Assessment of fertilizing ability of Merino ram semen cold-stored up to 48h by heterologous *in vitro* fertilization of bovine oocytes

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The use of cold-stored ram semen has been applied in sheep artificial insemination programs, since it preserves its fertilizing ability similar to fresh. Besides, the heterologous in vitro fertilization (IVF) has been successfully employed to assess semen fertilizing ability in several species. Hence, we aimed to evaluate the fertilizing ability of ram semen cold-stored up to 48h at 5 ºC by assessing heterologous IVF using bovine oocytes. Fifteen pools of three normospermic Merino ram (2-7 years) ejaculates were collected using artificial vagina, diluted to $200x10^6$ spermatozoa/ml with UHT-based extender (skim milk-6% egg yolk) and cold-stored up to 48h. *In vitro* matured zona-intact bovine oocytes were subjected to heterologous IVF using either fresh semen (FS, n=707), cold-stored to 24h (CS24, n=832) or cold-stored to 48h (CS48, n=611). In parallel, homologous IVF (Control, n=1356) and parthenogenesis (Parth Control non-fertilized oocytes n=334) were performed. Ram non-selected and selected (BoviPure) semen parameters were evaluated by CASA. Sperm-oocyte interaction was assessed at 2.5 hours post-insemination (hpi) by evaluating the number of bound spermatozoa whereas penetration and polyspermy were evaluated after 12 hpi. Presumptive zygotes were fixed and stained with Hoechst 33342 at 18, 20, 22, 24 and 26 hpi to assess pronuclear formation using phase contrast and confocal microscopy. Cleavage rate was evaluated in all groups at 48 hpi. Data obtained from 5 replicates was analysed using one-way ANOVA. Data was expressed as mean ± SEM. In terms of sperm storage time, non-selected semen showed a significant decrease ($p<0.05$) for CS24 and CS48h compared to FS on progressive motility [SPM (%): 52.30±4.1 and 36.9±5.5 *vs* 71.3±1.6] and straight-line velocity [VSL (mm/sec): 132.2±6.1 and 109.7±6.3 *vs* 176.7±4.3], respectively. However, selected semen showed a decrease ($p<0.05$) only for CS48h when compared to CS24h or FS on SPM (35.6±3.9 *vs* 56.1±6.91 and 59.3±2.6) and VSL (83.5±4.4 *vs* 105.3±6.5 and 110±2.0), respectively. No differences were observed between heterologous IVF groups in all parameters evaluated. Homologous IVF showed a higher percentage of penetration only when compared to heterologous FS group (44.4±6.8 *vs* 12.5±4.5%, $p<0.01$). The polyspermy was higher in heterologous CS24 group when compared to homologous IVF (11.4±3.4 *vs* 3.8±2.2, $p<0.05$). The homologous IVF group, as expected, showed the higher percentage of pronuclear formation at 18 hpi than heterologous IVF with FS (67.3±5.8 *vs* 35.2±5.6%), CS24 (72.1±4.5 *vs* 37.2±5.7%) and CS48 (63.0±6.0 *vs* 27.0±5.6%), respectively ($p<0.001$). Likewise, cleavage rate was higher in homologous group compared to heterologous IVF and parthenogenetic groups for FS (78.3±2.6.8 *vs* 46.3±3.2 and 7.0±2.3%), CS24 (78.4±2.6 *vs* 48.3±3.2 and 4.9±2.0%), and CS48 (78.4±3.3 *vs* 43.3±3.5 and 4.3±1.2%), respectively ($p<0.001$). In conclusion, Merino ram semen cold-stored up to 48h maintains its fertilization ability in the same extend as fresh and can be used for sheep crossbreeding programs.