Effects of thawing rate and three extenders on refreezing of equine semen for intracytoplasmic sperm injection

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Intracytoplasmic sperm injection (ICSI) is a reliable technique for assisted fertilization in horses. When frozen semen availability is limited, sperm can be thawed, diluted and refrozen to optimize their use for ICSI. We examined two thawing treatments and three commercial extenders for refreezing stallion sperm. Three 0.5 ml straws of frozen sperm from two different ejaculates were obtained for each of seven stallions; frozen at different facilities and in different extenders. For each ejaculate in the low temperature treatment (LT), one straw was thawed at 37°C for 12 sec (time required for straw to reach 5°C) and rapidly placed in a 5°C water bath. In a 5°C cold room, sperm were divided into three aliquots, diluted 4-fold in the treatment extenders (BOT, Botucro®, Betlabs, Lexington, KY; EZFMAX, E-Z Freezin® MFR5; and EZF, E-Z Freezin® MFR5, Animal Reproduction Systems, Chino, CA) and packaged in 0.5-ml straws. For the room temperature treatment (RT), a second straw was thawed at 37°C for 30 sec and at room temperature (22°C) the sperm divided into three aliquots, diluted and packaged as described. Straws were placed over nitrogen vapor for 15 min, before being plunged into liquid nitrogen. A third straw was used to evaluate original sperm parameters. Sperm were analyzed for total (TMOT) and progressive (PROG) motility by computer-assisted sperm analysis (Sperm Vision®, Minitube, Germany). Percentages of live/dead sperm (LIVE) (LIVE/DEAD® Sperm Viability Kit, Molecular ProbesTM, Eugene, OR) and sperm DNA fragmentation (DNA) (Sperm Chromatin Structure Assay, Acridine Orange, Sigma, St Louis, MO) were evaluated by flow cytometry. Results are presented as mean±SD. Analyses of variance and least squares means were performed for analyses. After refreezing, all sperm parameters were affected negatively (TMOT: 42±18 and 19±10; PROG: 33±16 and 13±10; LIVE: 37±5 and 10±8; DNA: 5±2 and 8±4; P<0.02, frozen and refrozen, respectively). Thawing treatments were also different for TMOT (LT: 23±11 and RT: 14±8; P<0.001) and PROG (LT: 17±10 and RT: 9±7; P=0.002) with higher sperm motility for sperm thawed only to 5°C. No differences were observed in LIVE (LT: 25±11 and RT: 21±9; P>0.05) or DNA (LT: 8±6 and RT: 7±2, P>0.05) between thawing treatments. Sperm refrozen in BOT had higher motility (TMOT 23±10; PROG 18±9) than sperm refrozen in EZF (TMOT 14±9; PROG 9±8, P=0.02), but was not different from sperm refrozen EZFMAX (TMOT 19±11; PROG 13±9). The percentage of LIVE and DNA were similar for extender (LIVE+ 22-24%; DNA- 7-8%). In conclusion, refreezing stallion sperm resulted in reduced motility, viability and DNA integrity, compared to sperm frozen only once. However, a modified thawing process, warming the sperm to only 5°C and processing at 5°C before refreezing in both BOT and EZFMAX resulted in sperm with the highest motility parameters, which are sufficient for ICSI.

Keywords: Stallion, semen, freezing, refreezing, thawing, ICSI