Torrance, CA, USA) to serve as a positive control. Media was then collected for detection of equine LH via RIA. There was a nine-fold increase in LH in GnRH vs control cells (mean ± S.E.M., 997.5 ± 114.8 vs 169.0 ± 59.2 ng/mL, $P < 0.006$), but there was no significant difference in the control vs KiSS-10 (182.4 ± 32.9 ng/mL) cells. To determine if cell density or KiSS-10 dose affected the response to KiSS-10, cells in January were also plated at two concentrations (6×10^5 vs 1.2×10^6/well), and we used three doses of KiSS-10 (1×10^2, 1×10^3, and 1×10^4 nM; $n = 3$ wells/treatment). In the absence of a significant trend in response using the two cell densities, we inferred that increasing cell density did not have an effect on gonadotrope cell function nor LH release. Nonetheless, there was a two-fold increase in LH following GnRH vs control (78.1 ± 9.3 vs 41.5 ± 6.2 ng/mL, $P < 0.005$). Interestingly, KiSS-10 also elicited a two-fold increase in LH vs control cells, regardless of dose ($P < 0.02$ for all treatments). Both the reduced LH response to GnRH and the reduced mean LH concentration obtained from non-treated control cells in January were consistent with decreased GnRH receptor number and LH content in the equine pituitary at the depth of the non-breeding season. The direct pituitary response to KiSS-10 in January may be explained by increased pituitary sensitivity to KiSS-10, mediating the ''gatekeeper'' effect in seasonal transition. Assessing seasonal changes in equine pituitary GPR54 may yield a more precise evaluation of pituitary sensitivity to KiSS-10 and delineate the hierarchal role of kisspeptins.

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Keywords: Kisspeptide; LH; GnRH; Pituitary culture; Horse

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Evaluation of cryopreserved-thawed stallion sperm before and after density gradient centrifugation with silane-coated silica particles (EquiPure®)

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By enhancing the sperm population used for AI, fewer cells can be used and dead or damaged sperm, which may have deleterious effects on the normal sperm, can be removed. Specifically, we hypothesized that the removal of dead or damaged sperm will increase the quality of live sperm after thawing of frozen semen, as measured by mitochondrial membrane potential, motility, and morphology of live sperm.

Semen from five mature stallions (two ejaculates each) was cryopreserved (in 0.5-mL straws) using a standard protocol, and stored in liquid nitrogen at −196 °C. For each ejaculate, four straws were thawed (37 °C for 30 s), diluted in a modified Tyrode's medium containing 0.1% polyvinyl alcohol (TALP-PVA), layered over an 80% EquiPure® gradient, and centrifuged at 200 × *g* for 35 min (EquiPure® sample). After centrifugation, sperm were collected from the bottom of the tube, resuspended in TALP-PVA, and washed once. Four additional straws were similarly thawed, diluted with TALP-PVA, and washed once to remove freezing extender (untreated sample). Sperm motility was determined by computerized semen analysis (CASA) and concentrations of both sperm preparations were adjusted to 5×10^6 sperm/mL. Aliquots of both preparations were analyzed for changes in membrane structure by staining with YoPro-I and propidium iodide (DNA stains with differential permeability across the plasma membrane). Mitochondrial function was assessed by staining with JC-1, a potentiometric probe which can be used to quantify mitochondrial membrane potential in equine sperm. Following incubation with YoPro-I, propidium iodide, and JC-1, samples were assessed with flow cytometry. Sperm morphology was assessed with differential interference contrast (DIC) microscopy (1000×) by an observer blinded to treatment group.

The separation of frozen-thawed equine sperm by centrifugation through the EquiPure® gradient increased ($P < 0.001$) total (80 ± 2.4% vs 41.7 ± 2.4%) and progressive sperm motility (69.5 ± 2.9% vs 31.5 ± 2.9%), as well as the percentage of morphologically normal sperm (45 ± 3.9% vs 27.7 ± 3.9%) compared to the control. In addition, the proportion of sperm in the treated sample with high mitochondrial membrane potential increased (81.6 ± 1.8% vs 42.1 ± 1.8%), as

did sperm viability (71.1 ± 2.4% vs 39.5 ± 2.4%) compared to the control. The proportion of sperm with altered membrane permeability was decreased in the EquiPure® sample (6.4 ± 1.1% vs 18.9 ± 1.1%).

In conclusion separation of frozen-thawed equine sperm via centrifugation through an EquiPure® density gradient increased total and progressive motility, normal morphology, mitochondrial membrane potential, and plasma membrane alteration of the separated sperm population (compared to the initial control). Although fertility was not examined in the present study, in specific situations, this separation procedure may improve fertility of frozen-thawed equine sperm.

Keywords: EquiPure®; Density gradient separation; Equine spermatozoa; Flow cytometry; Horse

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