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Study of sericin added to semen extender in sperm cryopreservation of Hungarian Giant Rabbit

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Abstract

Because of the dramatic decline of Hungarian Giant rabbit, we aim to create an *in vitro* sperm bank for safe gene preservation purposes. During testing different freezing protocols, we developed an effective extender for the cryopreservation of Hungarian Giant sperm. The present study investigated the effect of sericin as antioxidants on sperm freezability using the modified Lake's extender. Collected sperm from 10 bucks was diluted 1:4 with modified Lake's extender with 1% glycerol and 4% DMF (control group), containing sericin 0.1 and 0.3 w/v% (treated groups). Samples filled into 0.25 ml straws, and after equilibration at 5°C for 60 min static freezing (5 cm above liquid nitrogen for 15 minutes) was applied. The thawing was carried out at 40ºC for 30 sec. In the fresh and frozen/thawed sperm samples motility (CASA) and ratio of live/dead cells, sperm abnormalities (aniline blue-eosin staining) were checked. In testing two different sericin concentrations no significant difference was observed in the proportion of live, intact cells or in total and progressive motility, none of the tested concentrations of sericin supplementation improved sperm viability. Further studies are required to determine whether the concentrations were suboptimal or this rabbit breed responds poorly to these treatments.

Keywords: rabbit sperm; cryopreservation; sericin; Hungarian Giant

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Introduction

Despite the fact that research into rabbit semen cryopreservation has been ongoing for several decades, sperm cryopreservation is still applied mainly for experimental purposes and gene conservation. The dramatic decline of our native rabbit breed, the Hungarian Giant, highlights the need to establish an *in vitro* sperm bank to supplement the *in vivo* nucleus population already maintained at our Institute. The establishment and maintenance of an *in vitro* sperm bank enable the safe conservation of the breed's genetic material while also supporting the *in vivo* population.

To this end, in recent years, we have been working to develop an efficient freezing protocol for the Hungarian Giant. Following the comparison of different extenders and cryoprotectants finally we have developed an effective extender for Hungarian Giant sperm (KISSNÉ VÁRADI et al., 2026). However, as our cryopreservation protocol did not prove to be sufficiently effective during our fertilisation experiment, we considered it necessary to further improve the viability and motility parameters of frozen/thawed sperm.

Antioxidants used in semen extenders may improve semen freezability and protect sperm from damage occurring during the cryopreservation process; therefore, they may be suitable for improving the efficacy of sperm freezing protocols (KHAN, 2011; PEGG, 2015).

Sericin is a glycoprotein rich in amino acids that is extracted from the silkworm cocoon and is believed to have antioxidant and antimicrobial effects (ZHANG, 2002). Due to its bioactive, mechanical, and chemical properties, it is used in a wide range of fields, including the beauty, food, and pharmaceutical industries, as well as in various biological research applications (KUNDU et al., 2008). Since sericin is water-soluble, it can be easily applied as an antioxidant supplement in extenders during the sperm freezing of rabbits (RAZA et al., 2019) and other mammalian species (SASAKI et al., 2005). During the cryopreservation of stallion sperm, sericin supplementation increased DNA integrity and protected sperm from the damaging effects of reactive oxygen species (NASIRABADI et al., 2019). Similar protective effects have been observed during mouse and bull sperm freezing (GHASEMI, et.al. 2018; YANGNGAM et al., 2021).

Sericin may also offer a solution to a problem that arises during both cold storage and sperm freezing of rabbit semen, namely the 'dilution effect'. When semen is diluted to a greater extent, adverse changes occur in the structure and function of the spermatozoa's plasma membranes (AKSOY et al., 2010). Moreover, dilution may reduce the amount of plasma proteins available to sperm, which may lead to reduced fertility (DI IORIO et al., 2024). Due to the specific molecular properties of sericin, it may be suitable for repairing damage to the plasma membrane (AKSOY et al., 2024), particularly during treatment with extenders.

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In our study, we investigated the effect of sericin supplementation on sperm viability during cryopreservation and aimed to improve sperm motility by exploiting the potential protective effects of sericin. Improved sperm motility is essential for the successful use of frozen/thawed sperm in artificial insemination, which represents the ultimate purpose of sperm banking.

Material and methods

Animals and semen collection

The study was conducted at the National Centre for Biodiversity and Gene Conservation, Institute for Farm Animal Gene Conservation, Hungary. Ten sexually mature Hungarian Giant bucks, aged 10-22 months, were housed individually under 12L:12D photoperiod. The animals were fed 150-200g/day of a special rabbit breeding diet prepared according to the institutional recipe (17.38% crude protein, 10.35 AME/MJ, 3.04% crude fat, 16.79% crude fibre, 8.78% crude ashes). Hay and drinking water supplemented with probiotics and vitamins (Gastroferm M-vit, Premium Product Ltd. Hungary) were provided *ad libitum*.

Following a two-week training period, semen was collected once weekly from each of the 10 bucks using a pre-heated artificial vagina. Two consecutive ejaculates were obtained from each buck and evaluated.

Sperm qualification

Semen volume and motility were assessed in both ejaculates, and the gel plug was removed when present. Based on subjective motility scoring (scale 0-5), only samples of adequate quality (score 4-5) were pooled and subsequently analysed using a computer assisted semen analysis system (CASA, Microptic S.L. SCA®, Spain). The freezing experiments were performed in 10 replicates using pooled semen. Sperm concentration was determined using a Makler chamber (Sefi-Medical Instruments Ltd, Israel). Aniline blue-eosin (Aniline SIGMA-Aldrich, Eosin Y Merck Hungary) vital staining was used to assess the proportion of live and dead spermatozoa, with 200 cells evaluated per sample on smears (VÁRADI et al., 2013). The live/dead cell ratio was assessed in fresh pooled semen, after equilibration and after freezing/thawing. CASA parameters were determined before freezing (fresh samples) and after freezing/thawing in all experimental groups.

Sperm freezing protocol

Pooled semen samples were divided into three equal aliquots. The control sample was diluted with modified Lake's extender developed for rabbit semen (KISSNÉ VÁRADI et al., 2026) and supplemented with 1% glycerol and 4% dimethylformamide (DMF). The sericin-supplemented groups were extended with

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modified Lake's diluent supplemented with 0.1 and 0.3 w/v% sericin. The dilution ratio was 1:4 in all groups. Samples were loaded into 0.25 mL straws and equilibrated at 5°C for 60 minutes. Following equilibration, the straws were placed 5 cm above liquid nitrogen for 15 minutes. After freezing and a 30-minute stabilization period, the samples were thawed in a 40°C water bath for 30 seconds.

2.4. Statistical analysis

Statistical analyses were performed using StataNow 19.5 software (StataCorp LLC, College Station, TX, USA). For data expressed as percentages, arcsine transformation was applied prior to statistical analysis (REICZIGEL et al., 2007). Normally distributed data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. For non-normally distributed data, the Mann–Whitney U test was used to compare the groups.

Results and discussion

Following the cryopreservation procedure total and progressive motility showed a marked decline (Table 1). In contrast an earlier study on sperm cryopreservation with sericin pretreatment reported improvements in total and progressive motility in a dose dependent manner (RAZA et al., 2019). During 72 hours of cooling storage, the addition of 0.1% sericin also resulted in an increased sperm motility (AAD et al., 2024). The effectiveness of sericin supplementation appears to be concentration-dependent; in rabbits, concentration between 0.03-0.5% to be effective, whereas higher concentrations may lead to a decline in semen quality (ALI and KHATUN, 2026).

Table 1: Results of the CASA analysis of frozen/thawed semen samples supplemented with different concentrations of sericin.

	CASA analysis			
	Progressive (%)	**Non progressive (%)**	**Immotile (%)**	**Total motility (%)**
Fresh, diluted sample	76.1[a]	8.5 [a]	15.4[a]	84.6[a]
Frozen/thawed sample Control	3[b]	13 [b]	84[b]	16[b]
Frozen/thawed sample Sericin 0.1 w/v%	3.4[b]	14.1 [b]	82.5[b]	17.5[b]
Frozen/thawed sample Sericin 0.3 w/v%	3.8[b]	14.7 [b]	81.5[b]	18.5[b]

a-b-c-d indicate significant differences between the rows $P<0.05$

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In other species, sericin supplementation has been observed to increase total and progressive motility following the cryopreservation of bull, goat, boar and chicken sperm freezing (CHATURVEDI et al., 2022; KUMAR et al., 2024; RATCHAMAK et al., 2020, 2023). In contrast, in our previous studies on sperm cryopreservation in native Hungarian roosters, sericin supplementation proved ineffective (BÁNYÁSZ et al., 2025).

At the end of the equilibration period, no differences were detected in the proportion of live, intact cells, among the fresh (87.2%), control (diluted, without sericin: 86.5%), and sericin-supplemented groups (0.1 w/v%: 86.0%; 0.3 w/v%: 85.4%) (Figure 1). This finding contrasts with the results of RAZA et al. (2019), who reported that sericin pretreatment of fresh, diluted rabbit semen improved semen quality during cold storage. Increased sperm viability and a higher proportion of live, intact spermatozoa were observed during equilibration period following sericin treatment.

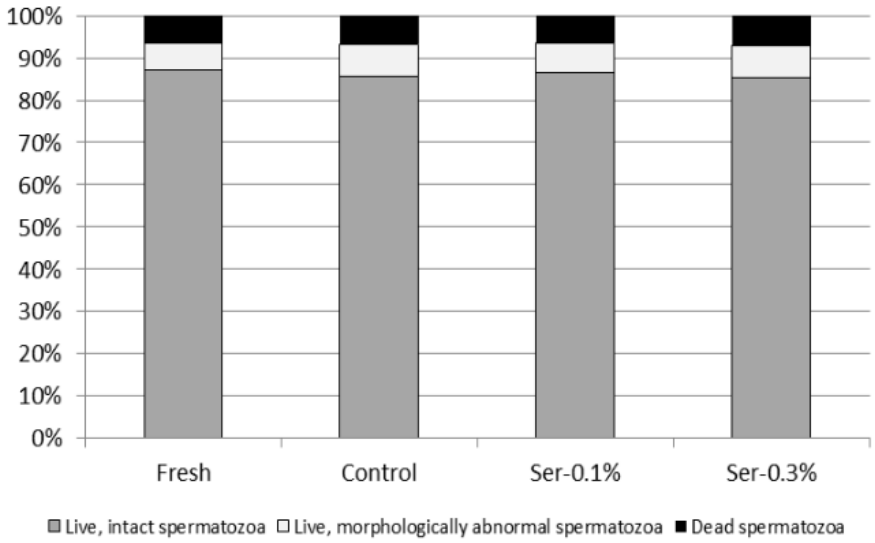


Figure 1: Changes in sperm quality following the equilibration period in samples supplemented with different concentrations of sericin.

Subsequent the freezing/thawing procedure, no differences in semen quality were found among the control and sericin-supplemented groups (Figure 2). The proportion of live, intact spermatozoa decreased significantly in all groups (Control: 33.3%; Ser 0.1 w/v%: 30.15%, Ser 0.3 w/v%: 30.17%), regardless of sericin supplementation.

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To the best of our knowledge, RAZA et al. (2019) are the only researchers to have investigated the effects of sericin supplementation during sperm cryopreservation in rabbits, reporting increased sperm viability and acrosome integrity following freezing and thawing. In other mammalian species, including mice, bull, goat, buffalo, and boar, improvements in sperm quality have also been demonstrated following the use of sericin during sperm cryopreservation (GHASEMI et al., 2018; KUMAR et al., 2015, 2024; VIJYETA et al. 2024; RATCHAMAK et al., 2020).

Unfortunately, the positive effects of sericin reported in other species were not evident during the cryopreservation of Hungarian Giant rabbit sperm. According to previous research, this may be related to the increased susceptibility of rabbit spermatozoa to oxidative damage (ROSATO el. al., 2011). However, as a positive effect has been observed in other rabbit breed (RAZA et al., 2019), it is possible that the effectiveness of antioxidant supplementation depends on breed-specific characteristics.

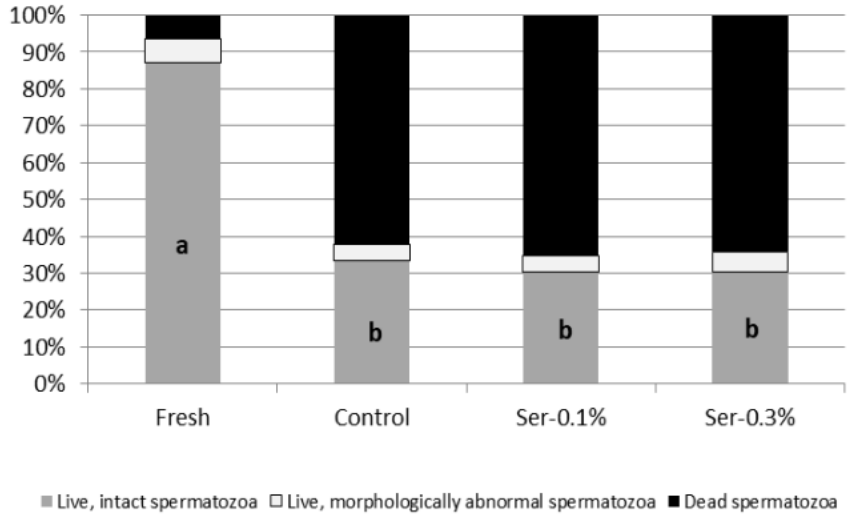


Figure 2: Proportions of live, membrane-intact; live, morphologically abnormal; and dead spermatozoa in fresh and frozen/thawed semen samples.
a-b indicate significant differences (P<0.05)

Conclusion and recommendation

At the concentrations tested (0.1 w/v% and 0.3 w/v%), sericin supplementation did not improve the outcome of sperm cryopreservation. Further studies are required to

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determine whether the tested concentrations were suboptimal or whether this rabbit breed responds poorly to sericin supplementation.

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