Investigations on antimicrobial properties of stallion seminal plasma

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When collecting semen from stallions, bacterial contamination cannot be completely avoided. To prolong the storage life of sperm, semen extenders therefore include antibiotics to inhibit bacterial growth. This approach, however, meets the definition of excessive or wrong antimicrobial use, considered a major cause for the development of antimicrobial resistance. We were therefore interested in characterizing antimicrobial properties of stallion seminal plasma (SP) with the final aim to further exploit such properties in cooled-stored stallion semen. Semen was collected by artificial vagina from 5 healthy normospermic stallions (6-29 years) of different breeds. For collection of SP, raw semen was centrifuged (10 min, 1800xg, 4 °C), the supernatant checked for absence of sperm, centrifuged again (10 min, 15000 x g, 4 °C) for removal of remaining bacteria and the supernatant then frozen at -80 °C. For extraction of antimicrobial peptides (AMP) from SP, ultrafiltration (10,000xg for 85min) was performed (omega membrane 10kDa; Pall Life Sciences, Portsmouth, UK), followed by electrophoresis (Tricine-SDS-PAGE) and Orbitrap LC-MS/MS analysis. The AMPs psoriasin and equine lysozyme were detected in very low abundance. For determination of antimicrobial effects of SP, bacterial isolates of *Streptococcus (Sc.) equi ssp. zooepidemicus* and *Escherichia (E.) coli* were used in a liquid growth inhibition assay (Schulze et al. 2019; Theriogenology 157:335-340) with minor modifications. The SP (from 2 ejaculates of each of 5 stallions) was diluted 1:1 (v:v) with tryptic soy broth (TSB), then 100 µl mixed with 800 µl bacterial suspension in TSB in two trials (starting concentrations of bacteria: $1x10^5$ and $1x10^3$ CFU, respectively). As controls, TSB with SP and bacterial suspension in TSB were used. Optical density (OD) was measured (spectrophotometer, 595 nm) at 0h and after 24h incubation (37°C). At 24h, serial dilutions were incubated at 37 °C on Columbia agar for 24h for determination of CFU. Statistical analysis of OD values was performed with two-way repeated measures ANOVA (IBM-SPSS statistics 29.0.1.0). For analysis of differences between CFU values, non-parametric tests were used. Based on changes in OD, there was a bactericidal effect of SP on growth of *Sc. zooepidemicus*, but only a minor effect on *E. coli* (time $p<0.001$, bacterial species $p<0.001$, time x bacterial species $p<0.001$) in both trials. When analysed by CFU, bacterial growth was inhibited in comparison to controls irrespective of the bacterial species ($p<0.01$). We conclude that stallion SP has antimicrobial properties effective against both, gram-positive and gram-negative bacteria. These are most likely not caused by the low concentrations of equine AMPs determined in this study. Stallion SP is therefore suggested to contain non-protein substances with antimicrobial activity. Further characterization of these substances may help to develop additives with antimicrobial properties to replace antibiotics in semen extenders.