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Spermatozoa DNA methylation patterns differ due to peripubertal age in bulls

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ABSTRACT

In the dairy industry, using semen as soon as the bull is mature enough to produce it is advantageous for breeding purposes. Mammalian spermatogenesis is a hormone-dependent developmental program in which a complex cascade of events must take place to ensure that germ cells reach the proper stage of development at the proper time. Conventional indicators of semen quality such as sperm cell motility and viability usually improve as bulls mature, meeting quality criteria satisfactorily at around 16 months. Using semen before that age may affect embryo viability, but other changes occurring during the peripubertal period should be considered. Although it is known that establishment of these patterns begins during foetal life, the extent to which sperm cell DNA methylation changes during puberty has not been studied. The aim of this study is to correlate the age of a young bull with the overall DNA methylation pattern of its spermatozoa.

Spermatozoa were collected from bulls at the ages of 10 months (early pubertal), 12 months (late pubertal) and 16 months (pubertal). Each animal (n = 4) was compared to itself with 16 months as control. Genome-wide DNA methylation was analyzed by microarray using the EmbryoGENE DNA Methylation Analysis platform. Using a fold change over 1.5 and a 5% FDR p-value correction, a total of 2602 differently methylated regions were found in common between 10 months of age and 16 months of age. No differently methylated regions between 12 months and 16 months of age were found at the same level of statistical significance. We conclude that spermatozoa from bulls aged 10 months have a different epigenetic profile, which could compromise their value.

1. Introduction

As in many other sectors of biology, understanding of fertility is being transformed by an emerging paradigm according to which responses to the environment experienced by previous generations become heritable through epigenetic phenomena that modulate the expression of the fundamental genetic legacy. This is thought to occur through phenotypic programming of gametes and embryos by the chemical and metabolic environment prevailing in the gonads, the oviduct and the uterus. Such programming is known to affect primarily the metabolic status of the offspring and appears to occur in all mammals studied so far [1,2]. In domestic animals in particular, fertility is a trait of low heritability and might be sensitive to parental metabolic status or environment. Some evidence suggests that programming of gametes or early embryos occurs in association with maternal dietary factors affecting blood pressure [3] and ovarian antral population [4]. Maternal lactation status at conception appears to have an impact on milk production by offspring [5]. In some species, the physiological environment of the male also might have an impact on offspring phenotype [6].

Alterations of DNA methylation patterns on the paternal genome have been investigated in numerous species. For example, inhibition of DNA methylation has been linked to a significant reduction in fertility in both genders. In studies of long-term exposure to the DNA methylation inhibitor 5-aza-2′-deoxycytidine, more extreme phenotypic expressions have been noted and a correlation has been established between DNA methylation status and embryo viability [7,8].

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A positive relationship between overall methylation of sperm cell DNA (measured by quantifying 5-methylcytosine) and cell count and motility has been established in humans [9]. The results of that study suggest that both overall and locus-specific methylation could be used as markers of testicular function and spermatogenesis, lack of methylation being associated with defective spermatogenesis. However, another human study has linked hypermethylation with semen of poor quality [10]. A particularly alarming aspect of spermatozoon epigenetic abnormality is that transmission of such defects to the offspring might influence their susceptibility to diseases [11,12]. Spermatozoa produced by young pubertal bulls reportedly have a higher percentage of head abnormality and proximal droplet. Count, motility and percent normality are correlated positively with the age of the bull [13]. Sperm cell morphology is considered one of the best predictors of calf output for bulls in multiple-sire herds, and reportedly affects in vitro fertilization success rate [14].

We have found no study of the correlation between animal age and DNA methylation in sperm cell chromatin. We hypothesized that the spermatozoon epigenetic profile evolves as the animal matures. The objective of this study was therefore to explore the overall DNA methylation patterns of spermatozoa obtained from bulls aged 10 months (early puberty), 12 months (late puberty) and 16 months (pubertal, considered adult).

To achieve this objective, we used the EDMA (EmbryoGENE DNA Methylation Analysis) platform, which allows genome-wide analysis. This tool combines four methodological principles: genomic fragmentation using restriction endonucleases, targeting of methylated regions using a combination of methyl-sensitive restriction endonucleases, amplification of methylated fragments using ligation-mediated PCR, and identification of the amplified fragment using a microarray [15]. The microarray slide contains 414,516 probes corresponding to 20,355 genes and 34,379 CpG islands. A bioinformatics data analysis pipeline was developed in parallel to complement the EDMA platform. This tool was built originally to assess the effect of the environment on early embryos, and its usefulness for evaluating genome-wide DNA methylation in smaller samples such as spermatozoa and oocytes has been demonstrated [15].

2. Materials and methods

2.1. Chemicals

All reagents and media supplements used in this study were of tissue culture grade and obtained from Sigma-Aldrich Co. unless otherwise specified.

2.2. Semen collection

Semen was collected from Holstein bulls (n = 4) at the ages of 10, 12 or 16 months using an artificial vagina, in compliance with the guidelines of the Canadian Council on Animal Care. Samples in straws containing about 5×10^7 spermatozoa were frozen in liquid nitrogen. All samples were obtained from L'Alliance Boviteq Inc (Saint-Hyacinthe, QC, Canada), a commercial provider of bovine gametes. Two straws from the same animal at the same age were pooled prior to the DNA extraction. A description of the semen at each stage and for each bull can be found in Supplementary Table 1.

2.3. DNA extraction

The DNA extraction protocol has been described previously [16]. Briefly, two units of extended semen were diluted with 1.0 mL of phosphate buffered saline (pH 7.4) containing 2% SDS in a 1.5 mL tube. The mixture was mixed by vortex for 5 s and centrifuged at 1,550×*g* for 5 min to pellet the spermatozoa. The supernatant was aspirated, and lysis buffer (450 μL of 10 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1.5% SDS, 100 mM NaCl) was added to the pellet, followed by 30 μL of 1 M dithiothreitol and 20 μL of proteinase K solution (20 mg/mL). The suspension was shaken for 16 h at 56 °C at 155 rpm with the tubes placed horizontally, and 160 μL of saturated NaCl solution were added, followed by mixing by vortex three times for 5 s each then centrifuging for 10 min at 15,500×*g*. The supernatant was removed carefully from the liquid pellet and transferred to 2 mL tubes. Ethanol at 4 °C was added (1.0 mL). The tubes were inverted gently 20 times to allow DNA to precipitate then centrifuged for 15 min at 16,000×*g* at 4 °C. The supernatant was discarded, 70% ethanol at 4 °C was added (500 μL) and the mixture was centrifuged for 5 min at 16,000×*g*. The supernatant was discarded, 50% ethanol at 4 °C (200 μL) was added and the mixture was centrifuged for 5 min at 16,000×*g*. The purified DNA was suspended in 100 μL of 10 mM Tris-HCl/1 mM EDTA buffer (pH 8.0). Quantity and purity were evaluated using a ND-1000 Nanodrop (Thermo Fisher) and validated by migration on 0.5% agarose gel at 75 V for 1 h. DNA in bull hair follicles and in granulosa cells was extracted using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions.

2.4. Methylation analysis

The methylation analysis was performed using the EmbryoGENE DNA methylation array (EDMA) platform (GEO #GPL18700). The methodology has been described in detail previously [15]. Briefly, 10 ng of genomic DNA sample and spike-in control were cleaved into fragments of about 160 bp using the Mse1 restriction enzyme (New England Biolabs). DNA was then precipitated using 1.0 μL of linear acrylamide (5 mg/mL) (Ambion), sodium acetate (3 M, pH 5.2) and absolute ethanol. Adapters were ligated to the cleaved DNA. Samples were then cleaved using methyl-sensitive restriction enzymes *Hpa*II, *Hin*P1I and Aci1 (Thermo Fisher), which target only un-methylated fragments, thus leaving only one adapter per fragment.

Cleavage efficiency was confirmed by analyzing the spike-in control added prior to treatment of the genomic DNA. For this purpose, a separate mixture containing forward and reverse primers targeting the *Hpa*II, *Hin*P1I and Aci1 sites was prepared for qPCR. Non-cleaved spike-in (1/100 dilution) was used as a positive control. The amplification plot and dissociation curve were evaluated for each primer set separately. DNA fragmentation was considered positive when the threshold cycle (ct) was greater than 5 compared to the undigested control.

2.5. Fragment selection by ligation-mediated PCR

DNA cleaved by methyl-sensitive enzymes (Mse1, *Hpa*II, HinP1I and Aci1) was precipitated in ethanol and re-dissolved in nuclease-free water. Selective amplification of methylated fragments was performed using two rounds of ligation-mediated PCR (LM-PCR). The PCR products were resolved on 0.8% agarose gel to assess the quality. The linkers were removed by Mse1 cleavage and the PCR fragments were purified using a QIAquick PCR purification kit (Qiagen). The DNA was quantified using a ND-1000 Nanodrop device.

2.6. Sample labelling and hybridization

DNA sample (2.0 μg) was labelled using the Genomic DNA High-Throughput ULS Labelling Kit (Agilent) according to the manufacturer's instructions. Non-reacted ULS-Cy3/5 was removed using a