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Frozen-thawed rhinoceros sperm exhibit DNA damage shortly after thawing when assessed by the sperm chromatin dispersion assay

T. Portas [a], S.D. Johnston [b,*], R. Hermes [c], F. Arroyo [d], C. López-Fernadez [d], B. Bryant [a], T.B. Hildebrandt [c], F. Göritz [c], J. Gosalvez [d]

[a] *Taronga Western Plains Zoo, Dubbo, NSW 2830, Australia*
[b] *School of Animal Studies, The University of Queensland, Gatton 4343, Australia*
[c] *Leibniz Institute for Zoo and Wildlife Research, Berlin 10252, Germany*
[d] *Autonomous University of Madrid, Cantoblanco 20849, Spain*

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Abstract

This study reports on the successful validation (via in situ nick translation and neutral comet assay) of the equine Sperm-Halomax kit as an appropriate methodology for the assessment of sperm DNA fragmentation in three species of rhinoceros. Rhinoceros sperm nuclei with fragmented DNA (validated using in situ nick translation) were evident as large halos with dispersed DNA fragments, whereas those with nonfragmented DNA displayed small halos of nondispersed DNA within the microgel. There was a high correlation (r) of 0.974 (R^2 value = 0.949; $P < 0.01$; n = 16) between the respective assessments of the Sperm Chromatin Dispersion test (SCDt) and the neutral comet assay. Application of the SCDt to determine the DNA fragmentation dynamics of rhinoceros (n = 6) sperm frozen in liquid nitrogen vapor and incubated postthaw at 37 °C for up to 48 h to mimic in vitro conditions in the female reproductive tract, revealed an increase (P = 0.001) in DNA damage, as soon as 4 h after the start of incubation. Linear regression equations were calculated for all six rhinoceroses over the first 6 h of incubation and revealed individual animal variation. Freshly collected and incubated (37 °C) rhinoceros (n = 3) sperm had no increase in the basal level of DNA fragmentation for up to 48 h, indicating that the cryopreservation of rhinoceros sperm in liquid nitrogen vapor, as used in this study, appeared to result in freeze-thaw DNA damage.

Keywords: Neutral comet assay; Rhinoceros; Sperm chromatin dispersion assay; Sperm cryopreservation; Sperm DNA fragmentation

1. Introduction

Of the five extant rhinoceros species, the International Union for Conservation of Nature (IUCN) Red List of Threatened Species [1] lists three as critically endangered (Javan rhinoceros, *Rhinoceros sondaicus*; Sumatran rhinoceros, *Dicerorhinus sumatrensis*; black rhinoceros, *Diceros bicornis*), one as endangered (greater one-horned rhinoceros, *Rhinoceros unicornis*), and the other as near threatened (white rhinoceros, *Ceratotherium simum*). Ex situ reproductive management, including assisted breeding, offers substantial advantages to the conservation of captive rhinoceros populations, but the current breeding programs are unfortunately characterized by a low level of reproductive success [2]. Despite investigations into some of the underlying causes of female rhinoceros infertility [3–6], there has been comparatively little consideration given to male factor infertility.

* Corresponding author. Tel.: +617 32026902; fax: +617 33655644.
E-mail address: stevejohnston@uqconnect.net (S.D. Johnston).

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Although the relationship between testosterone secretion and reproductive success in both captive [4] and wild [7,8] males has been investigated, only one study has attempted to assess male rhinoceros reproductive soundness in terms of pathology of the reproductive tract and semen evaluation [5]. Although the same study analyzed rhinoceros semen in terms of motility and morphologic abnormalities, there has been no attempt to characterize sperm DNA fragmentation in this taxon; such information has important implications for the development of assisted breeding technology in this species, as abnormal high levels of sperm DNA fragmentation in other mammalian species is typically associated with low fertility [9,10]. Sperm DNA fragmentation (sDF) has been the subject of numerous studies on human patients and domestic animals and is now accepted as a useful correlate of male factor infertility [11].

Sperm DNA fragmentation has traditionally been regarded as a constant parameter of sperm quality, without any qualification as to the interval that the gamete needs to be assessed prior to its use in assisted reproductive technology (ART). However, there are now numerous studies, which have shown that the sperm DNA fragmentation index (sDFI) can deteriorate rapidly if the semen sample is manipulated (chilled or frozen-thawed) prior to artificial insemination [12,13]. More interestingly, there appears to be differences in the rate of DNA fragmentation on a species-specific basis [14]. For example, both ram [15] and human [16] sperm exhibit a high rate of DNA fragmentation in the first 4 h of incubation at 37 °C after cryopreservation, whereas bull and boar sperm did not have a significant increase until 48 h [13,17].

Not only were there differences in DNA fragmentation dynamics among species, but also there appeared to be variation among individuals of the same species, which may be important when selecting males for ART. Consequently, it is necessary to standardize the assessment of sDFI with respect to the individual species and the time that semen is collected and evaluated. This phenomenon is likely to be further accentuated after incubation (37 °C) of cryopreserved sperm, as frozen-thawed sperm cells are more easily induced to undergo an acrosome reaction and have an unstable plasma membrane and a shorter life expectancy [18]. It is important to emphasize that the collection and cryopreservation of rhinoceros semen in this study was an opportunistic exercise, and unlike domestic species, these animals have not been selected for reproductive efficiency.

The objective of this study was to validate the Sperm Chromatin Dispersion test (SCDt) for the rhinoceros and establish the basal level of sperm DNA damage in three species housed at Taronga Western Plains Zoos (Dubbo, NSW, Australia) in order to determine the underlying DNA fragmentation dynamics of conventional liquid-nitrogen vapor frozen-thawed sperm. Given the unusually high incidence of DNA fragmentation in the sperm of some individual animals after cryopreservation and postthaw incubation, the rate of fragmentation of freshly collected incubated semen was also compared retrospectively for the black and greater one-horned rhinoceroses. The standardization of a methodology to assess sperm DNA damage in the rhinoceros will contribute to male reproductive management of these highly endangered species.

2. Materials and methods

2.1. Animals, semen collection, cryopreservation, and evaluation

Semen was collected from three white (W1, W2 and W3), two black (B1 and B2), and one greater one-horned (O1) rhinoceros. All animals used in this study were sexually mature and housed at Taronga Western Plains Zoo as part of an ex situ captive breeding program. At the time of collection in October 2003 (W1), October 2005 (B1), October 2006 (W3, O1), July 2006 (B2), October 2007 (W2), and June 2008 (B1, B2, and O1), all animals were clinically healthy. Both W1 and B1 had ultrasonographically detected testicular lesions, later characterized as seminomas [19] (T. Portas et al., personal observations). The semen evaluated and frozen from B1 was collected from the cauda epididymis after a hemicastration procedure. Although the three white and the two adult black rhinoceroses had previously sired offspring, the greater one-horned rhinoceros had not been given an opportunity to mate. Semen from B1 (hemicastrated), B2, and O1 was also collected by electroejaculation in June 2008 as part of a retrospective auxiliary study to assess the DNA fragmentation dynamics of fresh, noncryopreserved sperm.

Semen was collected from anesthetized rhinoceroses using a combination of ultrasound-directed electroejaculation and manual massage of the penile and pelvic urethra [5]. The electroejaculate was immediately diluted 1:1 into Berliner Cryomedium (BC), which was composed of 2.41% *n*-Tris(hydroxymethyl)methyl-2-aminethanesulfonic acid (TES), 0.58% Tris, 0.1% fructose, 5.5% lactose, 15.6% egg yolk, 6.25% dimethyl sulfoxide (DMSO), and 20 IU α-tocopherol/mL [5]. The diluted semen was centrifuged (800 × g) for 10 min to remove the seminal plasma and the sperm