Animal Science Papers and Reports vol. 42 (2024) no. 3, 241-254
DOI: 10.2478/aspr-2023-0034
Institute of Genetics and Animal Biotechnology
of the Polish Academy of Sciences, Jastrzębiec, Poland

sciendo

Long-term cryopreservation of bull semen as a main strategy for the *ex situ* conservation of endangered Polish Red cattle*

Magdalena Bryła[1], Monika Trzcińska[1], Marcin Kocik[2], Artur Burmańczuk[3]**

[1] Department of Reproductive Biotechnology and Cryoconservation, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice near Kraków, Poland

[2] Elanco Animal Health, Poland

[3] Department of Pharmacology, Toxicology and Environmental Protection Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Akademicka 12, 20-033 Lublin, Poland

(Accepted June 29, 2024)

Regular verification of the quality of cryopreserved semen derived from native cattle is one of the tasks performed at the bank as recommended by the Food and Agriculture Organization. The purpose of the present study was to evaluate the quality of semen from PR bulls stored for 40, 50 and 60 years in the BMB using standard evaluation parameters such as sperm motility as well as structural-functional parameters such as plasma membrane integrity, transmembrane mitochondrial potential and sperm chromatin damage. Semen pellets from 27 PR bulls (3 ejaculates/bull) were tested. The data were analysed by one-way and two-way ANOVA, and the significance of the difference (P≤ 0.01) between the means was determined using Duncan's test. Our study results revealed that the long-term storage of semen had no effect on sperm characteristics after thawing. However, statistically significant differences (P≤0.01) in sperm plasma membrane integrity and transmembrane mitochondrial potential, following storage in liquid nitrogen were noted between bulls at all time points. However, there were no significant differences (P>0.01) in sperm chromatin

*The present study was financially supported by statutory grant No. 04-19-12-11 from the National Research Institute of Animal Production in Balice near Kraków
**Corresponding author: magdalena.bryla@iz.edu.pl

damage between breeds or between different storage times, and the degree of DNA fragmentation ranged from 0.4 to 0.8%.

KEY WORDS: bull semen / cryopreservation/long-term storage / quality assessment/gen bank

Intensification of the production and creation of new breeds or lines of livestock with high genetic value or high productivity can significantly destabilize the global diversity of domesticated animals [Lauvie *et al*. 2011, Woelders *et al*. 2012]. The progressive decrease in the abundance and biodiversity of livestock breeds is often the result of harsh selection aimed at perfecting genotypes that result in the high performance value of specific genotypic and phenotypic traits from individual subpopulations and breeding herds [Leroy *et al*. 2020, Polak *et a*l. 2021]. Therefore, local breeds with more primitive traits are less common or are disappearing at the expense of high-yielding, widely distributed breeds [Kikuchi *et al*. 2016, Dua *et al.* 2021]. The main threats to declining animal biodiversity are environmental changes associated with the expansion of cultivated areas, as well as the introduction of nonnative species preferred by humans [Wood *et al.* 2018, Comizzoli and Holt 2019]. For these reasons, it is particularly important to protect the genetic resources of native breeds that are highly adapted to local, often harsh, climatic and ecosystem conditions and at the same time characterized by high resilience and good health [Trzcińska *et al.* 2023].

The globalization and intensification of livestock production poses a threat to the preservation of the genetic diversity and population variability of individual native bull breeds in Poland. Genetic resources are susceptible to loss and are rapidly declining due to habitat alteration/degradation, overexploitation, pollution, invasive alien species and climate change. According to Regulation (EU) 2016/1012 of the European Parliament and of the Council of 8 June 2016 on zootechnical and genealogical conditions for the breeding, trade in and entry into the Union of purebred breeding animals, hybrid breeding pigs and the germinal products thereof and amending Regulation (EU) No 652/2014, Council Directives 89/608/EEC and 90/425/EEC and repealing certain acts in the area of animal breeding; endangered breeds are "local breeds recognized by Member States as endangered, genetically adapted to one or more production systems in that country, whose endangered status has been scientifically confirmed by an authority with the necessary skills and knowledge in the field of endangered breeds." The status of endangered breeds in Poland is reported annually at http://bioroznorodnosc.izoo.krakow.pl/status-zagrozenia-ras. Current data for native cattle breeds indicate that the Polish Red (PR), Polish Black and White (ZB) and Polish Red and White (ZR) breeds are endangered and that White-backed cattle are critically endangered. In view of the above, the semen stored in the bank constitute a valuable and unique reserve of biological materials that can be used for the artificial insemination of cows in herds covered by the cattle genetic resources protection program. The collection of cryopreserved biological material in the form of bull semen is a fundamental component of the *ex situ* conservation strategy for livestock genetic resources [Delgado Bermejo *et al*. 2019].

Cryopreservation is the only method used to preserve the biodiversity of livestock and provides an opportunity to collect and store biological material with unique functional and adaptive traits for an unlimited duration. The cryopreservation program is designed to support the preservation of breeds *in situ* while determining their degree of endangerment, adaptation to the environment, economically important performance traits, unique traits, historical and cultural characteristics, genetic uniqueness and contribution to the preservation of the natural agroecosystem [Trzcińska *et al.* 2023]. The use of cryopreserved livestock semen allows the rational control of reproduction and significantly influences breeding progress and the preservation of genetic diversity. This applies to both native breeds and high-yielding commercial breeds that are at risk of losing genetic variability due to intensive selection or animal disease epidemics. Cryopreserved genetic material is not subjected to loss of variability caused by the effects of selection, genetic drift or increased inbreeding, as is the case in live animal populations [Sonesson *et al*. 2002].

The National Research Institute of Animal Production (NRIAP) in Poland has been approved by the Ministry of Agriculture and Rural Development (MARD) to carry out activities such as the coordination of tasks related to *in situ* and *ex situ* livestock genetic resource conservation programs. The origins of *ex situ* conservation of genetic resources at the NRIAP date back to the 1960s. In 1968, the Central Semen Bank (CSB) was established at the NRIAP.

The CSB's main responsibility was to store semen from young bulls that had been delivered to the bank from all insemination stations in Poland. In addition, due to the increasing import intensity of semen at the time, the MARD commissioned the CSB to conduct a quality evaluation of the semen and determine its qualification for artificial insemination procedures in the country. The first biological material (semen) of a PR bull was deposited at the CSB in 1968. Over the years during which the bank operated, its collection was expanded to include semen from more bulls of the PR breed as well as other native breeds: ZR, ZB and White-backed. Over the years, the only unique collection of biological material in Poland, both in terms of genotype and quantity of collected material, was created. To date, the bank's collection consists of 68 835 semen pellets from native cattle breeds, including 53 973 semen pellets from 144 PR bulls.

The PR is the only native breed of cattle in Poland, the breeding of which began at the end of the 19th century. The breed is derived from shorthorn taurine cattle, *Bos taurus brachyceros* [Czaja and Trela 1994]. The PR breed is characterized by several features typical of primitive populations, such as high vitality and fertility, calving ease, resistance to diseases and high nutritional value of milk. PR cattle are also known to adapt well to harsh environmental conditions, which is especially visible in their ability to limit their efficiency (enabling their survival in the face of seasonal feed deficiencies), as well as their relatively quick recovery after body condition loss. These features make the cattle of this breed well adapted to mountainous and submountainous living and production conditions [Szarek *et al*. 2004].

Consequently, the role of biological material banks is not only to systematically increase the pool of genetic reserves but also to periodically assess the quality of the stored material. According to the FAO guidelines contained in the guide "Cryopreservation of Animal Genetic Resources: FAO Animal Production and Health Guidelines", such an evaluation should be carried out once every 10 years of storage in liquid nitrogen. Such a procedure makes it possible to verify the quality of semen and determine its suitability for further storage in the bank and the possibility of using it in breeding practice.

Few studies in which semen quality was evaluated during long-term storage at -196°C indicate that bull spermatozoa demonstrate adequate motility 37 years after freezing [Leibo *et al.* 1994]. Moreover, Rofeim and Gilbert [2005] reported no significant reduction in the quality of human semen after 5 years of storage in liquid nitrogen. In contrast, Malik *et al.* [2015] reported that the viability and motility of thawed bull sperm stored in liquid nitrogen for 6 years were lower than those of thawed sperm stored for 1-2 years. Similarly, Akyol *et al*. [2018] observed that 30-year storage in liquid nitrogen had negative effect on the quality parameters of Brown Swiss bull semen.

This article presents the results obtained following an evaluation of the quality of bull semen from Poland's oldest native cattle breed stored at the BMB for 40, 50 and 60 years. To our knowledge, this is the first study in Poland on the quality of cryopreserved bull semen following FAO recommendations. The assessment was performed using a panel of methods for determining standard parameters such as motility, as well as for verifying sperm functional parameters such as plasma membrane integrity, transmembrane mitochondrial potential and sperm chromatin damage.

The aim of this study was to evaluate the functional parameters of semen and the effects of storage for 40, 50 and 60 years on the quality of the biological material that constitutes the genetic reserve of PR cattle in Poland.

Materials and methods

Animals

A total of 81 semen pellets from 27 PR bulls produced between 1963 and 1980 and stored up to the time of this study in liquid nitrogen (-196°C) in the Bank of Biological Materials (BMB) at the National Research Institute of Animal Production were used in this study. The sample pellets were divided into three groups based on the duration of cryostorage: Groups 1, 2, and 3, with semen stored in liquid nitrogen for 40, 50, and 60 years, respectively. The value of postthaw sperm motility before the commencement of storage in the BMB was evaluated by thawing one ejaculate pellet from each bull. Thawing was performed at 37°C for 10 minutes, and semen quality was assessed based on progressive motility (PM0) under a light microscope by one observer.

Evaluation of semen quality

After 10 min of incubation at 37°C, postthaw semen quality was evaluated based on sperm motility, viability, transmembrane mitochondrial potential and sperm chromatin damage.

Assessment of sperm motility using CASA

The pellets were thawed by placing them into a 0.7-ml OptiXcell extender (IMV, France) at 37°C, and postthaw sperm total (TM%) and progressive (PM%) motility were measured via computer-assisted sperm analysis (CASA) (Sperm Class Analyser, S.C.A. V5.1, Microptic, Barcelona, Spain). Thawed semen (3 µl) was placed in a Leja counting chamber (Leja Products B.V., GN Nieuw-Vennep, The Netherlands). Motility was measured twice in each sample, three fields were evaluated, and at least 1000 cells were counted in each analysis.

Fluorescence microscopy analysis of sperm viability and transmembrane mitochondrial potential

Sperm viability and the mitochondrial potential of thawed bull semen were evaluated under a Nikon Eclipse E600 compound microscope (Nikon Corp., Tokyo, Japan) equipped with PlanFluo 40×/0.75 DIC M objective lenses (Nikon). Fluorescence staining was assessed by microscopically observing at least 200 cells in one field per sample per slide by one observer.

A Vybrant Apoptosis Assay Kit #4 (Molecular Probes Inc., Eugene, USA) was used to detect changes in plasma membrane permeability to YO-PRO-1. Thawed spermatozoa were diluted in 1 mL of PBS (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and 2 µL of YO-PRO-1 (100 µmol/L) was added. The contents of tubes were gently mixed and incubated for 20 min at room temperature, after which 1 µmol/L propidium iodide (PI) was added to each tube. After the incubation period, at least 200 spermatozoa per sample were evaluated using appropriate filters for YO-PRO-1 (Ex:491 nm/Em:507 nm) and PI (Ex:538 nm/Em:619 nm). The results are presented as the percentage of viable spermatozoa (YO-PRO-1$^-$/PI$^-$), viable spermatozoa with apoptotic-like changes (YO-PRO-1+/PI-) and nonviable spermatozoa (YO-PRO-1$^+$/PI$^+$) in each semen sample [Trzcińska and Bryła 2015].

To evaluate transmembrane mitochondrial potential, spermatozoa were labelled with JC-1 dye (Molecular Probes Inc., Eugene, USA). Semen was centrifuged for 10 min at 300 g at room temperature, and the sperm pellet was then washed with PBS without calcium or magnesium (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). Finally, the sample was resuspended in 1 mL of PBS. The sample was stained with 10 µg/mL JC-1 (final concentration; stock solution 1 mg/mL in DMSO) at 37°C for 15 min. After incubation, the sperm were evaluated using a fluorescence microscope (Nikon Eclipse E600, Tokyo, Japan). At least 200 spermatozoa per sample were evaluated in one field per sample per slide by one observer. The percentages of sperm with high mitochondrial transmembrane potential (JC-1$^+$) in each semen sample were calculated [Trzcińska and Bryła 2015].

Cytometric assessment of sperm chromatin damage

For the assessment of sperm chromatin damage, the sperm chromatin structure assay (SCSA) protocol was used. Briefly, sperm chromatin denaturation was induced by incubating sperm cells with a mixture of HCl (Sigma-Aldrich Chemie GmbH) and Triton X-100 (Sigma–Aldrich Chemie GmbH) (pH = 1.2). After 30 sec, the spermatozoa were stained with acridine orange (Sigma–Aldrich Chemie GmbH) for 3 min. green (normal chromatin) and red (cells with fragmented DNA) fluorescence were quantified by flow cytometry. The percentage of sperm with a damaged chromatin structure was measured based on the DNA fragmentation index (DFI).

Statistical analysis

Percentage of mean values (±SD) for various parameters of semen quality were calculated. Traits that did not exhibit a normal distribution were logarithmically transformed, and then their differences were analysed using one-way and two-way ANOVA for frozen-thawed bull semen stored in liquid nitrogen for 40, 50 and 60 years. If a significant effect was detected using ANOVA, the significance of the difference between the means was determined using Duncan's test. The computations were performed using the Statistica 6.0 program (StatSoft, Tulsa, OK, USA). $P \leq 0.01$ value was considered statistically significant.

Results and discussion

Semen stored in the BMB not only constitute a genetic reserve in case factors threatening the biodiversity of Poland's cattle population emerge but are also used for *in situ* conservation programs for this livestock species. The storage of cryogenically preserved material from PR breed bulls is necessary because of the unique characteristics of the breed, such as good health, disease resistance and fertility, and thus longevity, as well as production traits such as a high fat content in milk, very good technological properties of milk as a raw material for cheese production, and high-quality indicators of meat as a culinary product [Szarek *et al*. 2004]. In addition, the collected material in the form of bull semen will allow us to rebuild the herd if necessary and continue breeding work in the pure breed. Until testing was carried out in accordance with BMB procedures, when bull semen was stored in the bank, the only way to verify its quality was to assess progressive sperm motility after thawing (PM0). For this purpose, one randomly selected pellet of semen was thawed, and sperm motility was evaluated under a light microscope. However, no quality evaluation of semen has been carried out at any point throughout its storage in liquid nitrogen until now. The effects of long-term storage for 40, 50 and 60 years on the functional parameters of bull semen are presented in Tables 1, 2 and 3, respectively. For the evaluation of progressive movement after thawing, there were no significant differences between the results from evaluations carried out at the time the biological material was received in the bank (PM0) and the evaluations carried out at present

(PM). However, significant differences (P≤0.01) in the percentage of sperm with progressive motility (PM0) were found between individual bulls whose semen had been stored for 50 years (Tab. 2). In contrast, no differences in these values were found between bulls whose semen had been stored for 40 and 60 years (Tab. 1 and 3).

Table 1. Mean values (SD) of quality parameters of bull semen stored in liquid nitrogen for 40 years

Bull no.	Motility (%)				Viability (%)			JC-1 (%)	SCSA
	progressiv emotility PM0	progressiv emotility PM	P-value	total motility TM	viable sperm (YO-PRO-1$^-$/PI$^-$)	viable sperm with apoptotic-like changes (YO-PRO-1$^+$/PI$^-$)	nonviable sperm (YO-PRO-1$^+$/PI$^+$)	high mitochondrial transmembrane potential JC-1$^+$	DFI (%)
1	55.0^A (5.0)	55.4^A (4.8)	0.208	70.2^A (2.2)	70.7^A (4.8)	8.0^B (1.3)	21.3^{AB} (3.5)	72.0^A (2.6)	0.5 (0.1)
2	51.7^A (2.9)	55.5^A (9.8)	0.545	66.2^A (5.9)	66.5^A (2.0)	7.5^B (1.5)	26.0^{AB} (0.5)	65.3^A (4.2)	0.6 (0.1)
3	51.7^A (5.8)	52.3^A (0.8)	0.316	70.0^A (1.3)	72.2^A (3.7)	8.3^B (1.6)	19.5^B (2.3)	70.3^A (3.5)	0.7 (0.2)
4	56.7^A (2.7)	57.5^A (2.7)	0.420	70.3^A (1.1)	67.5^A (4.8)	6.8^B (2.2)	25.7^{AB} (3.0)	71.3^A (3.0)	0.7 (0.1)
5	58.3^A (2.9)	56.1^A (4.0)	0.763	71.9^A (1.7)	64.3^A (2.2)	8.5^B (0.9)	27.2^{AB} (1.9)	68.3^A (5.5)	0.4 (0.1)
6	50.0^A (5.0)	54.1^A (6.5)	0.234	70.8^A (1.0)	67.8^A (2.4)	7.5^B (0.9)	24.7^{AB} (1.9)	63.0^A (6.2)	0.7 (0.1)
7	55.0^A (5.6)	60.8^A (7.3)	0.318	71.8^A (1.7)	52.5^B (1.0)	19.5^A (1.5)	28.0^{AB} (1.3)	66.3^A (3.8)	0.5 (0.1)
8	53.3^A (2.9)	55.2^A (4.3)	0.434	70.3^A (1.9)	65.2^A (2.0)	9.3^B (1.0)	25.5^{AB} (1.0)	73.0^A (7.2)	0.6 (0.1)
9	53.3^A (5.8)	60.8^A (3.2)	0.049	67.3^A (1.1)	46.3^B (1.7)	24.5^A (0.5)	29.2^A (1.0)	68.0^A (6.1)	0.8 (0.1)
Mean	**53.9 (4.5)**	**55.9 (4.3)**	**0.701**	**69.8 (2.8)**	**63.7 (9.9)**	**11.1 (6.2)**	**25.2 (3.4)**	**68.6 (5.2)**	**0.6 (0.1)**

^{AB}In columns means with different superscripts differ significantly at P≤0.01.

Table 2. Mean values (SD) of quality parameters of bull semen stored in liquid nitrogen for 50 years

Bull no.	Motility (%)				Viability (%)			JC-1 (%)	SCSA
	progressiv emotility PM0	progressiv emotility PM	P-value	total motility TM	viable sperm (YO-PRO-1$^-$/PI$^-$)	viable sperm with apoptotic-like changes (YO-PRO-1$^-$/PI$^-$)	nonviable sperm (YO-PRO-1$^+$/PI$^+$)	high mitochondrial transmembrane potential JC-1$^+$	DFI (%)
10	51.7^{AB} (2.9)	53.6^{A} (3.8)	0.309	68.9^{A} (2.7)	58.3^{A,B} (2.2)	10.0^{AB} (1.3)	31.5^{BC} (3.0)	68.7^{A} (2.1)	0.6 (0.1)
11	48.3^{AB} (7.6)	47.1^{A} (2.9)	0.814	53.8^{B} (9.0)	35.5^{C} (0.1)	18.5^{A} (1.3)	46.0^{A} (1.3)	50.3^{C} (5.0)	0.6 (0.1)
12	51.7^{AB} (2.9)	42.5^{A} (4.6)	0.350	58.7^{B} (6.2)	39.7^{C} (3.3)	19.5^{A} (1.7)	40.8^{A,B} (2.4)	53.7^{C} (4.5)	0.7 (0.1)
13	45.0^{B} (5.0)	49.0^{A} (1.2)	0.280	68.2^{A} (4.3)	53.0^{A,B} (1.3)	10.3^{A,B} (1.2)	36.7$^{A, B}$ (2.1)	61.3^{B} (5.5)	0.7 (0.0)
14	48.3^{A,B} (7.6)	51.6^{A} (7.4)	0.411	72.3^{A} (4.0)	60.2^{A} (2.1)	9.5^{B} (1.3)	30.3^{B,C} (1.9)	70.3^{A} (2.1)	0.6 (0.1)
15	47.7^{AB} (2.5)	46.8^{A} (5.9)	0.025	67.1^{A} (2.7)	59.2^{A,B} (2.5)	9.3^{B} (1.0)	31.5^{B,C} (2.0)	65.0^{A,B} (4.3)	0.5 (0.1)
16	53.3^{AB} (5.8)	51.6^{A} (4.9)	0.851	75.9^{A} (3.1)	67.7^{A} (1.7)	7.8^{B} (0.8)	24.5^{C} (1.0)	66.0^{A,B} (6.1)	0.5 (0.0)
17	56.7^{A} (7.6)	52.2^{A} (4.5)	0.070	71.7^{A} (4.2)	64.8^{A} (0.3)	10.0^{A,B} (0.5)	25.2^{B,C} (0.8)	70.0^{A} (2.0)	0.7 (0.1)
18	55.0^{A,B} (5.0)	47.6^{A} (2.3)	0.031	70.7^{A} (4.0)	61.5^{A} (1.5)	9.0^{B} (0.5)	29.5^{B,C} (1.3)	69.3^{A} (1.9)	0.8 (0.1)
Mean	**50.8 (5.9)**	**49.1 (5.0)**	**0.740**	**67.6 (7.8)**	**55.5 (10.7)**	**11.5 (4.2)**	**32.9 (6.9)**	**63.8 (7.9)**	**0.6 (1.3)**

^{ABC}In columns means with different superscripts differ significantly at P≤0.01.

Moreover, the results of the CASA showed no significant differences in the percentage of sperm with total (TM) or progressive (PM) motility between bulls regardless of storage time at -196°C.

Table 3. Mean values (SD) of quality parameters of bull semen stored in liquid nitrogen for 60 years

Bull no.	Motility (%)				Viability (%)			JC-1 (%)	SCSA
	progressiv emotility PM0	progressiv emotility PM	P-value	total motility TM	viable sperm (YO-PRO-1$^-$/PI$^-$)	viable sperm with apoptotic-like changes (YO-PRO-1$^+$/PI$^-$)	nonviable sperm (YO-PRO-1$^+$/PI$^+$)	high mitochondrial transmembrane potential JC-1$^+$	DFI (%)
19	52.5^A (2.9)	55.1^A (4.7)	0.552	68.6^A (3.0)	57.9^A (3.9)	9.8^B (1.0)	32.2^{A,B} (3.0)	70.7^A (1.5)	0.6 (0.2)
20	51.7^A (2.9)	55.9^A (9.6)	0.545	66.2^{A,B} (5.9)	57.0^A (2.3)	11.3^B (0.8)	31.7^{A,B} (3.0)	67.7^{A,B} (5.0)	0.4 (0.1)
21	54.7^A (4.5)	52.7^A (0.5)	0.417	68.3^{A,B} (1.2)	64.0^A (2.2)	9.7^B (1.2)	26.3^B (1.3)	69.0^B (1.0)	0.4 (0.2)
22	53.3^A (5.8)	47.5^A (2.6)	0.189	58.6^B (1.3)	36.2^B (4.0)	22.8^A (2.5)	40.9^A (1.5)	60.3^B (1.5)	0.3 (0.1)
23	55.0^A (5.0)	56.1^A (4.0)	0.387	74.6^A (1.3)	64.3^A (1.0)	9.8^B (1.0)	25.8^B (1.0)	70.7^A (8.5)	0.6 (0.2)
24	53.3^A (2.9)	50.9^A (5.9)	0.413	73.8^A (4.4)	63.8^A (1.6)	8.8^B (0.3)	27.3^B (1.5)	67.3^A (7.2)	0.5 (0.2)
25	51.7^A (2.9)	55.6^A (3.7)	0.295	67.6^{A,B} (3.5)	60.8^A (3.0)	9.0^B (0.5)	30.2^B (2.5)	62.6^{A,B} (3.2)	0.5 (0.1)
26	56.7^A (2.9)	56.3^A (1.8)	0.849	70.3^A (1.9)	64.3^A (0.6)	10.2^B (0.6)	25.5^B (1.0)	63.4^{A,B} (1.1)	0.4 (0.2)
27	55.0^A (0.5)	61.2^A (2.7)	0.258	72.6^A (4.1)	64.0^A (2.5)	7.5^B (1.5)	28.5^B (1.0)	69.0^{A,B} (4.3)	0.5 (0.2)
Mean	**53.8 (3.9)**	**54.6 (5.4)**	**0.825**	**68.9 (5.4)**	**59.0 (9.4)**	**11.0 (4.5)**	**29.9 (5.3)**	**66.7 (5.2)**	**0.6 (0.2)**

^{ABC}In columns means with different superscripts differ significantly at P≤0.01.

Cryopreservation in liquid nitrogen (-196°C) is the only method that allows the long-term storage of spermatozoa. Mazur and Kashimoto [2002] demonstrated that storing spermatozoa in liquid nitrogen is preferred because it prevents thermal reactions, and only slow background ionizing radiation can have a detrimental effect

on sperm function over a long period. Moreover, the cryopreservation of gametes ensures the cessation of ongoing life processes for a virtually unlimited time, with natural radiation being the only factor that can negatively affect the biological value of the stored material [Mazur 1984]. However, the effects of such radiation may only occur after 2,000-30,000 years [Lyon *at al.* 1977]. Nonetheless, for cryopreservation to be successful, it is necessary that spermatozoa are preserved for long periods without damaging their postthaw motility or fertilizing ability [Medeiros *et al*. 2002]. There are still inconsistent reports in the literature regarding the effect of semen storage time on sperm quality after thawing. Some studies have demonstrated a reduction in the fertilizing capacity of semen from bulls [Haugan *et al.* 2007] and boars [Kozumplík 1985] or a loss of human sperm motility after thawing [Smith and Steinberger 1973]. In addition, a study by Malik *et al.* [2015] showed that a significant decrease in bull sperm motility is associated with long-term storage of semen for 40-60 years. Moreover, studies on human biological material have shown that the long-term storage of semen does not affect clinical outcomes [Huang *et al*. 2019]. However, storing semen for more than 5 years negatively affects the quality of frozen and thawed donor semen samples [Huang *et al.* 2019]. Moreover, our results did not indicate a negative effect of different storage durations on the progressive motility of bull sperm, which is similar to the findings of Ramirez-Reveco *et al.* [2016].

The cryopreservation procedure not only reduces the percentage of motile sperm but also causes significant damage to the structural and functional parameters of spermatozoa [Salamon and Maxwell 1995, Yánez-Ortiz *et al*. 2022]. Indeed, sperm motility is not a sufficient indicator of biological properties since the loss of fertilization ability is not always related to a lack of functioning of the motility apparatus [Johnson *et al*. 2000]. In addition, there may also be "quasiapoptotic" sperm in the ejaculate [Sakkas *et al.* 1999], which appear in semen due to the lack of synchronization between spermatogenesis and apoptosis. In this case, the apoptosis of spermatogenic cells is not a complete process that eliminates damaged cells- and, therefore, leads to the occurrence of sperm with apoptotic changes in the ejaculate. Our previous results indicated that the detection of apoptotic changes in spermatozoa enables the determination of fertilization ability *in vivo* [Trzcinska *et al*. 2011, 2015, Trzcinska and Bryła 2015]. Therefore, in the present study, we also evaluated functional-structural parameters of sperm quality based on an analysis of sperm viability, an assessment of transmembrane mitochondrial potential and an assessment of sperm chromatin damage. The highest and lowest percentages of viable spermatozoa (YO-PRO-1$^-$/PI$^-$) (72.2±3.7 and 35.5±0.1) were noted in semen of bull nos. 3 and 11, which were stored for 40 years (Tab. 1) and 50 years (Tab. 2), respectively. An evaluation of apoptotic-like changes in spermatozoa revealed that the semen of bull nos. 7, 9, 11, 12 and 22 had a significantly greater percentage of apoptotic cells than did the semen of other bulls. The highest value was noted for bull number 9, where the percentage of YO-PRO-1$^+$/PI$^-$ sperm was 24.5%±0.5 (Tab. 1). Moreover, a significantly lower percentage of spermatozoa with TM and spermatozoa with high transmembrane mitochondrial potential was observed for bull

nos. 11 and 12 (Tab. 2). An assessment of transmembrane mitochondrial potential after thawing revealed no differences in the percentage of sperm with high mitochondrial potential (JC-1^{+}) among the bull semen pellets stored for 40 years (Tab. 1). In contrast, statistically significant differences were noted between bulls whose semen had been stored for 50 and 60 years (Tab. 2 and 3). In addition, a cytometric evaluation of sperm chromatin damage indicated no significant differences between the evaluated bulls regardless of the duration of semen storage in liquid nitrogen. An evaluation of sperm chromatin indicated that the DFI was low in all bulls studied regardless of semen storage time, with percentages ranging from 0.4 to 0.8%.

Our analysis of the structural and functional parameters of semen quality showed no differences due to the duration of semen storage in liquid nitrogen. Moreover, the cytometric analysis of sperm DNA integrity showed that in all semen samples analysed in the present study, the DFI of sperm was very low and did not exceed 1%, indicating high stability of sperm chromatin. The results obtained by Shoaib *et al.* [2013] demonstrated that microscopic analysis using toluidine blue staining technique of bull semen stored for three years in liquid nitrogen revealed 5.8% sperm with damaged DNA. Verification of sperm quality based on plasma membrane integrity, mitochondrial potential or chromatin damage is important. The previous study by Dogan *et al*. [2015] revealed that that reduced bull fertility is associated with defects in sperm chromatin structure. Additionally, our earlier studies on boar semen [Trzcińska *et al*. 2011] demonstrated that after insemination with semen with an elevated percentage of apoptotic spermatozoa, a greater number of degenerated embryos was obtained. Moreover, a study by Akyol *et al*. [2019] revealed that the use of Brown Swiss bull semen stored by 32 years with high percentage of necrotic sperm for *in vitro* fertilization results in lower cleavage ratio and blastocyst rates.

In present studies significant differences between bulls can be observed in the individual storage durations of frozen semen. In all analysed groups, regardless of the duration of storage in liquid nitrogen, there were few individuals with a high percentage of apoptotic sperm compared to the other bulls. This finding may reflect the individual susceptibility of each individual's semen to the freeze-thaw process rather than the duration of storage in liquid nitrogen. Moreover, this information in the aspect of both storage and suitability of bull semen deposited at BMB in breeding programs in Poland. However, because evaluation of semen quality of native PR breed was undertaken for the first time, we do not have a reference point and data to compare individual differences between bulls.

Conclusion

Based on the current research, long-term cryopreservation of PR bull semen had no effect on sperm characteristics/quality. The results revealed single individual variations in semen quality between males, but these differences in single structural-functional parameters were independent of the storage duration in liquid nitrogen.

In the future, periodic testing of cryopreserved bull sperm carried out at intervals recommended by the FAO will make it possible to determine more specifically whether the observed structural-functional changes in sperm are due to the individual susceptibility of the male donor to the semen cryopreservation process or are related to the period/time of semen storage in liquid nitrogen.

Acknowledgments. *The authors are grateful to Halina Korzonek, M.Sc. for assistance with statistical analysis.*

REFERENCES

1. AKYOL N., KIZIL S.H., SATILMIS M., KARAŞAHIN T., 2014 – Investigation of bull effect on *in vitro* embryo production. ***Kafkas Üniversitesi Veteriner Fakültesi Dergisi*** 20, 561-564.
2. AKYOL N., VARIŞLI Ö., KIZIL S.H., 2018 – Effects of Long-term Storage on Some Spermatological Parameters in Cryopreserved Bull Semen. ***CryoLetters*** 39, 354-358.
3. COMIZZOLI P., HOLT W.V., 2019 – Breakthroughs and new horizons in reproductive biology of rare and endangered animal species. ***Biology of Reproduction*** 101, 514-525.
4. CZAJA H., TRELA J.,1994 – Jak powstała populacja bydła polskiego czerwonego. In: Prace nad zachowaniem rzadkich ras zwierząt gospodarskich. Materiały Sympozium17-19 maja; Balice, 56-57.
5. DELGADO BERMEJO J.V., MARTÍNEZ MARTÍNEZ M.A., RODRÍGUEZ GALVÁN G., STEMMER A., NAVAS GONZÁLEZ F.J., CAMACHO VALLEJO M.E., 2019 – Organization and Management of Conservation Programs and Research in Domestic. ***Animal Genetic Resources Diversity*** 11, 235.
6. DOGAN S., VARGOVIC P., OLIVEIRA R., BELSER L.E., KAYA A., MOURA A., SUTOVSKY P., PARRISH J., TOPPER E., MEMILI E., 2015 – Sperm protamine-status correlates to the fertility of breeding bulls. ***Biology of Reproduction*** 92, 1-92.
7. DUA S., SHARMA P., SAINI M., RAWAT N., RAJENDRAN R., BANSAL S., WAKIL A.M., BENIWAL M., PARASHAR A., BAJWA K.K., et al. 2021 – Cryobanking of primary somatic cells of elite farm animals - A pilot study in domesticated water buffalo (Bubalus bubalis). ***Cryobiology*** 98, 139-145.
8. HAUGAN T., GRÖHN YT., KOMMISRUD E., ROPSTAD E., REKSEN O., 2007 – Effects of sperm concentration at semen collection and storage period of frozen semen on dairy cow conception. ***Animal Reproduction Science*** 97, 1-11.
9. HUANG C., LEI L., WU H.L., GAN R.X., YUAN X.B., FAN L.Q., ZHU W.B., 2019 – Long-term cryostorage of semen in a human sperm bank does not affect clinical outcomes. ***Fertility and Sterility*** 112, 663-669.
10. JOHNSON L., WEITZE K., FISER P., MAXWELL W.M., 2000 – Storage of boar semen. ***Animal Reproduction Science*** 62, 143-172.
11. KIKUCHI K., KANEKO H., NAKAI M., SOMFAI T., KASHIWAZAKI N., NAGAI T., 2016 – Contribution of in vitro systems to preservation and utilization of porcine genetic resources. ***Theriogenology*** 86, 170-175.
12. KOZUMPLÍK J., 1985 – Qualitative changes and the fertilizing capacity of sperm after 6 years of preservation of boar semen. ***Medycyna Weterynaryjna*** 30, 289-300.
13. LAUVIE A., AUDIOT A., COUIX N., CASABIANCA F., BRIVES H., VERRIER E., 2011 – Diversity of rare breed management programs: Between conservation and development. ***Livestock Science*** 140, 161-170.
14. LEIBO S.P., SEMPLE M.E., KROETSCH T.G.,1994 – In vitro fertilization of oocytes by 37-year-old cryopreserved bovine spermatozoa. ***Theriogenology*** 42,1257-1262.

15. LEROY G., GICQUEL E., BOETTCHER P., BESBES B., FURRE S., FERNANDEZ J., DANCHINBURGE C., ALNAHHAS N., BAUMUNG R., 2020 – Coancestry rate's estimate of effective population size for genetic variability monitoring. ***Conservation Genetics Resources*** 12, 275-283.
16. LYON M.F., WHITTINGHAM D.G., GLENISTER P., 1977 – Long-term storage of frozen mouse embryos under increased background irradiation. ***Ciba Found Symposium*** 52, 273-290.
17. MALIK A., LAILY M., ZAKIR M.I., 2015 – Effects of long term storage of semen in liquid nitrogen on the viability, motility and abnormality of frozen thawed Frisian Holstein bull spermatozoa. ***Asian Pacific Journal of Reproduction*** 4, 22-25.
18. MAZUR P., KOSHIMOTO C., 2002 – Is intracellular ice formation the cause of death of mouse sperm frozen at high cooling rates? ***Biology of Reproduction*** 66, 1485-1490.
19. MAZUR P.,1984 – Freezing of living cells: mechanisms and implications. ***American Journal of Physiology-Cell Physiology*** 247,125-142.
20. MEDEIROS C.M.O., FORELL F., OLIVEIRA A.T.D., RODRIGUES J.L., 2002 – Current status of sperm cryopreservation: why isn't it better? ***Theriogenology*** 57, 327-344.
21. POLAK G., KRUPIŃSKI J., MARTYNIUK E., CALIK J., KAWĘCKA A., KRAWCZYK J., MAJEWSKA A., SIKORA J., SOSIN-BZDUCHA E., SZYNDLER-NĘDZA M., TOMCZYK-WRONA I., 2021 – The risk status of Polish local breeds under conservation programmes - new approach. ***Annals of Animal Science*** 21, 125-140.
22. RAMÍREZ-REVECO A., HERNÁNDEZ J. L., AROS, P., 2016 – Long-Term Storing of Frozen Semen at −196°C does not Affect the Post-Thaw Sperm Quality of Bull Semen. ***IntechOpen*** doi: 10.5772/6494.
23. ROFEIM O., GILBERT B.R., 2005 – Long-term effects of cryopreservation on human spermatozoa. ***Fertility and Sterility*** 84, 536-7.
24. SAKKAS D., MARIETHOZ E., MANICARDI G., BIZZARO D., BIANCHI P.G., BIANCHI U., 1999 – Origin of DNA damage in ejaculated human spermatozoa. ***Reviews of Reproduction*** 4, 31-7.
25. SALAMON S., MAXWELL W.M.C., 1995 – Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. ***Animal Reproduction Science*** 37, 185-249.
26. SHOAIB M., AHMAD N., AHMAD M.,AHMAD I., IQBAL S., AKHTER S., 2014 – Effects of Storage Duration on the Quality and DNA Integrity of Nili-Ravi Bull Spermatozoa Frozen and Stored in Liquid Nitrogen. ***Pakistan Veterinary Journal*** 34, 205-208.
27. SMITH K.D., STEINBERGER E., 1973 – Steinberger Survival of spermatozoa in a human sperm bank: effects of long-term storage in liquid nitrogen. ***JAMA***, 223, 774-777.
28. SONESSON A.K., GODDARD M.E., MEUWISSEN T.H.E., 2002 – The use of frozen semen to minimize inbreeding in small populations. ***Genetical Research*** 80,27-30.
29. SZAREK J., ADAMCZYK K., FELENCZAK A., 2004 – Polish Red Cattie breeding: past and present. ***Animal Genetic Resources Information*** 35, 21-35.
30. TRZCIŃSKA M, BRYŁA M, SMORĄG Z., 2011– Apoptotic-like changes in the spermatozoa of fresh and stored boar semen and the quality of embryos produced *in vivo*. ***Animal Reproduction Science*** 124, 90-97.
31. TRZCIŃSKA M., BRYŁA M., 2015 – Apoptotic-like changes of boar spermatozoa in freezing media supplemented with different antioxidants. ***Polish Journal of Veterinary Sciences*** 18, 473-480.
32. TRZCIŃSKA M., BRYŁA M., GAJDA B., GOGOL P., 2015 – Fertility of boar semen cryopreserved in extender supplemented with butylated hydroxytoluene. ***Theriogenology*** 83, 307-313.
33. TRZCIŃSKA M., SAMIEC M., DUDA M., 2023 – Creating ex situ protected bioreservoirs as a powerful strategy for the reproductive biotechnology-mediated rescue of threatened polish livestock breeds. ***Agriculture*** 13, 1426.

34. WOELDERS H., WINDIG J., HIEMSTRA S.J., 2012 – How developments in cryobiology, reproductive technologies and conservation genomics could shape gene banking strategies for (farm) animals. ***Reproduction in Domestic Animals*** 47, 264-273.
35. WOOD K.A., STILMAN R.A., HILTON G.M., 2028 – Conservation in a changing world needs predictive models. ***Animal Conservation*** 21, 87-88.
36. YÁNEZ-ORTIZ I., CATALÁN J., RODRÍGUEZ-GIL J.E., MIRÓ J., YESTE M., 2022 – Advances in sperm cryopreservation in farm animals: Cattle, horse, pig and sheep. ***Animal Reproduction Science*** 246,106904.