A quantifiable biochemical marker for cold shock damage to porcine sperm

H. Galantino-Homer, M. Modelski, I. Dobrinski
University of Pennsylvania, School of Veterinary Medicine, Kennett Square, PA, USA

Porcine sperm are extremely sensitive to the damaging effects of cold shock. Accidental cooling below 15–17 °C, the temperature used to ship boar semen for AI, results in cold shock damage and decreased fertility. A method to evaluate the degree of cold shock damage in shipped boar semen would be of great benefit to swine producers. We previously reported a quantifiable increase in protein tyrosine phosphorylation (PY) following cold shock that correlated with temperature and the percentages of spontaneous acrosome reacted (AR) and dead sperm [1]. Therefore, we hypothesized that protein PY is a quantifiable biochemical marker of cold shock damage to porcine sperm. The purpose of this study was to localize cold shock- and capacitation-induced increases in protein PY, and to determine whether or not the same protein undergoes PY in both situations. The sperm-rich fraction was collected from three Yorkshire cross boars, filtered, extended, and washed by centrifugation. The pellet was re-suspended in capacitating medium containing 0.8 mM 2-hydroxypropyl-*b*-cyclodextrin (CD). Sperm in CD medium were washed in PBS, allowed to settle onto coverslips, fixed with 4% paraformaldehyde, permeabilized with methanol (−20 °C), stained with 1:200 antiphosphotyrosine antibody (4G10) and 1:200 Texas Red anti-mouse secondary antibody, and assessed via color fluorescence microscopy (300 cells/slide, 630 × magnification). Data are presented as means ± S.E.M. ($n = 5$, 3 boars used) and were evaluated by ANOVA, followed by Tukey's test (significance set at $P < 0.05$). Indirect immunofluorescence and differential interference contrast microscopy revealed that PY localized to the equatorial region (intact pattern) in intact sperm, to the equatorial and acrosomal regions (AR pattern) in sperm that have recently undergone the AR, but staining of the equatorial region was lost and acrosomal staining decreased (dead pattern) under conditions that promoted cell death (additional incubation following AR induction). The predominant pattern of staining shifted significantly from intact (90.0 ± 1.5% intact, 7.3 ± 1.2% AR, 2.7 ± 0.3% dead) at 0 h before cold shock or incubation to the AR pattern following cold shock (1.3 ± 0.3% intact, 88.0 ± 1.5% AR, 10.7 ± 1.8% dead) or AR induction in capacitated sperm (4.3 ± 3.8% intact, 80.3 ± 3.5% AR, 15.3 ± 5.2% dead). This acrosomal staining could represent proacrosin binding protein PY, as identified previously in capacitated porcine sperm. Sperm proteins extracted before and after cold shock or incubation to promote capacitation were evaluated by two-dimensional gel electrophoresis using the ZOOM™ system and reagents (Invitrogen, Carlsbad, CA, USA). Antiphosphotyrosine immunoblots revealed PY proteins migrating at identical relative molecular mass and isoelectric point after cold shock or capacitation. We inferred that protein tyrosine phosphorylation of an acrosomal protein was a sensitive and quantifiable marker of porcine sperm cold shock damage.

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Keywords: Boar sperm; Acrosome reaction; Tyrosine phosphorylation; Cold shock; Cryopreservation

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Effect of cryopreservation medium on post-thaw viability of equine testicular cells

M. Ferrer [a], B. Lutjemeier [b], Y. Lopez [b], M. Sanderson [a], J. Lillich [a], M. Weiss [b]
[a] *Department of Clinical Sciences, Kansas State University, School of Veterinary Medicine, 1800 Denison, Manhattan, KS, USA*
[b] *Department of Anatomy and Physiology, Kansas State University, School of Veterinary Medicine, 1800 Denison, Manhattan, KS, USA*

Although spermatogonial stem cell (SSC) cryopreservation has been proposed for preserving genetics from valuable stallions, a freezing protocol has not been developed for horses. Our objective was to evaluate the effect of three freezing media on post-thaw viability of equine testicular cells. Testes were obtained from 6- to 12-month old colts presented for routine castration ($n = 12$), and testicular cells were isolated by enzymatic digestion. The cell suspension was incubated overnight in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) at 38 °C in 5% CO_2 in air. The following day, non-adherent cells were recovered and divided into three aliquots. Each aliquot was frozen at a final volume and concentration of 1 mL and 3×10^6 cells/mL, respectively, in one of three freezing media: (A) DMEM with

10% FBS, 10% DMSO, and 0.07 M sucrose; (B) DMEM with 10% FBS and 10% DMSO; and (C) FBS with 10% DMSO. The vials were held at −80 °C for 24 h, and then transferred to liquid nitrogen (−196 °C). After 3 weeks, cells were thawed in a water bath at 38 °C for 2 min. After adding 2 mL of DMEM with 10% FBS, cells were centrifuged and resuspended to 0.5 mL in DMEM with 1% BSA. Cell viability was assessed by Trypan blue exclusion immediately after isolation (L_0), before adding the freezing medium (L_1), and after freezing and thawing (L_2). Survival rate (S) was defined as $L_2/L_1 \times 100$. The effect of treatment on L_2 or S was evaluated using one-way ANOVA. Cell viability after isolation, culture, and freezing and thawing (L_0, L_1 and L_2) was compared using ANOVA for repeated measurements (SAS Institute Inc., Cary, NC). There was no effect of treatment on L_2 or S. Overall mean values (±S.D.) of L_0, L_1 and L_2 were 99.1 ± 1.9, 78.5 ± 14.9 and $67.8 \pm 20.5\%$, respectively ($P < 0.05$). Overall survival rate was $86.4 \pm 21.5\%$. We concluded that the three freezing media tested were equally suited for freezing equine testicular cells. Enzymatic digestion yielded a cell suspension with excellent viability, which decreased after incubation and cryopreservation. Freezing equine testicular cells immediately after isolation may result in better post-thaw viability. However, functional assays are still required to positively identify SSCs among testicular cells, and to determine the ability of this cell population to survive freezing and thawing.

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Separation of viable, motile sperm from red blood cells and dead spermatozoa: A comparison of four density gradient centrifugation media in the dog

T.C. Phillips, G. Dhaliwal, J.P. Verstegen

Small Animal Reproduction Clinic, Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA

Prior to using semen form AI or another reproductive technique, it is essential that ejaculates are devoid of factors such as blood, urine, bacteria, or debris, which could reduce fertility. Our aim was to compare different commercial media [Isolate®, Percoll®, PureCeption®, PureSperm®] for their ability to optimally separate viable, motile sperm from non-motile sperm and red blood cells (RBC). The sperm-rich second fractions of four dogs were pooled. The pooled semen was analyzed with Spermvision (Minitube of America, Verona, WI, USA) for concentration, motility, morphology, and diluted to 300×10^6/mL with CaniPro® chilled extender (Minitube of America). Canine whole blood (10%, v/v) was added to semen to mimic contamination. An aliquot (1 mL) of the blood/sperm admixture was pipetted over 4 mL of a double layered-column of the following gradients: Isolate® (Irvine Scientific, Santa Ana, CA, USA); Percoll® (Pharmacia, Uppsala, Sweden); PureCeption® (SAGE In-Vitro Fertilization, Inc., Trumbull, CT, USA); and PureSperm® (Nidacon International AB, Molndal, Sweden). After centrifugation at $400 \times g$ for 20 min at 23 °C, the gradients were divided into 1 mL fractions (Top, B, C, D, E, and Pellet). The 1-mL fractions were analyzed with Spermvision. The fractions were stained with Dif Quik (Webster Veterinary Supply, Sterling, MA, USA), and assessed with light microscopy (1000X). For the RBC:sperm ratio, 100 cells were counted in three fields in each slide (and the average calculated). The study was repeated three times over a 2-week interval. Data were expressed as means ± S.D. and analyzed with GraphPad Instat3 (GraphPad Software, Inc., San Diego, CA, USA) using one way-ANOVA to compare sperm parameters and efficiency of each media for separation of contaminants. Following $P < 0.05$, two-by-two comparisons were done with the Bonferroni multiple comparisons test. The fraction with the highest concentration of motile sperm and the lowest ratio of RBC for each media was as follows: PureCeption® pellet; PureSperm® E; Percoll® D, pellet; and Isolate® D, pellet. The percent recovery of sperm cells from the fraction with the highest concentration in each media was as follows: PureCeption® ($93.0 \pm 2.3\%$) concentration, ($72.0 \pm 13.0\%$) motility; PureSperm® ($44.8 \pm 25.9\%$) concentration, ($53.5 \pm 17.1\%$) motility; Percoll®