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Cytosine methylation of sperm DNA in horse semen after cryopreservation

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ABSTRACT

Semen processing may contribute to epigenetic changes in spermatozoa. We have therefore addressed changes in sperm DNA cytosine methylation induced by cryopreservation of stallion semen. The relative amount of 5-methylcytosine relative to the genomic cytosine content of sperm DNA was analyzed by ELISA. In experiment 1, raw semen (n = 6 stallions, one ejaculate each) was shock-frozen. Postthaw semen motility and membrane integrity were completely absent, whereas DNA methylation was similar in raw (0.4 ± 0.2%) and shock-frozen (0.3 ± 0.1%) semen (not significant). In experiment 2, three ejaculates per stallion (n = 6) were included. Semen quality and DNA methylation was assessed before addition of the freezing extender and after freezing-thawing with either Ghent (G) or BotuCrio (BC) extender. Semen motility, morphology, and membrane integrity were significantly reduced by cryopreservation but not influenced by the extender (e.g., total motility: G 69.5 ± 2.0, BC 68.4 ± 2.2%; $P < 0.001$ vs. centrifugation). Cryopreservation significantly ($P < 0.01$) increased the level of DNA methylation (before freezing 0.6 ± 0.1%, postthaw G 6.4 ± 3.7, BC 4.4 ± 1.5%; $P < 0.01$), but no differences between the freezing extenders were seen. The level of DNA methylation was not correlated to semen motility, morphology, or membrane integrity. The results demonstrate that semen processing for cryopreservation increases the DNA methylation level in stallion semen. We conclude that assessment of sperm DNA methylation allows for evaluation of an additional parameter characterizing semen quality. The lower fertility rates of mares after insemination with frozen-thawed semen may at least in part be explained by cytosine methylation of sperm-DNA induced by the cryopreservation procedure.

1. Introduction

Because of the globalization of horse breeding, the significance of semen cryopreservation in horses is steadily increasing [1,2]. Although frozen semen is produced from a large number of stallions [3], with regard to cryosurvival of semen high variability exists among stallions [4]. Only approximately 50% of the stallions are considered acceptable for production of cryopreserved semen [3]. Cryoinjury of spermatozoa is mainly represented by membrane damage due to physical stress, i.e., osmotic stress due to extracellular ice formation, phase transition from liquid to crystalline and oxidative stress [4–6]. Moreover, cryopreservation also induces DNA fragmentation independent of membrane damage [7,8]. Cryopreserved semen of good *in vitro* quality may render low conception rates in the field

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[3]. Nevertheless, the possibility to predict semen fertility on the basis of laboratory assessment is still limited. Recently, aberrant methylation of sperm DNA has been suggested to affect fertilization and development of the preimplantation embryo [9–11]. Moreover, cryopreservation of semen contributes to dramatic changes in messenger RNA expression of epigenetic-related genes [12]. Assessment of DNA methylation has therefore been suggested as a new approach to evaluate the ability of spermatozoa to fertilize the oocyte and lead to normal embryo development [9].

In mammalian cells, epigenetic changes include DNA methylation, posttranslational histone modifications, chromatin remodeling, and production of small noncoding RNAs [13]. Among those, DNA methylation attracts specific interest for the assessment of epigenetic changes and is defined as the stable addition of a methyl group to cytosine, mainly in enriched CG (cytosine guanine) regions of the DNA, also described as CpG (5'—C—phosphate—G—3') islands. Methylation can represent a response to environmental cues and may modify gene expression [13]. Epigenetic modifications are generally removed and reestablished from one generation to the next [14]. During male germline development, paternal DNA methylation marks are erased and established through waves of demethylation and *de novo* methylation [15]. To the best of our knowledge, no information on DNA methylation of equine sperm is available so far.

In the present study, we have followed the hypothesis that DNA methylation of stallion sperm is affected by cryopreservation. We have therefore analyzed global 5-methylcytosine in DNA of equine semen before and after freezing.

2. Material and methods

2.1. Experimental animals and semen collection

The experiment was done in accordance with experimental animal legislation in Austria. Six healthy fertile Shetland pony stallions aged between 8 and 23 years (15.5 ± 3.2) with three stallions being younger than 12 and three older than 20 years of age were used for the study. Stallions' weight was between 116.0 and 183.5 kg (mean 156.0 ± 6.7 kg). All stallions were kept in groups in loose barns, fed hay twice daily, and had free access to water and minerals. Semen from the stallions was collected at regular intervals, i.e., two or three times per week with a Hannover artificial vagina (Minitube, Tiefenbach, Germany) on a dummy as described [16]. For semen collection, stallions were exposed to a teaser mare until erection and readiness to mount, followed by mounting of the dummy.

2.2. Experimental design

2.2.1. Experiment 1

For the first experiment, one ejaculate per stallion (n = 6) was collected. Immediately after semen collection, the gel fraction of the ejaculate was removed. Semen was filtered through sterile gauze, and volume, sperm concentration, total sperm count, as well as percentage of motile, progressively motile, and membrane-intact spermatozoa were evaluated (see Section 2.3 for further details). The ejaculate was then divided in two portions: from one portion, DNA was extracted without further semen processing, whereas from the second portion, 1.5-mL semen were filled in a tube and plunged into liquid nitrogen (−196 °C) for 15 minutes before thawing at 38 °C for 1 minute (shock-frozen semen), followed by DNA extraction.

2.2.2. Experiment 2

From each of the six stallions, three ejaculates were collected at 1-week intervals. Immediately after semen collection, the gel fraction of the ejaculate was removed. Semen was filtered through sterile gauze; and volume, sperm concentration, total sperm count, and percentage of motile, progressively motile, membrane-intact, and morphological intact spermatozoa were evaluated (see Section 2.3 for further details). Ejaculates were split in three aliquots. Two aliquots were diluted with EquiPlus extender (Minitube) and one with BotuSemen extender (Nidacon International, Mölndal, Sweden) to a concentration of 100 to 200 spermatozoa/mL, dependent on the volume and availability of semen, and filled into a sterile centrifugation tube. Centrifugation was performed with Androcoll (Minitube) for 20 minutes at 25 °C at 800 × *g* (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) for removal of somatic cells. The supernatant was discarded and the semen pellet submitted to further processing. One of the aliquots diluted with EquiPlus was used for analysis of semen quality and DNA extraction before freezing (final concentration: 50 million spermatozoa/mL). The second aliquot was cryopreserved with Ghent (G) extender, and the third aliquot cryopreserved with BotuCrio (BC) extender. In these aliquots, semen analysis and DNA extraction was performed after thawing of one straw each after at least 4 weeks of storage in liquid nitrogen (−196 °C).

2.3. Experimental procedures

2.3.1. Cryopreservation and thawing of semen

Processing of semen for cryopreservation was done at room temperature (20 °C). After centrifugation of extended semen, the supernatant was removed until approximately 1 cm above the surface of the sperm pellet. The sperm pellet was resuspended with the cryopreservation extender at a 1:1 ratio. The semen was filled in 0.5-mL straws and sealed automatically at room temperature (MPP Uno, Minitube). Straws were placed on a rack into the freezing chamber of a computer-controlled rate freezer at 20 °C (IceCube 14 M; Sylab, Purkersdorf, Austria). Semen was first cooled to 5 °C with a cooling rate of 0.3 °C/min, subsequently within 3 minutes to −25 °C (10 °C/min) and finally to −140 °C with a cooling rate of 25 °C/min. Straws were removed from the chamber and plunged directly into liquid nitrogen in which they were stored for at least 4 weeks before thawing at 38 °C for 15 seconds. Before further analysis, semen was held at room temperature for 15 minutes.

2.3.2. Semen analysis

Semen analysis was always performed by an experienced technician blinded to semen treatment. Sperm