Sperm RNA characteristics in stallion fresh semen are influenced by somatic cell lysis.

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Increased chromatin condensation and the reduced content of RNA render sperm challenging for RNA extraction and species-specific protocols are required. We aimed to optimize RNA isolation techniques in fresh stallion sperm, with particular focus on the relevance of somatic cell lysis.

Raw semen from 5 different stallions was diluted with EquiPlus extender. Sperm characteristics motility (CASA), morphology and DNA integrity (Aniline blue) were determined. With the remaining sample, density gradient centrifugation (Equipure) followed by two centrifugation steps in PBS was performed. One part was treated for somatic cell lysis (group SCL), the other was not (group NSCL). From each part, four portions were formed that underwent A) incubation with Trizol (room temperature); B) bead-based homogenization (3x) in Trizol; C) heated trizol (65°C 30 min); or D) bead-based homogenization (3x) + heated Trizol (65°C 30 min). In all samples, RNA was isolated using Direct-zol columns. RNA contamination by solvents (260/230 ratio) and by proteins (260/280 ratio) was considered, together with RNA integrity (DV200 Index) and RNA yield (TapeStation). Differences in the abundance of genes previously proven to be correlated with stallion fertility (*PRM1*, *PRM2*, *PRM3*, *CRISP3*, *PLCz1*, *ACR*, *ZPBP*, *PRDX5*, *NOX5*, *SOD)* with regard to treatment were determined by RT-qPCR (reference genes: *ACTB* and *RPL32)*. Statistical analysis was performed with two-way repeated measures ANOVA (IBM-SPSS statistics 29.0.1.0).

Somatic cell lysis influenced ($p<0.05$) RNA purity (260/280 ratio) and RNA integrity. Nevertheless, the relevance of 260/280 ratio remains questionable because numerical differences were low but still better for NSCL group considering 1.8-2.0 as the ideal range. With regard to RNA integrity, there was an effect of cell lysis ($p<0.05$) In both groups, lowest DV200 index was determined with heated Trizol (SCL: 39.86 ± 3.12; NSCL: 46.22 ± 6.29). With regard to RNA abundance, some of the genes (PRM1, PRM3, PLCz1, ACR) were overexpressed ($p<0.05$) in SCL, while SOD1 and CRISP3 were overexpressed ($p<0.05$) in NSCL. The mRNA from genes ZPBP, NOX5 and CRISP3 was not detected in many samples in SCL. Results suggest that the RNA extraction protocol has considerably effects

when analyzing mRNA abundance in sperm. The overexpression of CRISP3 in NSCL is therefore not considered reliable in this study.

This study confirmed that in stallion sperm, the inclusion of somatic cell lysis in RNA extraction improves RNA characteristics. The use of heated Trizol cannot be recommended. These protocols should also be tested in frozen-thawed semen where an increase in RNA disruption caused by the cryopreservation procedure must be considered.