

Artificial insemination success despite reduced post-thaw motility of cryopreserved semen in local rabbit breeds: Short communication

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Abstract: Cryopreservation of rabbit semen represents an important tool for the conservation of genetic resources; however, reduced post-thaw sperm motility may limit its practical application in artificial insemination (AI) programmes. The present study evaluated fertility outcomes following AI using cryopreserved semen from six local rabbit breeds selected to reflect biological variability under practical gene bank conditions. A total of 59 females were inseminated with heterospermic fresh or frozen–thawed doses standardised to approximately 0.2×10^9 spermatozoa per ml. Ovulation was hormonally induced prior to insemination. Cryopreservation resulted in a substantial reduction in sperm motility, with progressive motility decreasing from 52.6% in fresh semen to 23.7% after thawing and total motility from 75.2% to 42.7%. Despite this decline, conception rates remained high (85.3% in thawed semen vs 92.9% in fresh semen), and litter size was only slightly reduced (8.62 vs 9.25 kits). These findings indicate that cryopreserved rabbit semen exhibiting lower-moderate post-thaw motility can achieve high fertility under practical conditions and support its usability in genetic resource conservation programmes.

Keywords: conception rate; gene bank; genetic resources; litter size; spermatozoa

Artificial insemination (AI) represents a well-established reproductive biotechnology in rabbit production and breeding systems. Beyond its use in commercial meat production, AI plays an impor-

tant role in the conservation of local and endangered rabbit breeds through semen cryopreservation and gene bank programmes (Moce and Vicente 2009). The preservation of male germ cells is particularly

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valuable for maintaining genetic diversity and ensuring long-term sustainability of animal genetic resources.

However, rabbit spermatozoa are considered relatively sensitive to cryoinjury. The freezing–thawing process can induce structural and functional alterations, including damage to the plasma membrane, acrosome, and mitochondria, which ultimately results in reduced post-thaw motility and viability (Moce and Vicente 2009; Nishijima et al. 2021). Consequently, post-thaw sperm motility is commonly used as a primary indicator of semen quality and suitability for artificial insemination.

Despite the importance of laboratory semen evaluation, the predictive value of post-thaw motility for actual fertility outcomes remains complex. Fertilisation success depends not only on sperm motility but also on sperm dose, insemination management, synchronisation of ovulation, and female reproductive status. Recent studies have highlighted that optimisation of cryopreservation protocols and sperm concentration can partially compensate for reduced motility and improve overall functional competence of thawed spermatozoa (Nishijima et al. 2021; Di Iorio et al. 2023).

In practical breeding conditions, additional management strategies may further mitigate the negative effects of reduced sperm quality. The use of hormonally induced ovulation and standardised insemination doses increases the likelihood that a functionally competent sperm subpopulation reaches the site of fertilisation. Moreover, genetic variability among males and breeds influences semen freezability and post-thaw performance, which may contribute to variability in reproductive outcomes (Lavara et al. 2017). Therefore, evaluating multiple breeds may provide a broader assessment of the practical applicability of cryopreserved semen under field conditions.

Within gene bank systems, semen samples often display moderate post-thaw motility values, yet their real fertilising capacity under field conditions is not always systematically evaluated. Previous work on Slovak native rabbit breeds has focused primarily on semen storage and laboratory quality parameters (Kulikova et al. 2017), while fertility data obtained after insemination remain limited.

Therefore, evaluating reproductive performance following artificial insemination with cryopreserved semen exhibiting moderate post-thaw motility is essential for assessing the practical usability

of gene bank material. The aim of the present study was to assess fertility and litter size following artificial insemination using cryopreserved semen from six local rabbit breeds under standardised field conditions.

MATERIAL AND METHODS

Animals and housing. The study included 19 adult rabbit males representing six local breeds from the Central European region: Nitra rabbit ($n = 3$), Zobor rabbit ($n = 2$), Liptov baldspotted rabbit ($n = 3$), Zemplin rabbit ($n = 3$), Hungarian giant rabbit ($n = 4$), and Chrabrany rabbit ($n = 4$), as well as 59 females. Animals were 0.5–1.5 years of age at the time of the study.

Rabbits were housed individually under controlled environmental conditions at an ambient temperature of approximately 15 °C and a photoperiod of 12 h light per day. Animals had *ad libitum* access to water and were fed a standard commercial rabbit diet.

Ethical statement. All procedures involving animal handling and experimental manipulations were performed in accordance with the Slovak Animal Protection Regulation RD 377/12, which is harmonised with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. The experimental protocol was approved by the Ministry of Agriculture and Rural Development of the Slovak Republic (Permit No. SK U 18016). In addition, procedures carried out in Hungary were approved by the Directorate of Food Safety and Animal Health of the Government Office of Pest County, Hungary (PE/EA/729-8/2020), and by the Institutional Ethical Review Board.

Semen collection and evaluation. Semen was collected twice weekly using an artificial vagina (MiniTube, Germany) filled with warm water (approximately 50 °C). Semen was generally obtained after a single jump. If ejaculate was not obtained during the first attempt, the collection was repeated. Immediately after collection, ejaculates were evaluated using a computer-assisted sperm analysis (CASA) system (SpermVision™ software; Minitube, Germany) connected to a phase-contrast light microscope (AxioScope A1; Zeiss, Germany) at 200× magnification and equipped with a Makler counting chamber (Sefi Medical Instruments, Israel).

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The following parameters were assessed: total motility (TM; motility $>5 \mu\text{m s}^{-1}$), progressive motility (PM; motility $>20 \mu\text{m s}^{-1}$), and sperm concentration. Ejaculates exhibiting elevated levels of morphological abnormalities ($>20\%$) were excluded from further processing. CASA analysis was also performed after thawing to determine post-thaw sperm motility and concentration.

Semen processing and cryopreservation. Semen samples were diluted in BotuCrio (Nidacon, Sweden) freezing medium according to the manufacturer's recommendations. The final sperm concentration was standardised to approximately 0.2×10^9 spermatozoa per ml. Only ejaculates meeting the minimum quality criteria (TM: 50%, PM: 40%) were used for preparation of cryopreserved samples and insemination doses. Equal semen volumes from individual males were pooled before dilution and cryopreservation.

Extended semen was loaded into 0.25 ml plastic straws. Cryopreservation was performed using a manual freezing procedure. Straws were placed horizontally on a floating rack inside a liquid nitrogen container and exposed to nitrogen vapours for 15 min before being plunged into liquid nitrogen for storage.

For insemination, straws were thawed in a water bath at 40°C for 15 seconds. The thawed semen was used for insemination within a few minutes after thawing.

Artificial insemination and fertility evaluation. Fresh ejaculates were used for artificial insemination immediately after evaluation, while cryopreserved samples were thawed as described above.

Hormonal stimulation of females was initiated 48 h prior to artificial insemination by intramuscular administration of 25 IU pregnant mare serum gonadotropin (PMSG; Sargon, Bioveta, Czech Republic). Artificial insemination was performed using 0.5 ml of diluted semen administered with a plastic insemination pipette. Ovulation was induced at the time of insemination by intramuscular injection of $2.5 \mu\text{g}$ synthetic gonadotropin-releasing hormone (GnRH; Supergestran, Ferring-Léčiva, Czech Republic).

Pregnancy diagnosis was carried out by abdominal palpation on day 17 after insemination. Fertility was evaluated based on kindling rate, defined as the proportion of females that gave birth relative to the total number of inseminated females. The number of live-born and stillborn kits was re-

corded immediately after parturition. The insemination protocol followed the procedure described by Vozaf et al. (2024).

Statistical analysis. For comparative purposes, fertility outcomes obtained using frozen–thawed semen were evaluated alongside data obtained from artificial inseminations performed with fresh ejaculates. Descriptive statistics were calculated for all analysed parameters, including total motility, progressive motility, conception rate, and litter size. Differences between fresh and frozen–thawed semen groups were evaluated using non-parametric statistical tests due to the relatively small sample size. The Mann–Whitney U test was used to compare continuous variables, including sperm motility parameters and litter size. Differences in conception rates between groups were assessed using Fisher's exact test. All statistical analyses were performed using R statistical software (R Core Team, Austria), and results were considered statistically significant at $P < 0.05$. The column and bubble charts were generated using Microsoft Excel (Microsoft Corporation, WA, USA).

RESULTS

Semen quality before and after cryopreservation. Total and progressive sperm motility were significantly higher in fresh ejaculates compared with frozen–thawed semen samples ($P < 0.05$). Mean total motility of fresh semen reached approximately 75%, whereas frozen–thawed semen exhibited an average total motility of about 43%. Similarly, progressive motility decreased from approximately 53% in fresh ejaculates to about 24% after cryopreservation (Table 1). Total and progressive motility of fresh and frozen–thawed samples are illustrated in Figure 1, highlighting variability across samples.

Table 1. Mean sperm motility parameters in fresh and frozen–thawed rabbit semen samples

Parameter	Fresh semen (mean $\pm$ SD)	Frozen–thawed semen (mean $\pm$ SD)
Total motility (%)	75.2 $\pm$ 8.4 ^a	42.7 $\pm$ 9.6 ^b
Progressive motility (%)	52.6 $\pm$ 7.9 ^a	23.7 $\pm$ 6.8 ^b

^{a,b}Different superscripts within a row indicate significant differences ($P < 0.05$)

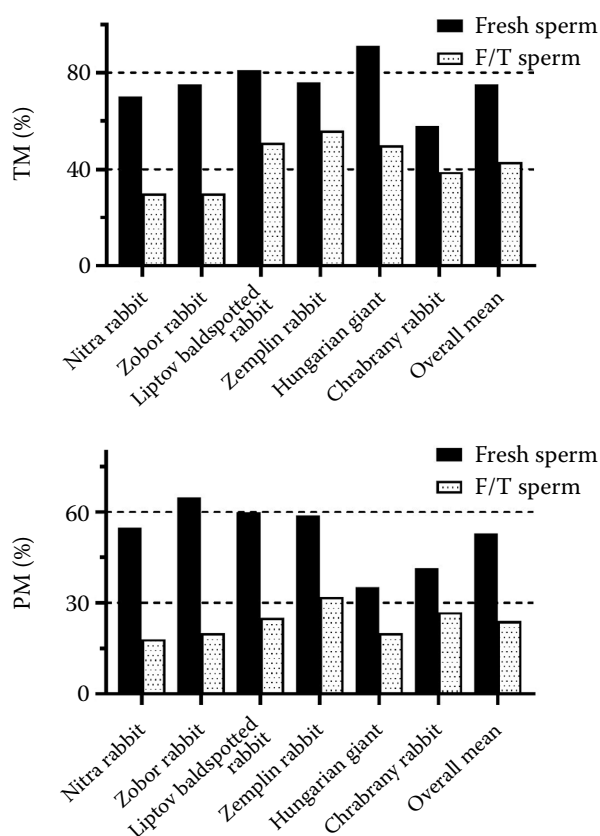


Figure 1. Total motility (TM) and progressive motility (PM) in fresh and frozen–thawed (F/T) rabbit semen samples

Data represent mean values for six local rabbit breeds from the Central European region and the overall mean across all samples. F/T samples exhibited significantly lower motility compared with fresh semen ($P < 0.05$)

These results confirm the substantial reduction in sperm motility associated with the freezing–thawing process.

Fertility outcomes. Despite the marked decrease in sperm motility after cryopreservation, fertility outcomes remained relatively high (Table 2). Artificial insemination using fresh semen resulted in a conception rate of approximately 93%, whereas frozen–thawed semen achieved a conception rate of about 85%.

The average litter size was slightly higher in inseminations performed with fresh semen (9.25 kits per litter) compared with frozen–thawed semen (8.62 kits per litter). No significant differences were observed between fresh and frozen–thawed semen in litter size ($P > 0.05$).

The relationship between post-thaw progressive sperm motility and reproductive performance

Table 2. Reproductive performance following artificial insemination using fresh and frozen–thawed rabbit semen

Parameter	Fresh semen	Frozen–thawed semen
Inseminated females	28	31
Conception rate	26/28 (92.9%)	26/31 (85.3%)
Litter size (kits)	9.25 ± 1.9	8.62 ± 2.1

No significant differences were detected between groups ($P > 0.05$)

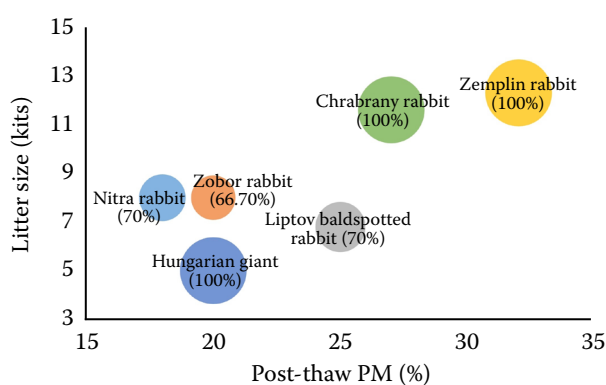


Figure 2. Relationship between post-thaw progressive motility (PM) and litter size following artificial insemination using cryopreserved rabbit semen from six local rabbit breeds

Bubble size represents conception rate (%). Each bubble represents mean values obtained for a specific breed

across analysed samples is illustrated in Figure 2. Bubble size represents conception rate. High conception rates were observed across most breeds despite moderate progressive motility values. The observed variability reflects differences among individual samples rather than systematic comparisons between breeds.

Overall, these findings indicate that even semen samples with moderate post-thaw motility can produce satisfactory reproductive outcomes under practical insemination conditions.

DISCUSSION

The present study demonstrated that satisfactory reproductive performance can be achieved after artificial insemination using cryopreserved rabbit semen despite relatively low post-thaw sperm motility. As expected, cryopreservation significantly reduced sperm motility parameters, particularly progressive motility. However, despite

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this reduction in semen quality, fertility outcomes remained relatively high. Similar observations have been reported in previous studies where moderate post-thaw motility values still resulted in acceptable fertility after artificial insemination in rabbits (Lavara et al. 2005; Moce and Vicente 2009).

The decrease in sperm motility observed after cryopreservation is consistent with previous reports describing the detrimental effects of freezing and thawing on spermatozoa. Cryopreservation exposes sperm cells to osmotic stress, oxidative damage, and membrane disruption, which impair sperm movement, viability and mitochondrial function (Watson 2000; Moce and Vicente 2009; Shi et al. 2024). Recent studies have also shown that supplementation of semen extenders with antioxidants such as astaxanthin may improve post-thaw sperm quality in rabbits (Luque et al. 2026). These processes are widely recognised as major factors responsible for reduced post-thaw sperm quality in many mammalian species.

Despite the reduction in sperm motility after cryopreservation, satisfactory reproductive outcomes were observed in the present study. Conception rates remained relatively high and litter sizes were only moderately reduced compared with inseminations performed using fresh semen. Notably, while progressive motility decreased by more than 50% following cryopreservation, the reduction in conception rate was relatively small (approximately 8 percentage points), indicating a disproportion between laboratory semen quality parameters and fertility outcomes. These findings suggest that a reduced proportion of motile spermatozoa may still be sufficient to ensure successful fertilisation, particularly under controlled insemination conditions with optimised sperm dose and ovulation synchronisation. Previous studies have shown that the relationship between semen quality parameters and fertility outcomes is often complex, and acceptable fertility may still be achieved even when motility values are relatively low (Lavara et al. 2005; Amann and Waberski 2014).

It should also be considered that litter size may be influenced by multiple biological and management-related factors. The variability observed in the present study may reflect inherent differences among animals as well as environmental and physiological factors rather than systematic differences between breeds. Previous studies have reported differences in reproductive traits among

rabbit breeds, including litter size and kit survival, indicating that genetic background can contribute to variability in reproductive performance (Fadare and Fatoba 2018; Ayyat et al. 2024). Therefore, part of the variation in litter size observed among breeds in the present study may reflect inherent breed-related differences rather than solely the quality of cryopreserved semen. It is also possible that differences in cryotolerance among breeds contributed to the observed variability in post-thaw sperm quality; however, this aspect was not specifically evaluated in the present study.

Another factor that may explain the satisfactory fertility observed in this study is the use of heterospermic insemination doses. By combining ejaculates from several males, heterospermia may reduce the impact of variability in semen quality among individual males and increase the likelihood that functionally competent spermatozoa are present in the insemination dose. Similar approaches are commonly used in rabbit artificial insemination programmes (Theau-Clement 2007; Viudes-De-Castro and Vicente 2023; Gimeno Martos et al. 2025).

The inclusion of several local rabbit breeds represents an additional relevant aspect of the present study. Conservation of animal genetic resources has become an important priority in livestock breeding programmes, and cryopreservation of semen represents an effective strategy for preserving valuable genetic material (Blackburn 2004; FAO Animal Production and Health 2012). Recent reviews on rabbit reproduction technologies have also emphasised the importance of cryopreserved semen in maintaining genetic diversity within local and endangered breeds (Viudes-De-Castro and Vicente 2023). The results obtained in this study therefore support the practical usability of cryopreserved semen in conservation programmes for local rabbit breeds.

However, some limitations of the present study should be acknowledged. The number of inseminated females per breed was relatively limited, which did not allow robust statistical comparisons among breeds. Consequently, breed was considered primarily as a source of biological variability rather than as a factor for formal statistical comparison. Future studies including larger datasets would be beneficial to further clarify potential sources of variability in post-thaw semen quality and fertility outcomes.

CONCLUSION

The present study demonstrated that although cryopreservation substantially reduced sperm motility in rabbit semen, satisfactory reproductive performance was still achieved after artificial insemination. Conception rates remained high and litter sizes were only slightly lower compared with inseminations performed using fresh ejaculates. These findings indicate that lower-moderate post-thaw sperm motility may still be sufficient for successful artificial insemination in rabbits. The results further support the practical usability of cryopreserved semen in breeding programmes and genetic resource conservation of local rabbit breeds. Future studies involving larger datasets may help to better clarify potential breed-related differences in post-thaw semen quality and fertility.

Conflict of interest

The authors declare no conflict of interest.

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