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Selection of frozen-thawed semen for standard *in vitro* fertilization

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Since the report of a repeatable method for standard *in vitro* fertilization in the horse (IVF) using prolonged sperm pre-incubation (Felix et al., Biol. Reprod. 107:1551-1564, 2022), efforts have been directed toward applying the IVF procedure clinically. A major barrier to clinical use is the need for fresh semen. In this study, we compared three techniques – swim-up (SU), colloid centrifugation (CG), and microfluidics (MFL) – to select frozen-thawed sperm for standard IVF. Media were modified TALP (TALP-R and FT-PHE, Felix et al., 2022). Straws of frozen semen from one fertile stallion were used. For SU, 200 µL frozen-thawed semen was layered under 1 mL FT-PHE in each of two 5-mL tubes. The tubes were incubated for 30 min in 5% CO_2 in air, then the uppermost 750 µL of medium was collected, combined, centrifuged and the pellet resuspended in TALP-R to ~10 million/mL. The suspension was added to 45-µL droplets of FERT-TALP to a concentration of 1 million sperm/mL (in 50 µL) and the droplets were incubated in 5% CO_2 in air. For CG, frozen-thawed semen (400µL) was layered on 3 mL of Equipure™ colloid and centrifuged for 20 min at 200g. The sperm pellet was washed once in FT-PHE, then resuspended in TALP-R and sperm added to droplets as above. For MFL, 400µL frozen-thawed semen was combined with 500µL of FT-PHE and this suspension was placed into the loading chamber of a commercial microfluidic device. FT-PHE (700µL) was placed into the collection chamber. The device was incubated for 30 min in 5% CO_2 in air, during which time sperm migrated through a porous membrane into the collection chamber. After incubation, 500µL fluid from the collection chamber was combined with 500µL FT-PHE then centrifuged for 5 minutes at 300g. The pellet was resuspended with TALP-R and sperm added to droplets as above. After droplets were incubated for 10h, *in vitro*-matured equine cumulus-oocyte complexes (COCs) were added. After 3h co-incubation, the COCs were transferred to embryo culture medium for 32h, then denuded and stained with DAPI. Cleaved embryos with two or more normal nuclei were considered to represent fertilization. Sperm selection by MFL resulted in a higher cleavage rate per co-cultured oocyte (23/32, 71.9%) than did the other two selection methods (8/35, 22.9% and 5/31, 16.1% for SU and CG, respectively). We conclude that standard equine IVF can be achieved with frozen-thawed semen and that sperm selection using a microfluidic device is associated with a higher cleavage rate than is selection using swim-up or colloid centrifugation.