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The effects of Trolox on the quality of sperm from captive squirrel monkey during liquefaction in the extender ACP-118™

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Summary

The aim of this study was to evaluate the effect of incubating semen for different periods (90, 270 or 450 min) with or without Trolox® (100 or 150 µM) on the quality of sperm from *Saimiri collinsi*. Sperm motility, vigour, and plasma membrane integrity (PMI) were evaluated in both fresh semen and semen incubated for different time periods, i.e. 90, 270 or 450 min of incubation. Supplementation of semen extender with Trolox® 100 µM improved sperm motility, vigour and PMI for up to 270 min of incubation.

Introduction

A limiting factor for biotechnology in non-human primates (NHP) is handling of semen, usually ejaculated as liquid or coagulated fractions, the last fraction containing a substantial amount of viable sperm (Oliveira *et al*., 2016a, 2016b; Lima *et al*., 2017). Semen coagulum from *Saimiri* genus can be partially liquefied by using extender ACP-118™ (ACP Biotecnologia™, Fortaleza, Ceará, Brazil) without causing sperm damage (Oliveira *et al*., 2016a, 2016b). However the time taken remains a challenge, as at least 90 min are necessary for partial liquefaction (Oliveira *et al*., 2016b), and this brings risks of oxidative stress if more time is needed to increase the amount of liquefied semen (Chatterjee and Gagnon, 2001). In NHP, the use of antioxidants resulted in improved post-thaw sperm motility even from ejaculates of low freezability (Dong *et al*., 2010). Among the available antioxidants, Trolox®, an analogue of vitamin E, has the ability to permeate cells and to protect the cell membrane due to its liposoluble and hydrosoluble properties. Extender supplementation with Trolox® improves human (40 µM Trolox®; Minaei *et al*., 2012) and feline (5 mM Trolox®; Thuwanut *et al*., 2008) sperm viability and motility after cryopreservation. To improve liquefaction outcome with long incubation times, we evaluated if Trolox® (100 or 150 µM) supported *S. collinsi* sperm quality after semen incubation in ACP-118™ for 90, 270 or 450 min.

Materials and methods

Five healthy, sexually mature (>5 years) *S. collinsi* males were selected based on body weight (1000 ± 21 g), and testes consistency, symmetry and mobility. Males were housed in cages of 4.74 × 1.45 × 2.26 m (length, width, and height, respectively), under a natural photoperiod (12 h of light and 12 h of dark). The diet consisted of fresh fruits, vegetables, milk, cricket larvae (*Zophobas morio*), and a commercial pellet chow for primates. Water was available *ad libitum*. Semen was collected in the morning before feeding. For this, males were anaesthetised with ketamine hydrochloride [15 mg/kg, intramuscularly (IM); Vetanarcol; Köning S.A., Avellaneda, Argentina] and xylazine hydrochloride (1 mg/kg, IM; Köning S.A.). Under anaesthesia, the genital region was sanitized with a mild soap and distilled water (1:10) and gauze, and males were stimulated by electroejaculation (EEJ) (Autojac; Neovet, Uberaba, Brazil) (Oliveira *et al*., 2015). If a male was unable to ejaculate after the session, no further attempts were made. Intervals between collections were of at least 30 days. In total, 19 ejaculates (three or four samples per animal) were collected. Immediately after ejaculation, conical tubes (1.5 ml) containing the semen were placed in a water bath at 37°C. Volumes of liquid and coagulated fractions were assessed in a graduated tube. After this step, each ejaculate was divided into three equal aliquots, which were diluted (1:1) in ACP-118™ alone or combined with 100 or 150 µM Trolox®. Sperm parameters were evaluated before (only liquid fraction) and after partial dilution (liquefaction) of the obtained coagula. Liquefaction was performed for three different incubation times (90, 270 or 450 min). These exposure times

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Table 1. Percentages [mean ± standard error of the mean (SEM)] of sperm motility and vigour, and plasma membrane integrity observed in the control, and after incubation in ACP-118 supplemented or not with Trolox (100 or 150 µM)

Incubation (min)	Sperm motility			Sperm vigour			Plasma membrane integrity		
	Control	Trolox		Control	Trolox		Control	Trolox	
		100 µM	150 µM		100 µM	150 µM		100 µM	150 µM
0	$44 \pm 8.4^{a,A}$	$51 \pm 7.2^{a,A}$	$51 \pm 6.0^{a,A}$	$2.6 \pm 0.5^{a,A}$	$3.2 \pm 0.3^{a,A}$	$2.8 \pm 0.4^{a,A}$	$61 \pm 3.1^{a,A}$	$62 \pm 3.8^{a,A}$	$58 \pm 5.6^{a,A}$
90	$37 \pm 8.9^{a,A}$	$52 \pm 9.1^{a,A}$	$43 \pm 9.1^{a,A}$	$2.0 \pm 0.5^{a,A}$	$2.9 \pm 0.5^{a,A}$	$2.5 \pm 0.5^{a,A}$	$48 \pm 6.3^{a,A,B}$	$64 \pm 4.2^{b,A}$	$49 \pm 4.2^{a,A}$
270	$12 \pm 4.8^{a,B}$	$36 \pm 8.2^{b,A}$	$23 \pm 7.3^{a,b,B}$	$1.0 \pm 0.3^{a,B}$	$2.0 \pm 0.5^{b,A,B}$	$1.7 \pm 0.5^{a,b,A,,B}$	$36 \pm .3^{a,B,C}$	$56 \pm 5.3^{b,A}$	$41 \pm 5.3^{a,A,B}$
450	$4.0 \pm 1.9^{a,B}$	$27 \pm 7.1^{b,A}$	$10 \pm 4.3^{a,b,B}$	$0.5 \pm 0.2^{a,B}$	$1.5 \pm 0.4^{a,B}$	1.0 ± 0.3^{aB}	$23 \pm 6.6^{a,C}$	$49 \pm 6.1^{b,A}$	$29 \pm 5.0^{a,B}$
P-values									
Treatment	< 0.0001			0.0001			< 0.0001		
Incubation	0.0037			0.0188			0.0002		
Interaction	0.8573			< 0.9979			0.3914		

^{a,b}Different lowercase letters in the same row indicate differences among treatments within the same incubation time and evaluated parameter ($P < 0.05$).
^{A,B}Different uppercase letters in the same column indicate differences among incubation times within the same treatments and evaluated parameters ($P < 0.05$).

were selected based on experience under field conditions. Sperm concentration, motility, vigour and PMI were determined as previously described (Oliveira *et al*., 2015). Dead sperm were labelled with propidium iodide (0.5 mg/ml; Sigma Chemical Co., St. Louis, MO, USA) and acrosome membrane damage was accessed with fluorescein-conjugated *Pisum sativum* agglutinin (FITC–PSA; 100 µg/ml; Sigma). Hoechst 33342 stain (40 µg/ml; Sigma) was added to detect the sperm under the microscope (Celeghini *et al*., 2007). Data were analysed using the StatView 5.0 program (SAS Institute Inc., Cary, NC, USA). The animal was kept in the experimental unit. The effects of Trolox® concentration and incubation times on sperm motility, vigour, PMI and acrosome integrity were evaluated by two-way analysis of variance (ANOVA) with Tukey as the post-hoc test. Differences were considered significant when *P*-values were <0.05.

Results and Discussion

Means [± standard error of the mean (SEM)] of the collected volumes of liquid and coagulated seminal fractions were 85 ± 10 µl (0–200 µl) and 280 ± 51 µl (0–600 µl), respectively. Both fractions were transparent or opaque, and colourless, whitish or yellowish. Total sperm concentration was $24 \pm 19 \times 10^6$ sperm/ml. None of the tested treatments was effective to completely liquefy seminal coagulum and no differences in liquefied volumes were observed. This result indicated that extender should be supplemented with other compounds rather than only with antioxidant. Importantly, extender supplementation with antioxidant affected sperm parameters more significantly than did incubation time, and there was no interaction between time of liquefaction and treatment. It was possible to maintain sperm motility and vigour after 270 min of incubation at control levels only when extender was supplemented with Trolox® 100 µM. The same was observed for PMI after 450 min of incubation (Table 1). Acrosome membrane integrity was not affected by incubation time or treatment. The present results of Trolox®-supplemented extender were superior to those reported previously for *Sapajus apella* without Trolox® (Oliveira *et al*., 2011). The effect of Trolox® on sperm motility is concentration dependent. Extender supplementation with Trolox® 60 µM was detrimental to human sperm motility, whereas no free radical activity was detected in the supplemented sperm samples (Donnelly *et al*., 1999). Possibly, there are differences in sperm susceptibility to lipid peroxidation, the set of intracellular and extracellular antioxidant systems that presents in semen differs between species, plus concentration of antioxidant added to the extender was too high for human sperm, leading to a pro-oxidant effect (Cao and Cutler, 1997) as observed with 150 µM Trolox®. Prevention of lipid peroxidation in the sperm membrane was reported in samples treated with Trolox® (Sarlós *et al*., 2002). Lipid peroxidation caused failures in the metabolic rate mechanism resulting in cell death (Benzie, 1996). Once dead, the sperm released enzymes that had toxic effects on living sperm, causing changes in kinematic parameters such as decrease in sperm motility (Shannon and Curson, 1972). Therefore Trolox® could have helped motility indirectly by decreasing cell death. In conclusion, ACP-118^{TM} with 100 µM Trolox® maintains sperm quality (motility, vigour and PMI) for at least 270 min of semen incubation at 37°C. Nevertheless, seminal coagulum was not completely liquefied even after 450 min.

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Conflicts of interests. The authors declare that there is no conflict of interest that can be perceived as prejudicing the impartiality of the research reported.

Ethical standards. All experimental protocols were approved by the System of Authorization and Information in Biodiversity (SISBIO/ICMBio/MMA no. 31542-2) and by the Ethical Committee in Animal Research of Evandro Chagas Institute (no. 0010/2011/CEPAN/IEC/SVS/MS).

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