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RESEARCH ARTICLE

Characteristics of diluted-stored and post-thawed semen of Hutsul stallions

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ABSTRACT

The use of frozen semen lowers the risk of disease transmission, eliminates geographical limitations and supports the implementation of genetic resource protection programs. However, due to the very rare use of frozen semen from Hutsul 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. The aim of this study was to analyse basic parameters such as sperm motility, vitality and morphology in diluted-stored and post-thawed Hutsul semen, using a CASA system. There were no differences in sperm motility ($P = 0.3372$) or morphology between the groups, although the progressive motility was higher in thawed semen ($P = 0.0151$), while the sperm vitality was higher in diluted-stored semen ($P = 0.00517$). This study demonstrates that semen from Hutsul horses is suitable for cryopreservation, thus supporting the creation of a sperm bank as a genetic reserve for representatives of this breed.

KEYWORDS

Hutsul horses, semen, morphology, motility, vitality, CASA system

INTRODUCTION

The use of frozen stallion semen has increased in recent decades and is closely related to the routine use of artificial insemination (AI) in equine reproduction (Govaere et al., 2014; Huber et al., 2019). The use of frozen semen lowers the risk of disease transmission, eliminates geographical limitations, and supports the implementation of genetic resource protection programs (Alvarenga et al., 2016; Huber et al., 2019). Although there are many published examples of semen cryopreservation in many breeds of horses, these do not include Polish primitive breeds, i.e. the Hutsul and Polish Konik. According to Posta et al. (2020), in Europe there are populations of Hutsul horses from Slovakia (Pjontek et al., 2012), Poland (Mackowski et al., 2015), Romania (Posta et al., 2020) and Hungary (Somogyvári et al., 2018). Currently, the population of these horses is about 5,000 broodmares, which contributed to the recognition of this breed as endangered with extinction (Posta et al., 2020). Insemination in this breed is only allowed under exceptional and justifiable circumstances and requires the approval of the Studbook Commission. Due to these limitations, the stallions most frequently used for insemination are those to which breeders have the easiest access, and so this breed is at risk of increased inbreeding as well as low population efficiency. The genetic material of this breed is not secured in sperm banks, so it is necessary to create a collection of their semen to address these risks. Moreover, due to the very rare use of frozen semen in this breed,

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information about Hutsul horse semen parameters, such as its suitability for cryopreservation and sperm survival rates after thawing, is not available. Therefore, the aim of the study was to analyse the parameters of diluted-stored and frozen-thawed semen from Hutsul horses using a CASA system.

MATERIALS AND METHODS

Study design

Semen for testing was obtained from 12 Hutsul stallions. Ejaculates were collected from each stallion twice, one day apart. The ejaculate on the second day was intended for research. Each ejaculate was divided into two parts, one of which was cryopreserved (Fig. 1). Parameters such as sperm motility, morphology and vitality were analyzed using the SCA automated semen analysis system (Microptic, S.L., Barcelona, Spain). In order to minimise errors in the analysis, the cryopreserved semen was tested in duplicate, using the semen from two randomly selected straws. The results are presented as the average of the duplicate assessments.

Semen collection

The semen was obtained from 12 adult Hutsul stallions with confirmed fertility using an artificial open vagina. The stallions were stationed at the same stud where hand mating is used and the collections were made in February. The sperm-rich fraction of the ejaculate was used in this study.

Semen cryopreservation

After collection, the semen was preliminarily assessed (volume, colour, general sperm movement), and then diluted 1:5 with EquiPlus extender (Minitube, Germany), preheated to 37 °C. This sample was then divided in half, one part was

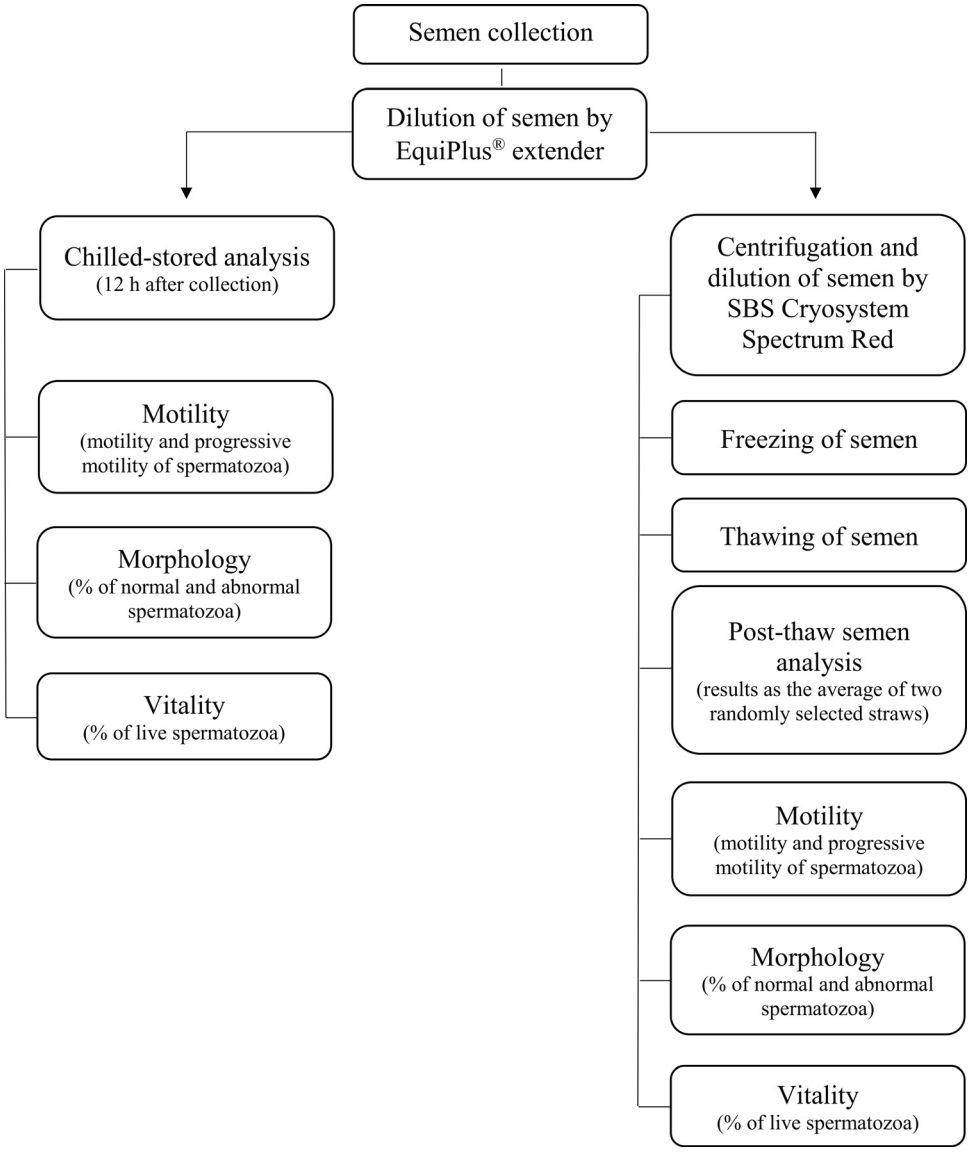

Fig. 1. The scheme of testing diluted-chilled and cryopreserved Hutsul stallion semen

intended for freezing and the rest of the semen was stored at 5 °C for 12 h.

The semen intended for freezing was centrifuged (336 RCF for 7.5 min) and additionally diluted (final concentration 38×10^6/ml) with SBS Cryosystem Spectrum Red extender (Minitube, Germany, Ref. 13572/0030) and 0.25-ml aliquots were loaded into cryostraws (Minitube, Germany). The straws were cooled at 5 °C/60 min, and then placed in liquid nitrogen vapour on a commercial metal float for freezing (freezing unit, Minitube, Germany, Ref. 15043/0736) for 5 min before being immersed in liquid nitrogen for storage until analysis.

Compared to those likely required for successful insemination, the low volume and cell count of the semen solution placed in the cryostraws were selected with the aim of post-freezing analyses in our mind.

Semen preparation for analysis using the SCA system

For analysis, 200 µl of thawed (37 °C/30 s) or diluted-stored semen was mixed with 1 ml PBS (Bioshop, Canada, Ref. PBS408.500) warmed to 37 °C. The sample was then centrifuged at 336 RCF for 7.5 min. The supernatant was discarded and the pellet was resuspended in 1 ml PBS at 37 °C (Bioshop, Canada, Ref. PBS408.500). Further procedures depended upon the parameter being tested.

Slide preparation for sperm motility analysis

For sperm motility assessment, extended and thawed semen samples were diluted 1:20 in PBS (37 °C) and loaded onto Golcyto microscope slides (Microptic, S.L., Barcelona, Spain; 10 µl per chamber). Sperm motility was determined via the 'Motility' module of the SCA system, using 10 × magnification and phase contrast optics. For all stallions, sperm motility and progressive motility in diluted-chilled semen were determined 12 h after collection, because the laboratory, where the assessments were completed, was far away from the stud. Randomly selected fields (minimum 300 sperm) were analysed. In the case of thawed spermatozoa, the average of the results from two independent samples (300 sperm for each) was used for assessment. The motility and progressive motility of spermatozoa from diluted-chilled and thawed semen were compared using Student's *t*-test for correlated pairs (P value = 0.05).

Slide preparation for the analysis of sperm morphology

For morphology assessment, 10 µl prepared semen was smeared onto a microscope slide and allowed to dry. The slides were then stained with Rapid Sperm Blue (Microptic, S.L., Barcelona, Spain), following the manufacturer's instructions. When dried, the preparations were covered with SPX mountant and a coverslip applied. Sperm morphology was assessed via the 'Morphology' module of the SCA system, using 60 × magnification and white light with a blue filter. A total of 100 randomly selected spermatozoa were assessed for each preparation. The morphological parameters related to the sperm head, acrosome, midpiece and tail were determined automatically. In the case of thawed semen samples, where two straws from the same ejaculate were analysed, the mean results for each sample were used. The results were compared using Student's *t*-test for correlated pairs (P value = 0.05).

Slide preparation for sperm vitality analysis

Ten microlitres of previously prepared semen was combined with 40 µl Bright Vit dye (Microptic, S.L., Barcelona, Spain) and incubated at 37 °C for 5 min before being applied to a microscope slide, smeared and allowed to dry. The vitality analysis was performed via the 'Vitality' module of the SCA system, using a 20 × objective with negative phase contrast. Two hundred spermatozoa per preparation were analysed. Sperm heads stained pink were counted as dead, while those that were unstained were counted as live. For slides made using frozen semen, the analysis was carried out using two different straws of the same sample, and the result was presented as an average. The percentage of live and dead sperm before and after freezing was compared using Student's *t*-test for correlated pairs (P value = 0.05).

Statistical analyses

Statistical analyses were performed using Statistica 13.0 (StatSoftland) software. Normality of distributions were examined by the Kolmogorov–Smirnov test. The level of significance of the tests were was set at $P = 0.05$.

RESULTS

Motility

There was no significant difference in the proportion of motile spermatozoa in the diluted or frozen semen groups (47.3% vs. 41.8%, respectively, $P = 0.3372$; Fig. 2).

However, there was a difference in the proportion of progressively motile spermatozoa, which was significantly higher for the thawed semen samples (15.3% vs. 7.1% for diluted-stored semen, $P = 0.0151$; Fig. 2).

Vitality

There was a significant effect of freezing/thawing on sperm vitality, with a significant reduction in the proportion of live spermatozoa (44.3% vs. 56.8% in diluted-stored semen, $P = 0.00517$; Fig. 3). There was a corresponding increase in the proportion of dead spermatozoa in the frozen-thawed samples ($P = 0.00831$; Fig. 3).

Morphology

There was no effect of freezing on the proportion of spermatozoa with normal morphology (45.4% vs. 66.7% for diluted-stored semen, $P = 0.30103$; Fig. 4). Similarly, there

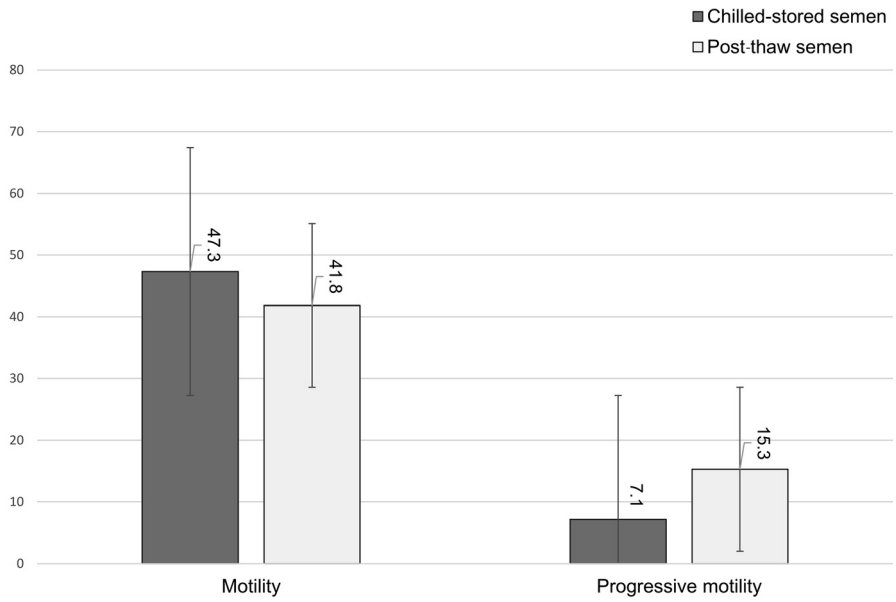

Fig. 2. Diluted-chilled and post-thaw semen motility parameters (n = 12). Data are presented as percentages. Values with different letters (a, b) differ between the groups ($P < 0.05$)

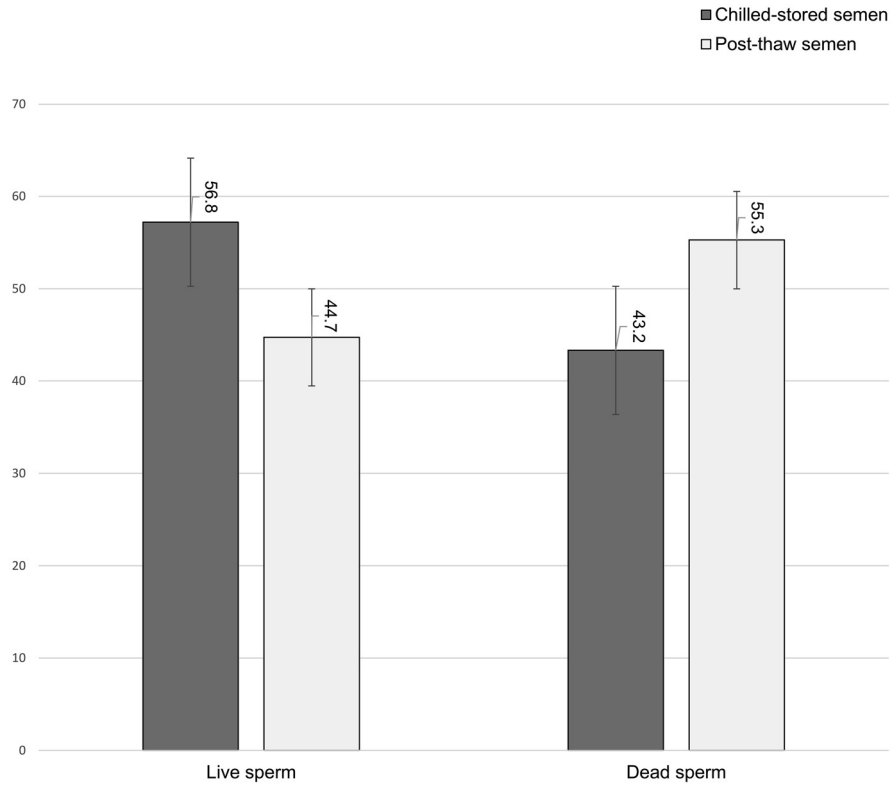

Fig. 3. Diluted-chilled and post-thaw semen vitality parameters (n = 12). Data are presented as percentages. Values with different letters (a, b) differ between the groups ($P < 0.05$)

was no significant effect on the proportion of spermatozoa with abnormal morphology (P = 0.32652; Fig. 4).

Table 1 presents a comparison of the semen parameters between stallions with higher (>35%) or lower (<35%) progressive movement of spermatozoa.

DISCUSSION

This is the first report of the analysis of semen parameters for Hutsul stallions. The sperm motility was similar for

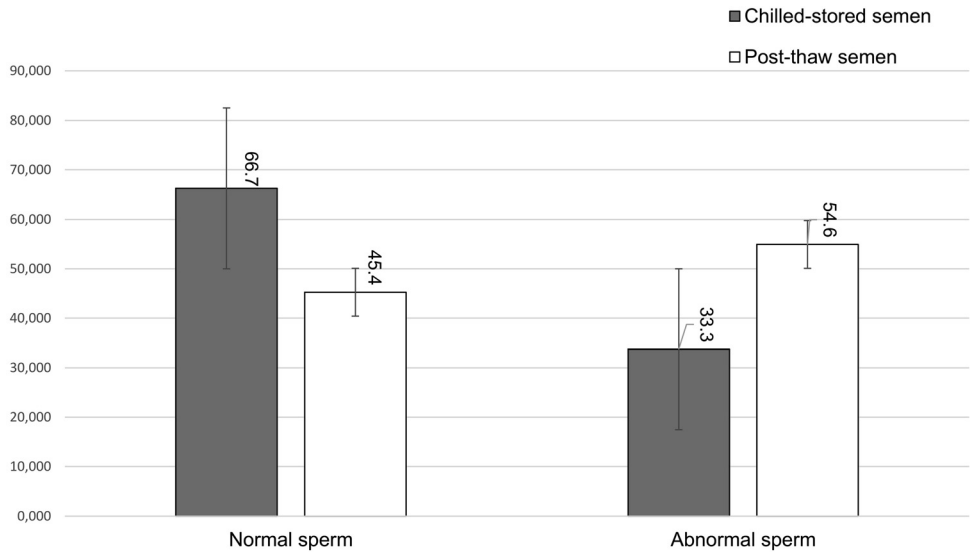

Fig. 4. Diluted-chilled and post-thaw semen morphology parameters (n = 12). Data are presented as percentages. Values with different letters (a, b) differ between the groups (P < 0.05)

Table 1. Semen parameters of Hutsul stallions with >35% and <35% post-thaw motility. Results are presented as percentages

Parameter	Stallions with >35% post-thaw motility value (n = 8)	Stallions with <35% post-thaw motility value (n = 4)
Motility (%)	49.3	27
Progressive motility (%)	18.7	8.6
Vitality (% of live spermatozoa)	48	36.9
Morphology (% of normal spermatozoa)	44.5	54.1

diluted-stored and frozen-thawed samples (P > 0.05, Fig. 2), indicating the suitability of this semen for freezing. The recommended threshold for post-thaw motility of stallion spermatozoa is at least 35% (Miller, 2008). In our research, 8 out of the 12 tested stallions showed sperm motility above 35%. Interestingly, thawed sperm had significantly higher progressive motility than sperm from diluted semen (Fig. 2). This phenomenon can be explained by differences in the time of assessment of diluted semen. While the semen was frozen immediately after collection, stopping sperm metabolism, sperm motility in the diluted-stored semen was assessed 12 h after the semen was collected, which could have influenced the result. Nevertheless, the motility results for diluted-stored and thawed Hutsul spermatozoa are in line with the standards adopted for frozen stallion semen. A very similar level of sperm motility was demonstrated by Dini et al. (2020) in fresh Shetland horse semen (59%), although much higher sperm motility results were obtained in the fresh semen of Koninklijk Warmblood Paardenstamboek Nederland (KWPN) horse (74%) and Friesian horse (67%). Another study of 27 stallions used commercially in Sweden, Finland, Italy and the USA reported a mean post-thaw sperm motility of 37% (Kuisma et al., 2006), which is comparable to the results obtained in the present study. Additionally, what is interesting, among the 8 stallions showing motility above 35%, the progressive movement was 18.7%. However, in the case of the remaining stallions, the progressive movement was 8.6% (Table 1).

There were significant differences in the sperm vitality results between diluted-stored and thawed Hutsul horse semen (56.8% vs. 43.2% live spermatozoa, respectively; Fig. 3). Moreover, in the stallions whose semen freezes better, 48% of the sperm are alive, compared to 36.9% in the other stallions (Table 1). A reduction in the proportion of live spermatozoa after thawing is normal, as sperm cell membranes are particularly sensitive to the freezing process and can be damaged (Watson, 1995; Ortega-Ferrusola et al., 2007). In addition, after thawing the sperm cell membrane has a lower ability to cope with osmotic stress than spermatozoa from fresh semen (Schweisguth and Hammerstedt, 1992). Another study of sperm vitality in frozen-thawed semen of 'Selle Francais' stallions reported a range of 54–59% live spermatozoa, depending on the extender used (Vidament, 2005). However, this analysis was performed by flow cytometry and fluorescent staining with carboxy-fluorescein diacetate and propidium iodide. In our study, the vitality analysis was based on the sperm blue dye, which is the equivalent of eosin staining, so it is difficult to compare the results of the studies. Additionally, using flow cytometry results in more precise analyses.

The vitality of sperm in fresh semen was tested by Dini et al. (2020) using similar staining as that used in our study. They reported 62% live spermatozoa in the semen of

Shetland horses, and a range of 72–75% live spermatozoa in the semen of KWPN and Friesian horses.

The final parameter assessed in this study was morphology, with no significant difference observed in the proportion of normal spermatozoa in the diluted-stored semen and thawed semen (66.7% vs. 45.4%, respectively; Fig. 4). Similar results were found between stallions with motility >35% and <35%, the percentage of normal spermatozoa was 44.5% vs. 54.1%, respectively (Table 1). It has been reported that a normal spermiogram for stallions is one in which 50% of the sperm have normal morphology (Card, 2005; Phetudomsinsuk et al., 2008), so our results show that the percentage of normal sperm in the diluted semen of Hutsul stallions is normal. However, Neild et al. (2000) showed that semen with 43.4% normal spermatozoa had an acceptable level of fertility, while others determined the ranges for normal sperm to be 39–67% (Dini et al., 2020) and 51–89% (Kuisma et al., 2006). Therefore, while 45% of the thawed spermatozoa in our study had normal morphology, they could still be considered potentially fertile.

As indicated in the literature, semen quality is not only correlated with the number of normal spermatozoa in terms of morphology, but also with the percentage of individual sperm defects (Jaśko et al., 1990, 1992; Brito, 2007). Brito (2007) describes that a higher percentage of sperm with a head defect in semen will negatively affect the fertility of stallions. On the other hand, Love (2011) shows that there is no correlation between sperm head defects, the occurrence of cytoplasmic droplets, and the parameters of motility. The authors emphasise that such disorders may occur in non-motile sperm and sperm exhibiting various types of movement (fast, slow, linear, nonlinear) (Love, 2011). However, it is worth emphasising that head defects may affect the ability to penetrate the zona pellucida of the oocyte, and thus reduce fertility (Pesch and Bergmann, 2006). Tail defects negatively affect the movement of spermatozoa and are also associated with reduced fertility; however, due to the fact that they show poor movement, they do not distort the results.

Morphology has been positively related to motility (Jaśko et al., 1990, 1992), and the number of morphologically normal sperm in the ejaculate may indicate the fertility of stallions. The number of normal spermatozoa provides more information about stallion fertility than the number of abnormal spermatozoa (Varner, 2008); however, it is also important to realise that different sperm defects might also have different effects on fertility even if the proportion of normal spermatozoa are the same (Brito, 2007).

In conclusion, our study showed that semen from Hutsul horses is suitable for cryopreservation, and thus it is feasible to create a sperm bank for representatives of this breed as a genetic reserve.

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