups, this divergence may not be sufficient to significantly affect the expressions of histones measured by western blotting between the two groups. An abnormal ratio of histones to protamines in spermatozoa has been claimed to negatively affect sperm chromatin. For example, Hammoud *et al.* (2011) suggested that histone retention was the main reason for different localizations of histones in the sperm cells of fertile men compared with their infertile counterparts. Moreover, subfertile men with increased levels of spermatozoa stained by ANBL displayed qualitative alterations of histones related to acetylation sites in their sperm cells (Paradowska *et al.* 2012). The causes of excessive histone retention in sperm cells are still unclear, but the changes in testosterone and FSH levels and molecular defects might be among the contributing factors (Gill-Sharma *et al.* 2012). Meyer-Ficca *et al.* (2009, 2011) reported that the deficiency of poly(ADP-ribose) glycohydrolase – an enzyme playing a role in DNA repair during spermatogenesis – in a subfertile phenotype increased the levels of phosphorylated H2AFX as well as of abnormal retention of the core histones in mouse sperm cells.

Using bioinformatic analysis, we demonstrated that H3.3 and H4 proteins were identical among bulls, mice and humans, while H2B was mostly conserved between bulls and humans compared with their mouse counterparts. These results suggest that H3.3 and H4 proteins might also be functionally identical among the species. However, we suggest here that H2B variants might have different functions among mammals. Bovine models are commonly used in diverse areas of reproductive science such as embryo cryopreservation (Huang *et al.* 2007), genetic tests (Almodin *et al.* 2005), and sperm cytotoxic assays (Cesari *et al.* 2007). Because valuable fertility data

are available from the cattle AI industry (DeJarnette *et al.* 2004), the bull is a suitable model for studies related to genetic and epigenetic aspects of male fertility. The AI centers not only carry out analyses of more than 100 inseminations for each male, but also distribute the males in distinct levels of fertility instead of only classifying them as fertile and infertile phenotypes. In addition, techniques for *in vitro* production of bovine embryos are well established and are suitable for evaluating the impact of male epigenome on mammalian embryonic development (Machaty *et al.* 2012). In this study, we also showed the conservation and evolutionary relationships of the histone proteins among bulls, mice and humans in order to further the value of bull sperm cells for studies on the integrity of mammalian sperm chromatin.

This study is the first to evaluate the differences in bull fertility as related to the expression of sperm histones. The small sample size used for the immunofluorescence analysis limited the statistical power needed to detect significant differences within the two groups. However, these results showed variations among the sperm populations that can be furthered using a large sample size for clinical or diagnostic purposes (Fig. 4). Although PTMs in histones modulate the sperm epigenome, our study did not focus on these traits (Jenkis & Carrell 2012). Thus, a qualitative analysis of PTMs in histones of bulls can yield new perspectives to better understand epigenetic regulations affecting sire fertility. Despite using the current semen analysis methods, the prediction of bull fertility is still far from the demands of the cattle industry. In humans, the paternal histone contribution to zygotes has been examined, which demonstrated a possible influence of the male epigenome on embryonic development (van der Heijden *et al.* 2008). In this context, the evaluation of paternal epigenome can yield new perspectives for both producers and consumers in bull selection. In conclusion, our results showed that bull fertility was associated with chromatin condensation status changed by histone retention in a sperm population. We did not find any relationship between the expression levels of specific histones (H2B, H3.3, and H4) and bull fertility. In addition, using immunofluorescence analysis, we demonstrated distinct sperm populations with regard to nucleohistone signals.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-12-0399>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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