

PRELIMINARY ASSESSMENT OF BASIC SEMEN QUALITY PARAMETERS IN STALLIONS WITH POTENTIAL RELEVANCE TO CRYOPRESERVATION

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ABSTRACT

The aim of this preliminary study was to characterize basic semen quality parameters in stallions and to assess their potential relevance to semen cryopreservation. Semen samples were collected from five stallions of different breeds: two stallions of the Slovak national breed Norik of Muráň aged 13 and 20 years (Jantár, Nerot), two stallions of the Slovak national breed Hucul aged 11 and 19 years (Gurgul, Oušor) and one stallion of the Czech national breed Norik Silesian (Reny) aged 5 years. The collected samples were analysed using Computer-Assisted Sperm Analysis (CASA) and flow cytometry. Total motility (TM), progressive motility (PM) and sperm functional parameters, including viability, apoptosis (YO-PRO-1) and acrosomal integrity (PNA AF647), were evaluated.

The results showed individual variability among stallions. The highest semen quality was observed in Hucul breed stallions Gurgul and Oušor, with TM values ranging from 85 to 88 % and PM - from 72 to 73 %. In contrast, the stallion Reny exhibited the lowest motility (TM - 48 %, PM - 31 %) and reduced viability. Flow cytometry confirmed that higher motility corresponded with a higher proportion of live (42-87 %) and viable (38-56 %) spermatozoa. However, increased levels of early apoptosis were detected in some stallions with otherwise favourable parameters, indicating possible sublethal cellular damage. The proportion of sperm with damaged acrosomes remained below 2 % in all samples.

In conclusion, the Hucul breed stallions Gurgul and Oušor showed the most favourable sperm quality profiles in this preliminary assessment, suggesting potential suitability for further evaluation of semen cryopreservation, while the Norik Silesian stallion Reny exhibited parameters indicating poor cryopreservation potential. The combination of CASA and flow cytometry provided complementary information on sperm motility, viability and cellular integrity; however, further studies including post-thaw assessment and fertility data are required to determine their predictive value for cryotolerance and reproductive outcome.

Keywords: semen, freezing, CASA, flow cytometry

INTRODUCTION

Artificial insemination (AI) is widely applied for improving productivity and health in livestock breeding globally, including horses. Recognizing its significant benefits, the equine industry has increasingly embraced cooled-transported semen as a desired breeding method (Khalil *et al.*, 2025).

Cryopreservation remains the only reliable method for long-term storage of male germplasm, supporting livestock breeding programmes and the conservation of endangered species. However, freezing and thawing impose additional stress through ice crystal formation, osmotic imbalance and oxidative damage, also remodelling sperm membrane proteins in a manner that mimics premature capacitation (Boni *et al.*, 2025).

The cryopreservation of semen may impair the process of capacitation and, consequently, the acrosome reaction, which is a process necessary for the proper fertilisation process. The acrosome reaction in equine spermatozoa is known to be more difficult to induce compared to other species, and substances such as calcium ionophores, progesterone, or zona pellucida glycoproteins have been used in various protocols to trigger this reaction (Bugno *et al.*, 2025).

Cryopreservation of equine semen remains challenging due to pronounced individual variability in cryotolerance, because freezing induces oxidative stress and spermatozoa are particularly susceptible to such damage (Boni *et al.*, 2025). It is known that sperm morphometric parameters significantly correlate with fertility in horses and other mammals. The size and shape of sperm can profoundly influence its motility, capacitation and fertilisation capabilities (Nooroozian *et al.*, 2024).

The aim of this preliminary assessment was to characterize basic semen quality parameters in a small group of stallions and to evaluate whether these parameters may provide an initial indication of their potential relevance to cryopreservation and subsequent storage in a gene bank.

MATERIAL AND METHODS

Biological material and semen collection

This study was designed as a pilot assessment of semen quality parameters in a small number of stallions. Two stallions of the Slovak national breed Norik of Muráň aged 13 and 20 years (Jantár, Nerot), two stallions of the Slovak national breed Hucul aged 11 and 19 years (Gurgul, Oušor) and one stallion of the Czech national breed Norik Silesian (Reny) aged 5 years were used. Semen was collected at the National Stud Farm in Topoľčianky and at the Provincial Stud Farm Tlumačov using a Missouri-model artificial vagina (AV). Stallions were transported from their home farm and ejaculate was collected during a phantom jump on the stud farm premises. Qualified personnel were responsible for collecting the semen. Immediately after collection the semen was diluted at a 1:1 ratio with a Botusemen commercial extender (Botupharma, USA) and transported to the laboratory at room temperature (RT). Ejaculate was collected only once from each stallion.

Stallion semen analysis

Sperm concentration and motility parameters were evaluated using the SpermVision™ Computer-Assisted Sperm Analysis (CASA) system (Minitube, Germany). Samples were analysed using a Makler counting chamber maintained at 37 °C. The SpermVision™ settings included a field-of-view depth of 20 µm and a sperm cell identification area of 14–80 µm². Sperm trajectories were smoothed using five points. According to the software classification settings, spermatozoa with an average orientation change (AOC) < 9.5 were classified as immotile, whereas motile spermatozoa with a distance straight line (DSL) < 6 were classified as locally motile; the remaining motile spermatozoa were classified as progressively motile. For each semen sample, two technical measurements were performed, with seven microscopic fields evaluated per measurement, resulting in a total of 14 analysed fields per sample. The values obtained from the two

measurements were averaged and the resulting mean values were used for descriptive evaluation.

Flow cytometric evaluation of sperm characteristics was performed using a CytoFLEX S flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with Near UV (375 nm), Violet (405 nm), Blue (488 nm) and Red (638 nm) lasers. Data acquisition and analysis were carried out using CytExpert software (Beckman Coulter, Brea, CA, USA).

Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA) was used for DNA staining and identification of spermatozoa. It was excited by the Near UV laser (375 nm) with emission detected in the 450/45 nm channel. The samples were first incubated with Hoechst 33342 for 15 minutes in the dark at RT.

Yo-Pro-1 fluorochrome (0.1 µM; Thermo Fisher Scientific, Waltham, MA, USA) was used to detect early apoptotic-like changes. It was excited by the Blue laser (488 nm) with fluorescence detected in the 525/40 nm channel. Samples were incubated for 15 minutes at RT in the dark.

Propidium iodide (Thermo Fisher Scientific, Waltham, MA, USA) was used to identify membrane-compromised (non-viable) spermatozoa. It was excited by the

Blue laser (488) with emission collected in the 610/20 nm channel. PI was added immediately prior to flow cytometric measurement.

Acrosomal integrity was evaluated using PNA AF647 (0.5 mg/mL, Thermo Fisher Scientific, Waltham, MA, USA). When the spermatozoa are not fixed (as in this study), this fluorochrome binds to the acrosome of spermatozoa with a damaged plasma membrane, whilst the acrosome of the sperm with intact plasma membrane remains inaccessible to PNA and, therefore, unstained. This fluorochrome was excited by the Red laser (638 nm) with emission detected in the 660/20 nm channel. Unfixed samples were incubated for 15 minutes at RT in the dark.

All staining procedures were performed in the dark under controlled temperature conditions to prevent photobleaching. After incubation, samples were immediately analysed by flow cytometry. For each sample, at least 10,000 events were recorded at a low flow rate. Sperm populations were initially gated based on forward scatter (FSC) and side scatter (SSC) characteristics to exclude debris. Hoechst 33342-positive events were further selected to confirm sperm-specific populations.

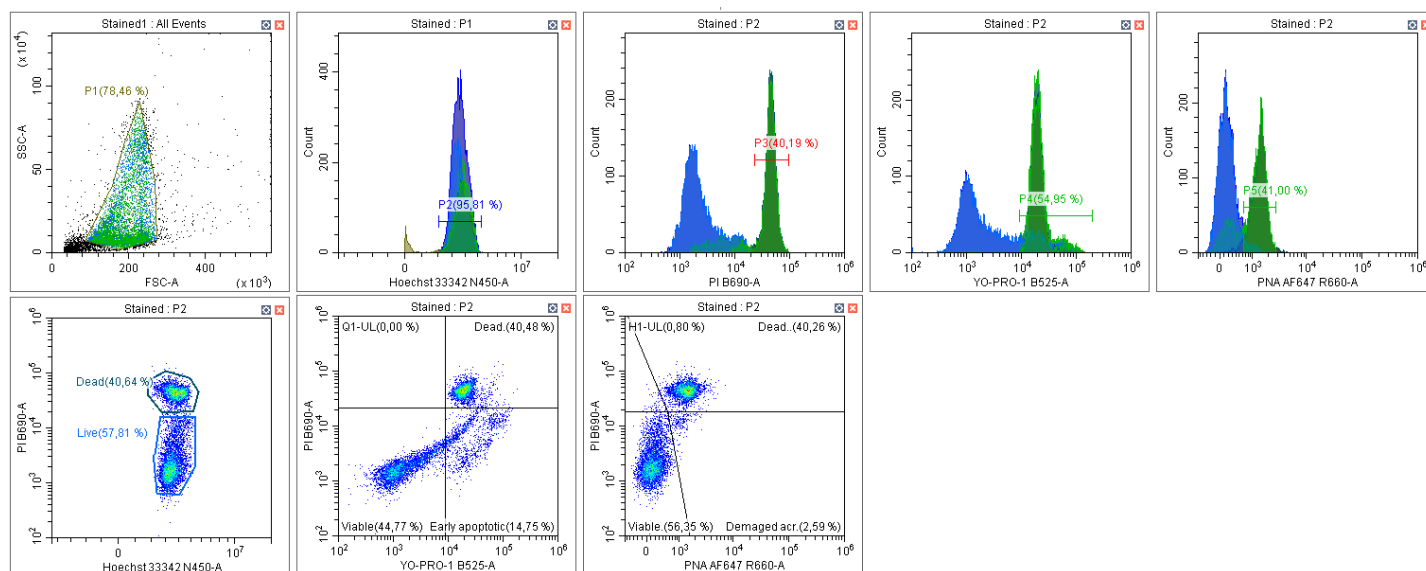


Figure 1 Flow cytometry gating strategy

We determined the populations as live (PI-negative), viable (PI-negative, Yo-Pro-1-negative), apoptotic-like (Yo-Pro-1-positive), and plasma membrane-damaged (PNA-positive) spermatozoa.

RESULTS

The best sperm motility results were observed in the stallions Oušor and Gurgul, with the total motility (TM) within the range of 85–88 % and progressive motility (PM) in the range of 72–73 % (Fig. 2). Jantár and Nerot had slightly reduced sperm motility (TM value between 54 and 64 % and PM value between 45 and 54 %), while Reny showed the lowest motility values (TM - 48 % and PM - 31 %).

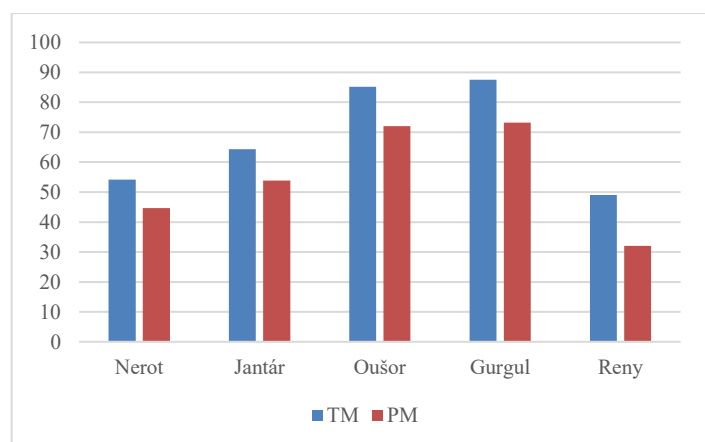


Figure 2 Total (TM) and progressive (PM) motility of stallion semen

Figure 3 shows that Gurgul had the highest proportion of live (87 %) and viable (56 %) sperm. At the same time, he had a higher proportion of apoptotic cells (21 %). Oušor had high proportions of live (72 %) and viable (50 %) sperm values, but the highest proportion of apoptosis (29 %). Jantár had medium live (60 %) and

viable (45 %) values and lower apoptosis values (13 %), which represented a stable, although not optimal, profile. Nerot had live (53 %) and viable (48 %) values and low apoptosis values (7 %). Reny had the lowest live (42 %) and viable (38 %) values and low to medium apoptosis values (11 %). This was consistent with the lower motility observed in this stallion.

Staining with PNA AF647 showed that the number of sperm with a disrupted plasma membrane did not exceed 2 % in any stallion.

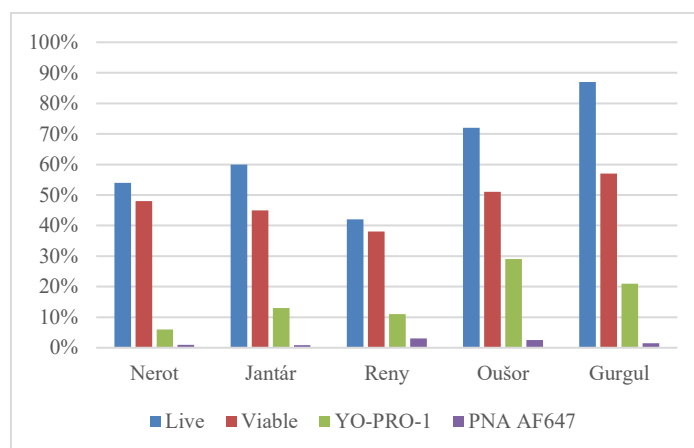


Figure 3 Sperm viability parameters for each stallion (Flow cytometry results)

DISCUSSION

Sperm motility results showed individual differences between stallions, with the highest values of total (TM) and progressive (PM) motility being recorded in Gurgul and Oušor stallions. Motility values in the Hucul breed are comparable to those reported in the study by Bugno *et al.* (2025). The use of frozen semen lowers the risk of disease transmission, eliminates geographical limitations and supports the implementation of genetic resource protection programmes. However, due to

the very rare use of frozen semen from Hucul stallions, their genetic material is not secured in sperm banks, and very little information is available about their semen, including its suitability for cryopreservation, and sperm survival rates after thawing (Bugno *et al.*, 2021).

Conversely, the lowest values were observed in the Reny stallion. It is generally stated that total motility should exceed 70 % and progressive motility reach the range of 50 – 60 % in fresh ejaculate of a normal stallion (Egyptien *et al.*, 2023), with the minimum acceptable value of TM being above 50 % (Bitton *et al.*, 2025). Based on these criteria, most of the stallions studied met the requirements for quality semen, except for the Reny stallion, which did not reach optimal values, which may indicate a less favourable profile for cryopreservation; however, post-thaw sperm survival was not assessed in the present study.

However, from the point of view of current knowledge, it is emphasized that motility itself is not the only decisive factor in fertility, but the total number of progressively motile sperm in the insemination dose is especially key (Egyptien *et al.*, 2023). This means that even with lower motility, fertility can be maintained if there is enough quality sperm, which needs to be considered when interpreting the results.

The results of flow cytometry provided a more detailed view of sperm quality at the cellular level. The highest proportion of live and viable sperm was recorded for the Gurgul stallion, which correlated with his high motility. A similar trend was also observed for the Ouřor stallion. On the contrary, the Reny stallion showed the lowest proportion of live and viable sperm, which was consistent with his low motility. These results confirm that the integrity of the cell membrane and the viability of sperm are closely related to their motor activity.

An interesting finding is the increased proportion of apoptotic cells (Yo-Pro-1-positive) in some stallions with otherwise good parameters (e.g. Ouřor and Gurgul). This phenomenon may indicate ongoing sublethal cell damage, which may not yet be manifested by a decrease in motility, but may affect the fertilising ability of sperm. In this context, it has been reported that microbial contamination may increase the production of reactive oxygen species, especially H₂O₂, in refrigerated semen, leading to reduced tolerance to cryostorage (Boni *et al.*, 2025). Oxidative stress may, therefore, be one of the factors influencing the observed differences between individuals.

In addition, the age of the stallion has a significant impact on sperm quality, affecting almost all evaluated parameters (Boni *et al.*, 2025). Although age was not explicitly analysed in this study, it may represent an important factor explaining individual differences between stallions, especially in the case of lower motility and viability values.

Several limitations should be considered when interpreting these findings. The study included only five stallions, with a single ejaculate collected from each individual, and was therefore designed as a preliminary assessment rather than a definitive evaluation of semen cryotolerance. In addition, the stallions differed in breed and age, and these factors were not analysed independently. Importantly, semen was not subjected to cryopreservation and post-thaw sperm quality or fertility outcomes were not evaluated. Therefore, the present findings cannot establish the predictive value of the assessed parameters for cryosurvival or fertility. Larger studies involving repeated ejaculates, controlled cryopreservation protocols, post-thaw assessment and, where possible, reproductive outcomes are required to validate the observations reported here.

Overall, the Hucul stallions showed the most favourable semen quality profiles in this preliminary assessment. These findings suggest that their ejaculates may warrant further evaluation using actual cryopreservation and post-thaw analyses. The lower motility and viability observed in Reny (Silesian Norik) suggest a less favourable baseline semen quality profile; however, his actual cryotolerance cannot be determined from the present data. The combination of classical motility assessment and flow cytometry provides complementary information on sperm quality and may be useful for the preliminary characterization of ejaculates prior to cryopreservation. Its value for predicting post-thaw sperm survival or fertility should be evaluated in larger studies including cryopreservation outcomes and reproductive data.

CONCLUSION

The present study demonstrated individual variability in semen quality among stallions of different breeds, which may strongly influence their suitability for cryopreservation. The Hucul stallions Gurgul and Ouřor exhibited the most favourable ejaculate characteristics, including high total and progressive motility, as well as a high proportion of live and viable spermatozoa, suggesting a favourable baseline profile for further cryopreservation assessment and potential use in genetic resource preservation. In contrast, the Norik Silesian stallion Reny showed reduced motility and viability values, suggesting a lower tolerance to cryopreservation procedures.

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REFERENCES

- Bitton, A., Frishling, A., Kalo, D., Roth, Z. & Arav, A. (2025). The Impact of Precisely Controlled Pre-Freezing Cooling Rates on Post-Thaw Stallion Sperm. *Animals*, 16 (1). <https://doi.org/10.3390/ani16010021>
- Bugno, M., Bielecka, M., Pietras, N., Kij-Mitka, B., Podstawski, Z. & Dlugosz, B. (2025). Influence of Cryopreservation on the Acrosome Reaction in Hucul Stallion Spermatozoa. *Animals*, 15 (13). <https://doi.org/10.3390/ani15131915>
- Bugno, M., Kij-Mitka, B., Podstawski, Z., Dlugosz, B., Lasek, O., Mrowiec-Kucharska, P. & Tischner, M. (2021). Characteristics of diluted-stored and post-thawed semen of Hutsul stallions. *Acta Veterinaria Hungarica*, 70 (1), 51-57. <https://doi.org/10.1556/004.2021.00050>
- Boni, R., Ruggiero, R., De Luca, F., Preziosi, G., Ferrara, M. A., Ostuni, A., Guerreiro, S., Gallo, A., Murano, C. & Cecchini, S. (2025). Enhancing Stallion Semen Cryopreservation: Selected Antioxidant Extracts and Sperm Freezability. *Antioxidants*, 14 (11). <https://doi.org/10.3390/antiox14111363>
- Egyptien, S., Deleuze, S., Joy, L. & Ponthier, J. (2023). Sperm Quality Assessment in Stallions: How to Choose Relevant Assays to Answer Clinical Questions. *Animals*, 13 (19). <https://doi.org/10.3390/ani13191123>
- Khalil, W. A., Mostafa, H. E., Derbala, M. K., Alfattah, M. A., Alhujaili, W., Hassan, M. A. E., El-Harairy, M. A. & Abdelnour, S. A. (2025). Efficacy of butylated hydroxytoluene nanoparticles in enhancing the quality and preservation of stallion chilled semen. *Veterinary Research Communications*, 50 (1). <https://doi.org/10.1007/s11259-025-10977-5>
- Nooroozian, A., Goodarzi, N. & Tafti, R.D. (2024). Morphology and morphometry of sperm in Kurdish stallions, a local breed from western Iran. *Reproduction in Domestic Animals*, 59 (1). <https://doi.org/10.1111/rda.14534>