98 Efficacy of commercial equine semen freezing extenders for cryopreservation of southern white rhinoceros (*Ceratotherium simum simum*) sperm

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Abstract

Once nearly extinct in the wild, the southern white rhinoceros is currently listed as near threatened by IUCN. This status is likely to change as poaching continues to escalate. To preserve the species' current genetic diversity, cryopreserving and biobanking white rhinoceros sperm is imperative. The horse is the closest domestic relative of the rhinoceros and a useful model for the development of assisted reproductive technologies, including semen cryopreservation. Two equine semen cryopreservation protocols were compared to a common rhinoceros freezing method. Semen was collected from a single male on 3 occasions by electroejaculation. Initial semen parameters were 86% motility; speed 3.2 (scale 1-5); 89% plasma membrane integrity; and 95% intact acrosomes. Semen was extended 1:1 in INRA 96 (IMV Technologies, L'Aigle, France) before centrifugation at 400 × *g* for 10 min. Supernatant was removed and the sperm pellet was subjected to 1 of 2 treatments: resuspension in 500 µL of either BotuCrio (Botupharma, Botucatu, Brazil) or Cryomax (ARS Inc., Chino, CA, USA), both containing a proprietary combination of glycerol and an amide as cryoprotectants. Following a 40-min cool at 4°C, extended semen was frozen in vials at a cooling rate of 30°C/min for 3 min before LN submersion. Control semen was extended 1:1 in TEST-Y buffer without cryoprotectant and cooled for 2.5 h before adding glycerol to a final concentration of 4%. Extended sperm (500 µL) was frozen in vials at 12.5°C/min for 15 min before LN submersion. Initial motility score (IMS; % motile × speed of progression^2), plasma membrane integrity (IPL), and acrosome integrity (IAC) were recorded after extension. All vials were thawed at 37°C for 60 s and the cryoprotectant was removed by centrifugation. Sperm pellets were resupended in M199 + HEPES and sperm was evaluated for the characteristics described above at 37°C at 0, 30, and 60 min (T0, T30, T60) post-thaw. All data are expressed as a percentage of initial (%IMS, %IPL, and %IAC) to account for the differences in sperm parameters between ejaculates. Cryopreservation protocol significantly affected %IMS at T0 ($P = 0.0131$, Table 1). Although the differences were significant only at T0, sperm frozen in Botucrio or Cryomax tended to maintain a higher %IMS than the control freeze at all time points. However, sperm frozen in Cryomax lost a greater percentage of %IMS over time (67% from T0 to T60 *v.* 44 and 46% for Botucrio and TEST-Y, respectively). Cryopreservation protocol did not affect %IAC or %IPL at any time point, but again Cryomax and Botucrio tended to be higher than TEST-Y. This study indicates that rhinoceros sperm may suffer less cryodamage in Botucrio or Cryomax frozen at 30°C/min than in the conventional TEST-Y frozen at 12.5°C/min.

Table 1. Percent of initial motility score (IMS), plasma membrane integrity (IPL), and acrosome integrity (IAC) at 0, 30, and 60 min post-thaw (T0, T30, and T60, respectively)

Extender	Freeze rate	T0%IMS	T30%IMS	T60%IMS	T0%IPL	T30%IPL	T60%IPL	T0%IAC
Cryomax	30°C/min	63.0[a]	40.6[a]	20.6[a]	80.3[a]	80.3[a]	66.3[a]	81.3[a]
Botucrio	30°C/min	40.7[ab]	34.5[a]	22.7[a]	86.9[a]	70.6[a]	65.4[a]	79.7[a]
TEST-Y	12.5°C/min	27.8[b]	21.9[a]	15.1[a]	77.5[a]	61.3[a]	60.2[a]	83.4[a]
ANOVA		0.0131	0.2258	0.5541	0.8736	0.5563	0.7764	0.7743

[a,b]Within each column, means without a common letter differ ($P < 0.05$).