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INFLUENCE OF THE CRYOPRESERVATION PROCESS ON DNA STABILITY IN HUCUL STALLION SPERM

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Abstract

Hucul horses, which are covered by the Genetic Resources Conservation Program, belong to one of the oldest breeds in Poland. Due to the nature of the breeding program, which heavily favors natural mating, semen from Hucul stallions has not been tested previously in terms of the level of sperm chromatin damage and its impact on fertilizing ability. The aim of this study was to assess the level of DNA fragmentation in Hucul stallion sperm, both in chilled semen and after cryopreservation, using the Halomax® test. The study identified highly significant differences in sperm DNA fragmentation ($p<0.01$) between chilled and thawed semen, with the cryopreservation process significantly reducing semen quality.

Keywords: Hucul horses, semen, DNA fragmentation in spermatozoa

1. Introduction

Sperm DNA fragmentation (SDF), defined as a single or double stranded DNA break, could be one of the causes of infertility. The main mechanisms causing DNA damage include apoptosis during spermatogenesis (Muratori et al., 2019), DNA strand breaks caused by chromatin remodeling during spermiogenesis (Sakkas and Alvarez, 2010), and DNA fragmentation induced by oxidative stress (Gil-Guzman et al., 2001). Endogenous caspases and endonucleases, the use of radio- and chemotherapy, as well as the influence of toxins from the environment might also be responsible for DNA damage (Sakkas and Alvarez, 2010).

Sperm DNA damage can also occur during sperm and semen preparation procedures used in assisted reproduction. For example, incubation of semen at 37.8°C after centrifugation may increase the level of SDF. Also, the cryopreservation process can lead to an increase in the degree of DNA fragmentation, especially in individuals with reduced fertility (Donnelly et al., 2001).

DNA fragmentation, which is a permanent, irreversible phenomenon, independent of other parameters, is the result of the inability of spermatozoa to repair damaged DNA (Simon et al., 2019). Although sperm with damaged DNA can fertilize an oocyte, this damage can

affect preimplantation embryo development, with a high risk of early miscarriage for any embryos that do implant (Robinson et al., 2012).

Hucul horses, with their unique features, constitute an invaluable genetic resource and are included in the Genetic Resources Conservation Program. As a result, the breeding of Hucul horses is based on natural mating as the standard practice, with artificial insemination with fresh, chilled or frozen semen, as well as transfer of embryos, allowed only with the approval of the Stud Book Commission. Due to these requirements of the breeding program, the current state of knowledge on the semen parameters of Hucul stallions is very limited, as this material is not routinely available, nor subject to the cryopreservation process. With conservation breeds, the priority is to create a gene bank, and semen, which is the most valuable biological material, is its essential element.

The aim of this study was to assess the level of DNA fragmentation, using the Halomax® test and the SCA® automatic semen analysis system, in chilled and cryopreserved sperm samples from Hucul stallions.

2. Material and methods

2.1. Animals

All the stallions from which the semen was obtained came from one stud, the Hucul Horse Stud in Gładyszów, where they were maintained under uniform environmental and nutritional conditions, and where standard breeding methods are routinely used. The animal study protocol was approved by the Institutional Commission for Animal Welfare of the Department of Animal Husbandry and Biology. All animal procedures were approved by the Local Ethics Committee in Krakow, resolution No. 98/2021, in accordance with the legal regulations of the European Union.

2.2. Sperm collection

The analysis included semen from 15 Hucul stallions (stallions marked with Roman numerals from I to XV), aged between 4 and 18 years. A single ejaculate was collected from the stallions, using an open-ended artificial vagina (Ar Model – University of Agriculture in Krakow) which allowed the separation of the sperm-rich fraction from the gel fraction and/or to avoid contamination of the semen with blood, urine, or bacteria. The collected ejaculates were diluted with the INRA96 diluent in a 1:1 ratio, secured in sterile 50 ml Falcon tubes and transported at +4°C to the laboratory within a maximum of 6 hours, where they were subjected to a detailed evaluation and cryopreservation.

2.3. Evaluation of sperm concentration and motility

Prior to the cryopreservation process and evaluation of the level of SDF, each ejaculate was analyzed for sperm concentration and motility. For each specimen, extended semen (2 µl) was loaded into a GoldCyto® slide chamber (TK Biotech, Warsaw, Poland) and analyzed under phase contrast microscopy using final magnification 100× and the Motility module of the SCA® system.

2.4. Semen cryopreservation

After the assessment of sperm concentration and motility, the extended semen was cryopreserved. After centrifugation (8 minutes/2000 rpm), to isolate sperm from extended seminal plasma, each ejaculate was combined with the Spectrum Red extender (SBS CryoSystem Spectrum Freezing Extender; Insatex-MT, Poznań, Poland) in an amount appropriate for the concentration and mixed, then transferred to 0.5 ml straws and equilibrated for 30 minutes in

a refrigerator. Then the straws were placed on a float and chilled in liquid nitrogen vapor for 15 min, before being transferred directly to liquid nitrogen and stored until analysis.

2.5. Semen preparation for fragmentation level analysis using Halomax®

To analyze the level of SDF, chilled semen, obtained 6 hours earlier and stored in a refrigerator, and the same semen subjected to the cryopreservation process, were used. The chilled semen from each stallion was transferred into an 1.5 ml Eppendorf tube and then a 1x PBS solution was added to it. The amount of semen and 1x PBS were selected in such a way that the final sperm concentration was 15–25 × 10^6 spermatozoa/ml, which allowed for proper analysis. The suspension prepared in this way was centrifuged for 8 minutes/2000 rpm. The supernatant was aspirated, retained and used for further preparation of samples for the Halomax® DNA fragmentation assay.

One straw of frozen semen from each stallion was used for testing. The straws were removed from liquid nitrogen and thawed at 37°C for 30 seconds. The thawed semen was then transferred to an Eppendorf tube and processed in the same way as the chilled semen.

2.6. DNA fragmentation analysis – Halomax®

To assess the level of SDF, the Equus-Halomax® kit (Halotech DNA SL, Madrid, Spain) was used. This kit is intended for the analysis of stallion spermatozoa, in both fresh and thawed semen samples. For each ejaculate, 25 µl of prepared semen was mixed with 50 µl of agarose solution and incubated at 37°C. Then, 1.5–2 µl volumes of each sample were placed in the wells of the test slide, covered with a coverslip, and transferred to the refrigerator for 5 minutes. After the agarose and semen solidified, the coverslip was removed and the 8-well slide was placed horizontally in 10 ml lysis solution for 5 minutes. The slides were then washed in distilled water for 5 minutes and dehydrated using a 70% and 100% ethanol series, for 2 minutes each.

Staining was performed just prior to the actual evaluation. Dyes provided by the manufacturer (Halosperm G2®) were used for light microscopy analysis. Previously prepared slides were incubated for 7 minutes in red Staining Solution A (SSA) and then for another 7 minutes in blue Staining Solution B (SSB) and left to dry. DNA fragmentation was evaluated using Nikon Eclipse E200-LED microscopy. A minimum of 300 spermatozoa from each semen specimen were evaluated.

2.7. Statistical analysis

The statistical significance of the results was determined using the SAS Enterprise Guide program. Data distribution was verified using the Shapiro–Wilk test. Because the data did not follow a normal distribution, a non-parametric sign test was used to compare chilled and thawed semen samples. For DNA mobility and fragmentation (Halomax®), a sign test was chosen, which is a non-parametric equivalent of the Student's t-test for matched pairs. Linear regression was used to determine the relationship between the stallion age and the level of SDF, and the correlation was determined using the Spearman correlation coefficient in the OriginLab 2021b program.

3. Results

3.1. Effect of stallion age

We first investigated whether the age of the stallion had an influence on the incidence of SDF. Based on the linear regression analysis, there was no significant relationship between stallion age and SDF level in either chilled semen or thawed semen (both $p>0.05$).

Similarly, the correlation between age and SDF (r = 0.03) was low for chilled semen (r = 0.03) and for thawed semen (r = 0.09) (Figure 1).

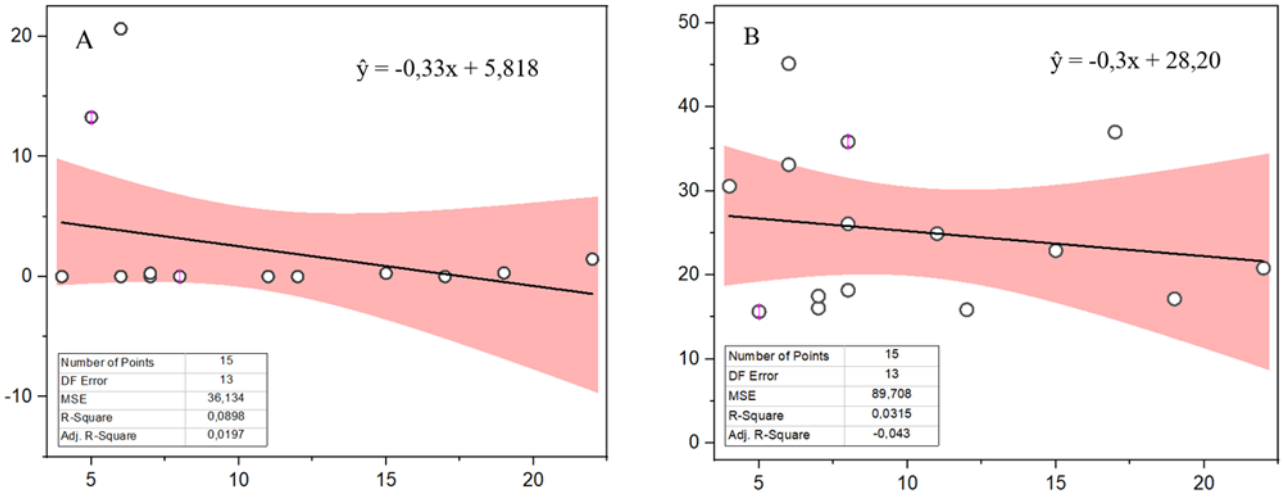

Figure 1. The relationship between the level of SDF and stallion age, in chilled semen (A) and in thawed semen (B)

3.2. Motility

Depending on the sample concentration, the number of areas of the slide assessed for the motility analysis varied from a few to around a dozen. Results were generated using the SCA® system, and are presented in Figure 2.

All specimens assessed showed expected levels of sperm motility in chilled semen (no data for stallion III). In the thawed semen of eight stallions (I, II, V, VI, VII, IX, XI, XIV) the motility was lower than normal (≤40%).

Overall, the cryopreserved spermatozoa had significantly lower motility than the chilled spermatozoa ($p<0.01$). This parameter has significantly decreased.

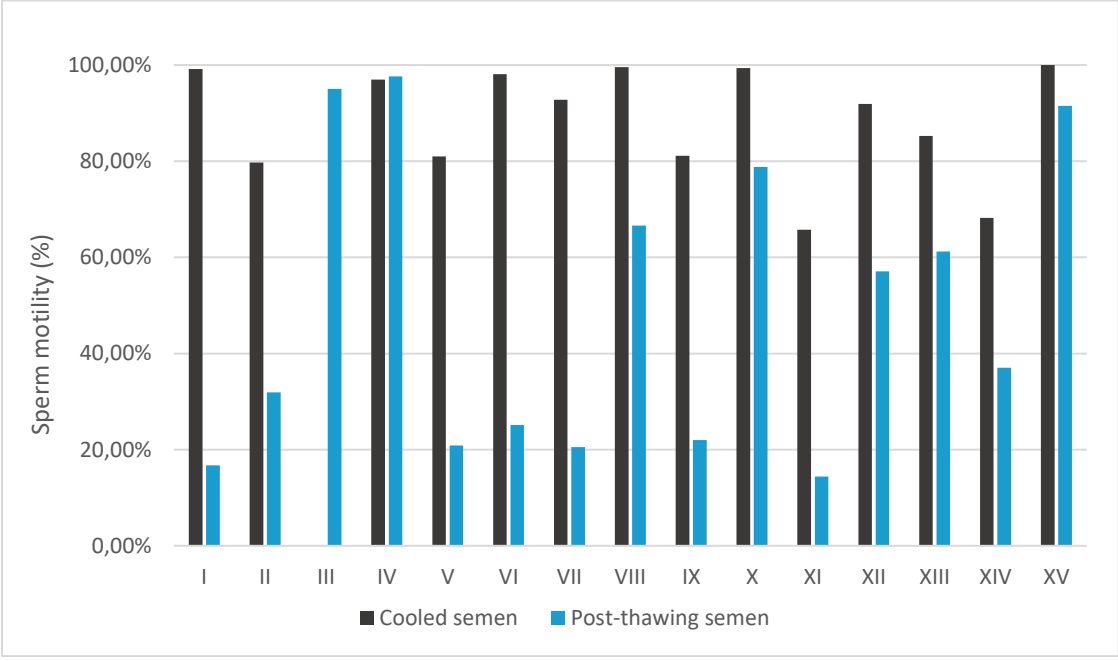

Figure 2. Comparison of sperm motility in chilled semen and in thawed semen

3.3. DNA fragmentation

The DNA Fragmentation module of the SCA system was used to determine the proportion of spermatozoa with DNA fragmentation (Figure 3). In all stallions, the proportion of spermatozoa without DNA fragmentation in chilled semen was normal (≥70%). In frozen semen, the level of SDF was below the normal range for five stallions (I, IX, XI, XII and XV), while the remainder had an increased level of SDF, but within the normal range.

Using the sign test, highly significant differences ($p<0.01$) were found in the level of DNA fragmentation in sperm between chilled semen and thawed semen.

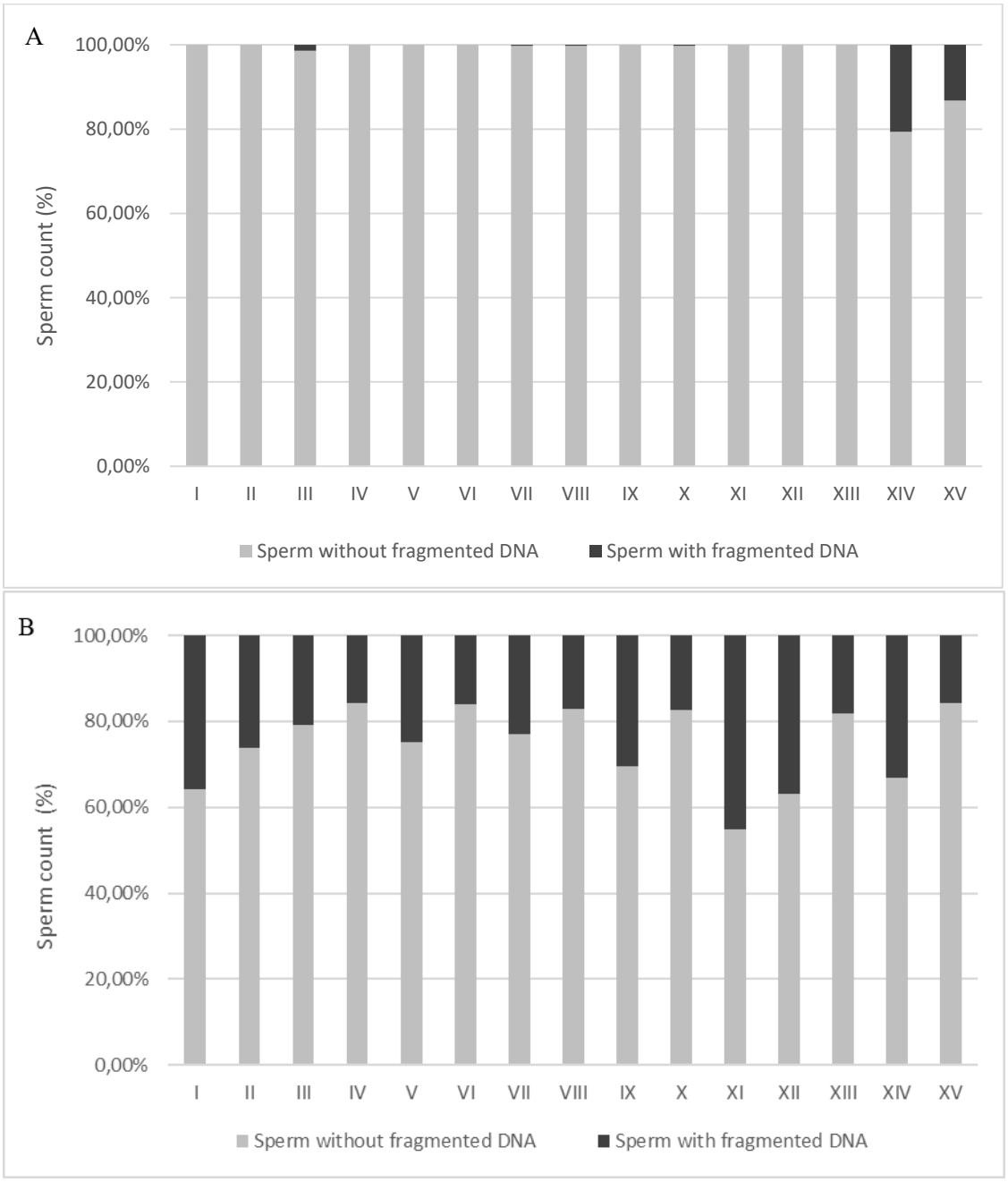

Figure 3. The level of DNA fragmentation in chilled semen (A) and in thawed semen (B)

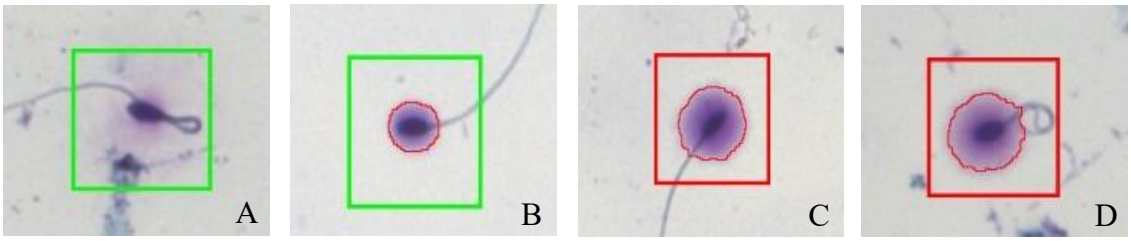

Figure 4. Sperm DNA fragmentation using the Halomax® test and the SCA® system. Non-fragmented spermatozoa are marked in green squares (a – no fragmentation, b – small halo), in red – spermatozoa with DNA fragmentation (c – medium halo, d – big halo)

4. Discussion

Increasingly, studies indicate that the incidence of SDF plays a significant role in male infertility and thus in reproductive success (Agarwal et al., 2016; Aitken, 2017; Bui et al., 2018; Esteves et al., 2020). This process may negatively affect the fertilizing capacity of spermatozoa when DNA strand damage is high (González-Marín et al., 2012). Reproductive success will therefore depend on the extent of this damage and the ability of the oocyte to repair single-strand DNA breaks as a possible alternative (Menezo Jr et al., 2007; González-Marín et al., 2012).

In animals, male fertility disorders are diagnosed on the basis of microscopic assessment of sperm concentration, motility, morphology, and vitality in the ejaculate. This analysis provides essential information on semen quality and is the basis for diagnosing possible fertility problems (González-Marín et al., 2012). However, the qualitative characteristics of both fresh and frozen semen vary widely between horse breeds (Paccamonti et al., 1999; Gottschalk et al., 2016; Greiser et al., 2020).

Currently, a great challenge in equine assisted reproduction is the high variability of sperm cryosurvival in the cryopreservation process (Ferrer et al., 2021). The cryopreservation process has been shown to cause sperm damage, with significant reductions in sperm function in thawed semen (Maziero et al., 2019). As a result, many stallions have poor post-thaw sperm motility, which makes it impossible to use their semen for commercial purposes. According to available literature, chilled stallion semen can generally maintain acceptable motility and fertilizing ability for 24–48 hours, depending on the extender used and storage conditions (Loomis and Graham, 2008). However, studies conducted by Bugno-Poniewierska et al. (2021) on ejaculates from Hucul stallions showed no significant difference in the number of motile spermatozoa after the cryopreservation process in relation to diluted semen ($p = 0.3372$), which differs from results obtained in this experiment ($p<0.01$), showing that this parameter may depend on many factors, including the individuals included in the experiment, or the timing of cryopreservation (in the 2021 study, cryopreservation was carried out immediately after semen collection and evaluation, while in this study, cryopreservation was carried out on semen chilled for 6 hours), which suggests that the first hours of semen storage may represent a critical window for DNA degradation, which requires further investigation in Hucul stallions. The results also indicate substantial individual variability in sperm motility after thawing. In several stallions, post-thaw motility remained relatively high, which may reflect individual differences in sperm resistance to cryoinjury.

Previous studies assessing the level of chromatin damage in stallion sperm have used methods such as the Halo test or single-cell gel electrophoresis (Comet Assay) (Linfor and Meyers, 2002; López-Fernández et al., 2007; Castro et al., 2020), and have illustrated how the

phenomenon of sperm DNA fragmentation is shaped in the semen of stallions of different populations. In the case of DNA fragmentation, activities performed to preserve semen intended for artificial insemination can contribute to the increase in DNA damage in stallion spermatozoa, and this damage may be an indirect consequence of stress factors caused by temperature changes or instability of the plasma membrane (López-Fernández et al., 2007). Although stallion semen parameters change during the year, and its quality is best during the breeding season, seasonal differences in the percentage of sperm with damaged chromatin are small (López-Fernández et al., 2007). In addition, it has been shown that sperm DNA is fragmented at a different rate and it depends not only on the storage temperature, but also on the fertility status of the stallions (Love et al., 2002).

In this study, there was a low correlation between stallion age and the level of SDF, both in chilled semen samples ($r = 0.03$) and after cryopreservation ($r = 0.09$). This result makes it possible to rule out age as a factor influencing the increase in DNA destabilization in spermatozoa, which is also confirmed by studies conducted by Greiser et al. (2020). In turn, Castro et al. (2020) showed that SDF was higher in older Chilean purebred stallions (CPS) than in younger stallions. This diversity of results indicates a high inter-individual variability in terms of stallion age. The relatively stable level of DNA fragmentation observed across different age groups may be related to the specific characteristics of the Hucul breed. As a primitive and highly resilient breed adapted to harsh environmental conditions, Hucul horses may exhibit greater stability of sperm chromatin structure compared with more intensively selected breeds (López-Fernández et al., 2007; Castro et al., 2020, Greiser et al., 2020).

Our results indicated that the cryopreservation process significantly reduced the quality of Hucul stallion semen. For both motility and DNA fragmentation (Halomax®), highly significant differences ($p<0.01$) were found between chilled and frozen semen. In their study of Arabian stallion semen using the TUNEL test, Shojaeian et al. (2018) found that the cryopreservation process contributed to an increase in SDF, which confirms the consistency of our results. The range of non-fragmented spermatozoa obtained after the cryopreservation process oscillated between 15.63% and 45.15%, which is higher than in the study conducted by López-Fernández et al. (2007), where the differences ranged from 8.3 to 26.3%. Atroshchenko et al. (2019) reported relatively high SDF in fresh semen of Arabian stallions, which was further increased after the cryopreservation process. In contrast, Blottner et al. (2001) found that the percentage of sperm with damaged DNA was minimal and differences between fresh and thawed semen were small. In turn, Linfor and Meyers (2002) analyzed the level of SDF using the SCGE test to assess the effect of the length of storage of samples at reduced temperatures, and their results were similar to ours.

Due to the nature of the breeding program, the semen of Hucul stallions has not previously been subjected to a detailed analysis in terms of DNA fragmentation, and these results, to our knowledge, are the first to assess the impact of the cryopreservation process on the DNA stability of Hucul stallion sperm. It should be emphasized that semen cryopreservation is a necessary process for the preservation of the genetic resources of animals, including horses, and an effective strategy for the protection of endangered species. Creating gene banks is an important element of securing gamete reserves and increasing biodiversity (Ali et al., 2017).

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