Theriogenology 117 (2018) 34–39

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Effects of seminal plasma and flash-freezing on DNA structure of stallion epididymal sperm exposed to different potentiators of DNA damage

R. Serafini*, D.D. Varner, T.L. Blanchard, S.R. Teague, K. LaCaze, C.C. Love

Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, TX, USA

ARTICLE INFO

Article history:
Received 12 December 2017
Received in revised form
17 May 2018
Accepted 18 May 2018
Available online 24 May 2018

Keywords:
DNA structure
Sperm
Seminal plasma
Flash-freezing
Potentiators

ABSTRACT

The tolerance of sperm DNA structure to seminal plasma and freezing conditions has both clinical and basic biologic relevance. In this study, fresh (FS) or flash-frozen (FZ) stallion epididymal sperm were exposed (SP^+) or unexposed (SP^-) to seminal plasma. Sperm were then evaluated to monitor the degree of change in DNA structure following challenge with chemical (dithiothreitol-DTT), oxidative (iron sulfate; $FeSO_4$) or enzymatic (DNase I) potentiators of DNA damage. For sperm not treated with potentiators (controls), there was no effect of SP treatment (SP^- vs. SP^+) or freezing treatment (FS vs. FZ; non-significant) on measures of any DNA assays (i.e., 8-hydroxy, 2′deoxyguanosine [8OHdG], TUNEL, or sperm chromatin structure [SCSA] assays). Group FZ was more susceptible than Group FS to potentiators of DNA damage. Percent 8OHdG-positive sperm was higher in Group FZ/SP^- treated with $FeSO_4$ than all other groups ($P < 0.05$). Percent TUNEL-positive sperm was similar among FZ/SP^- groups treated with DTT, $FeSO_4$, or DNase (non-significant) and was higher in these groups than all other treatments ($P < 0.05$). Percent COMP-α_t was higher following treatment with DNase or DTT, as compared to their respective controls, regardless of prior exposure to SP ($P < 0.05$). Overall, sperm DNA structure was unaffected by seminal plasma or freezing treatment when samples were not exposed to potentiators of sperm DNA damage; however, marked differences were identified in DNA structure when sperm were challenged with chemical, oxidative or enzymatic treatments. These results highlight the importance of challenging DNA structure prior to analysis. The use of potentiators of DNA damage provided a model to evaluate sperm DNA structure following exposure of sperm to various experimental treatments.

1. Introduction

Sperm DNA is a tightly compacted structure compared to somatic cells and is considered to be more resistant to stressors [1]. Some assays of DNA quality (e.g., terminal deoxyribonucleotidyl transferase-mediated fluorescein-dUTP nick end-labeling [TUNEL] and 8-hydroxy, 2′deoxyguanosine [8OHdG] assays) are marketed as tests for somatic cell DNA quality; however, their use with sperm may be inadequate without species-specific modifications to decondense the DNA and to maximize accessibility to assay binding sites.

Various DNA assays (i.e., 8OHdG, TUNEL, and sperm chromatin structure [SCSA] assays) have been developed to evaluate different types of structural changes in the DNA. Base oxidation is reported to be the first DNA lesion that occurs following oxidative stress and results in the formation of the 8OHdG adduct in mitochondrial DNA [2]. Oxidative stress may also lead to destabilization of the nuclear DNA and increased susceptibility of the DNA to acid hydrolysis, resulting in formation of single-stranded DNA (as measured by the SCSA) or creation of DNA nicks (as measured by the TUNEL assay), [3].

Mixing of seminal plasma with epididymal sperm prior to commercial cryopreservation has been reported to increase [4–7]; decrease [8] or have no effect [9] on post-thaw stallion sperm motility. The effect of seminal plasma exposure on DNA quality of fresh or flash-frozen stallion sperm has not been reported.

The preservation of frozen-thawed stallion sperm harvested from the ductus deferens and cauda epididymis is commonly performed in stallions that die prematurely. It is unclear whether seminal plasma should be added prior to cryopreservation to

* Corresponding author.
E-mail address: rosanna.serafini@gmail.com (R. Serafini).

https://doi.org/10.1016/j.theriogenology.2018.05.023
0093-691X/

resemble an ejaculated semen sample. Fertility outcomes using epididymal sperm have been reported to be similar to that of ejaculated sperm, both in vivo [10,11] or after intra-cytoplasmic sperm injection (ICSI), [12,13]. However, Heise et al. [14] reported that fertility of commercially frozen-thawed epididymal sperm supplemented with seminal plasma was higher than epididymal sperm unexposed to seminal plasma. Conversely, Monteiro et al. [10], Papa et al. [15] and Melo et al. [16] reported high pregnancy rates with commercially frozen-thawed epididymal sperm unexposed to seminal plasma. Seminal plasma, in a dose dependent manner, has been shown to reduce features of sperm quality (motility, viability, and DNA structure) following cooled storage [17].

It is unknown whether sperm subjected to flash-freezing yields similar results to fresh semen for DNA analysis. This is an important topic because sperm generally need to be frozen for shipment to a reference laboratory for DNA analysis. Evenson et al. [18] indicated that semen samples may be flash-frozen prior to analysis by SCSA, as values were similar between flash-frozen and fresh semen samples. The effect of flash-freezing on the results of other DNA assays is unknown, and this is pertinent because these assays identify different aspects of DNA structure. Such findings may have implications for studying the effects of commercial cryopreservation methods on DNA structure.

The current study examines the effects of sperm exposure to seminal plasma or flash-freezing on DNA structure following treatment with chemical (dithiothreitol-DTT), oxidative ($FeSO_4$) or enzymatic (DNase I) stressors known to induce structural changes in DNA. The intent of applying a chemical stressor (DTT) is to allow DNA decondensation by disulfide bond reduction among protamine molecules. Decondensation allows increased access of the fluorescent probes to the DNA core. Subsequently, the stressors challenge the native DNA structure, oxidatively or enzymatically, for evaluation using the 8OHdG, TUNEL or SCSA assays. As such, these experimental conditions can be applied to evaluate treatment conditions, such as seminal plasma exposure and flash-freezing. To date, there are no published data on stallion DNA quality of fresh or flash-frozen epididymal sperm following seminal plasma exposure. The aims of this study were to: 1) create experimental conditions to evaluate structural changes in sperm DNA following exposure to chemical, oxidative, or enzymatic treatments, and 2) examine the effects of seminal plasma exposure and flash-freezing on changes in DNA structure.

2. Materials and methods

All experimental procedures were performed according to the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training and were approved by the Laboratory Animal Care Committee at Texas A&M University.

Twelve healthy light-breed stallions, 2–6 years of age, were castrated following general anesthesia. Following castration, testes and epididymides were immediately transferred to an adjacent laboratory in a styrofoam box, and placed in a refrigerator until dissection. Epididymal sperm were harvested within 12 h from castration. The epididymides were dissected from the testes and sperm were collected from each cauda epididymis and ductus deferens by retrograde flushing with air. Aliquots of 20 μL of epididymal sperm were immediately diluted in 980 μL (1/50; v/v) of Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO, USA) and incubated for 15 min at room temperature. Sperm concentration was measured with a fluorescence-based cell counter (NucleoCounter-SP100™, Chemometec, A/S, Allerød, Denmark). Additional aliquots of epididymal sperm were diluted (1/50; v/v) with frozen-thawed seminal plasma (SP) and also incubated for 15 min at room temperature. The SP was obtained by centrifugation of a single ejaculate from a fertile stallion (70% pregnancy rate per cycle) at 2000 g × 10 min at room temperature and filtering the supernatant through tandem 5.0- and 1.2-μm nylon filters (Maine Manufacturing LLC, ME, USA). One-mL aliquots were placed in capped tubes and stored at −60 °C in a freezer prior to thawing for use.

2.1. Exposure to potentiators

Epididymal sperm samples were further diluted to a concentration of 2×10^6 sperm/mL in DPBS. Fresh (unfrozen) sperm (FS) exposed (SP^+) or unexposed (SP^-) to SP were aliquoted into 1.5-mL conical-bottom microfuge tubes (1.5 mL, VWR International, LLC Radnor, PA, USA) and processed immediately, whereas 1-mL aliquots of sperm were also flash-frozen (FZ) at −60 °C in a freezer for storage prior to thawing (37 °C for 30 s) and further processing. All samples (FS and FZ, SP^+ and SP^-) were centrifuged at 400 g × 5 min and the resulting supernatant was removed. Sperm were resuspended in DPBS to 2×10^6 sperm/mL and then exposed to the following treatments for 1 h at 37 °C: 1) Control (sperm with no exposure to potentiators of DNA damage); 2) sperm were exposed to 2 mM DTT [Sigma-Aldrich, St. Louis, MO, USA]) for 30 min at 37 °C; DTT; 3) sperm were exposed to a solution containing 10 μM iron sulfate ($FeSO_4$, Sigma-Aldrich, St. Louis, MO, USA) + 20 μM hydrogen peroxide (H_2O_2, 35 wt. % in H_2O, Sigma-Aldrich, St. Louis, MO, USA; $FeSO_4$); 4) sperm were exposed to a solution containing 200 U/mL of DNase I (Roche Diagnostics, Basel, Switzerland) + 5 μM of magnesium chloride ($MgCl_2$, Sigma-Aldrich, St. Louis, MO, USA; DNase). All sperm samples were then centrifuged at 400 g for 5 min. Supernatant was removed, then sperm pellets in treatments 3 and 4 were resuspended in DPBS and further exposed to 2 mM DTT for 30 min at 37 °C. Samples were washed one time in DPBS, then fixed in 2% (w/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and stored in 0.1 M glycine (Amresco, Astral Scientific Pty Ltd, Gymea, NSW, Australia) at 4 °C for 24 h prior to analysis by flow cytometry.

2.2. 8-Hydroxy, 2′deoxyguanosine (8OHdG) assay

Oxidative DNA injury was evaluated by measuring the percentage of 8OHdG-positive sperm. The protocol was modified from that previously described [19] and utilized a commercially available kit (OxyDNA assay kit; Calbiochem, CA, USA) whereby a fluorescein isothiocyanate (FITC)-conjugated protein binds to 8OHdG adducts (products of oxidative injury) resulting in fluorescent emission. Preliminary experiments were performed in our laboratory to optimize sperm concentration, stain dilution, incubation temperature and time, and titrations of various potentiators of DNA damage on this experimental measure. Specifically, we determined appropriate concentrations of $FeSO_4$, DNase I and DTT to ensure maintenance of intactness of sperm shape following treatment, while also eliciting a measurable change in 8OHdG adducts. Intactness of sperm shape was monitored by phase contrast microscopy (Nikon Eclipse E200, Melville, NY, USA, 400× magnification) and by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA, USA; FC) comparing the flow rate of the untreated to treated samples. Following incubation with 10 μM $FeSO_4$ + 20 μM H_2O_2, sperm were still motile and there was no change in intactness of sperm shape or expected sperm number. Sperm dissolution was not detected with DNAse I or DTT when using concentrations selected for this study. A confocal microscope (Zeiss LSM 8 Family with Airyscan, Jena, Germany) was used to localize the FITC-conjugated 8OHdG along the sperm membrane (Fig. 1).