APPLICATION OF HIGH HYDROSTATIC PRESSURE (HHP) TO IMPROVE CRYOPRESERVATION OF YOUNG BULL SEMEN*

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Abstract

The objective of the study was to determine the effect of high hydrostatic pressure (HHP) on quality of cryopreserved semen of young bulls. Semen for this study was collected from 8 bulls aged between 13 and 18 months at monthly intervals, from June to September. After collection, semen was diluted in a commercial Bioxcell® extender (one part at 1:1 and a second part to give a sperm concentration of 20 million/0.2 mL), filled into straws and treated with HHP at 30 MPa for 90 min. After HHP treatment, pre-diluted semen (1:1) was diluted to a sperm concentration 20 million/0.2 mL and filled into straws. In addition, part of the semen diluted to a concentration of 20 million/0.2 mL was not treated with HHP (control). All of it was held at +4°C and frozen in a freezer after 2.5-h equilibration. Semen was thawed in a water bath at 38°C and subjected to estimation of the percentage of motile sperm both subjectively and using a computer-assisted semen analyzer and cytometric assessment of sperm cell membrane integrity. Subjective motility and fast progressive motility were significantly higher with pre-diluted (1:1) and HHP treated semen compared to control ($P<0.05$). No significant differences were observed in percentage of membrane-intact spermatozoa between control and experimental groups. Additionally, the influence of HHP on the sperm of individual bulls was assessed. In bull number 2, the HHP treatment after semen pre-dilution significantly improved progressive motility from 54.1 to 63.4 percent ($P<0.05$). In bull number 4, the HHP treatment after semen pre-dilution significantly improved subjective motility, rapid motility and progressive motility by 12.5, 16.8 and 16.3 percent, respectively ($P<0.05$). No effect was seen for 6 bulls. It is concluded that for some bulls, the application of HHP before semen freezing may improve the cryopreservation outcome. However, this requires further research in this area, also to determine the fertilizing capacity of bull semen exposed to high hydrostatic pressure.

Key words: young bull, frozen semen, hydrostatic pressure, motility, membrane integrity

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Bull spermatozoa have a relatively high tolerance for freezing and thawing, and frozen semen has been used in cattle breeding for more than half a century. The bull semen cryopreservation technique currently uses commercial extenders containing tris, citric acid, fructose and glycerol supplemented with a hen's egg yolk (Biladyl® and Triladyl® – Mini-tüb, Germany, Optidyl® – Cryovet, France) or soy lecithin (Biociphos-Plus® and Bioxcell® – IMV, France, Andromed® – Mini-tüb, Germany) (Layek et al., 2016). Cryopreservation of bull semen based on commercial extenders allows for generally satisfactory fertility results after insemination with frozen semen. Nevertheless, some males produce semen with low freezing capacity (poor freezability), that is, one that has poor quality scores after freezing and thawing (Rodríguez-Martínez and Peña Vega, 2013). It has also been observed that quality of young bull semen is generally lower than that of mature bull semen (Murphy et al., 2018). After introducing genomic evaluation of cattle, which provides information about an animal's breeding value right after birth, early use of bulls for reproduction and the associated semen cryopreservation became necessary. Therefore, attempts are made to improve the quality of frozen semen by using additional cryoprotective agents (Taşdemira et al., 2013), supplementing the diluent with antioxidants (Ansari et al., 2012; Tuncer et al., 2010), cholesterol (Amorim et al., 2009; Moraes et al., 2010), n-3 and n-6 polyunsaturated fatty acids (Kaka et al., 2015 a, b; Takahashi et al., 2012; Szczęśniak-Fabiańczyk et al., 2020), and modifying the cryopreservation procedure (Takahashi et al., 2012; Szczęśniak-Fabiańczyk et al., 2020).

One of the technological solutions tested in semen cryopreservation is to use high hydrostatic pressure (HHP) generated in the pressure chamber. In the context of bull semen cryopreservation this solution was first proposed by Pribenszky et al. (2007). He reported that HHP (30 MPa for 90 min) significantly improves the post-thaw motility of the frozen semen. The device, filled with distilled water, executes a program according to the set parameters, such as magnitude and duration of the pressure, and treatment temperature (Pribenszky et al., 2010). The bull semen program allows for hydrostatic pressure of 10-80 MPa and duration of 30–120 minutes. With proper magnitude and duration, high hydrostatic pressure induces a defensive reaction to stress, thus increasing resistance to a further stress of cryopreservation. HHP-treated cells activate chaperone proteins, which counteract adverse changes caused by a stress factor and ensure safe stress levels. The chaperone proteins in cells are involved in chromatin and cytoskeleton stabilization, cell cycle control, redox regulation, fatty acid and lipid metabolism, and elimination of damaged proteins (Kültz, 2003).

The aim of this study was to determine the effect of high hydrostatic pressure on the quality of cryopreserved semen of young bulls.

Material and methods

Bulls

The study used 8 young bulls aged from 13 to 18 months, which were maintained at the Małopolska Biotechnical Centre in Krasne, Poland: 4 Polish Red-and-White

(RW), 2 Polish Red (PR) and 2 Simmental (SM) bulls. All the animals were kept under the same housing and feeding conditions. For experimental purposes, semen was obtained from the above bulls at monthly intervals from June to September. Semen was collected using an artificial vagina.

Semen handling

Immediately upon collection, ejaculate volume, sperm concentration and sperm motility were assessed. Sperm concentration was estimated by an Accucell spectrophotometer (IMV Technologies, L'Aigle, France), while motility was evaluated subjectively under a Nikon optical phase-contrast microscope with heating table (38°C) at 100x magnification (standard procedure in the insemination station). Only ejaculates with sperm concentrations >800 million/ml and motility >60% were used for further processing. Next, 0.5 mL of semen was collected, diluted 1:1 with a commercial diluent (Bioxcell®, IMV, France), and filled into two 0.5-mL midi-straws (Cassou, France). The remainder of the ejaculate was extended also with Bioxcell® diluent to give a total sperm concentration of at least 20 million per 0.2 mL. Semen was packed into standard 0.25-mL mini-straws (Cassou, France). Two straws were put aside and the rest were transferred to a refrigerator compartment at +4°C. After 2.5-h equilibration, semen was frozen in a freezer and transferred to liquid nitrogen (control).

The midi-straws filled with pre-diluted semen and the put-aside mini-straws with finally diluted semen of the same bull were put into the chamber of a high hydrostatic pressure device (Cryo-Innovation Ltd., Budapest, Hungary). The remaining free space in the chamber was filled with redistilled water whose temperature corresponded to that of the ambient temperature of the laboratory, and the device's operating parameters were set as follows: pressure – 30 MPa, duration – 90 minutes, temperature – 22°C (equal to that of the ambient temperature of the laboratory). The HHP device was switched on and after 90 min of pressure treatment, the straws with semen were removed from the chamber and thoroughly dried. Mini-straws were put aside, the semen from midi-straws was transferred to a test tube and further diluted with Bioxcell® for a final concentration of at least 20 million per 0.2 mL. Next, semen was filled into standard 0.25-mL mini-straws (Cassou, France), transferred together with those that had been put aside (after removal from the HHP device) to a refrigerator compartment at +4°C, frozen after 2.5-h equilibration in a freezer, and later transferred to liquid nitrogen where they were stored until analysis.

Semen assessment

After thawing in a water bath at 38°C for 30 sec, semen was assessed at a laboratory specializing in andrological diagnosis at the Department of Reproductive Biotechnology and Cryoconservation, National Research Institute of Animal Production in Balice, Poland. The assessment included estimation of the percentage of normal motile sperm, CASA motility assessment (rapid motility, fast progressive motility, progressive motility) and cytometric assessment of sperm cell membrane integrity (percentage of membrane-intact spermatozoa). The estimate was performed collectively by two individuals with long-standing expertise. Semen motility was evalu-

ated subjectively under a Nikon optical phase-contrast microscope with heating table (38°C) at 100x magnification.

Rapid motility, fast progressive motility and progressive motility were assessed using a computer-assisted semen analyzer (Sperm Class Analyzer, S.C.A. V5.1, Microptic, Barcelona, Spain). Semen was assessed in 20 micron Leja 8-chamber slides (Leja Products B.V., The Netherlands). A 1 µL sample of thawed semen was dripped into the chamber. Five fields were evaluated in the preparation. The computer program counted the average values. Instrument settings were 25 frames per second and 25 images per sample. Regarding the analysis settings, sperm with VAP >50 µm/s were classified as rapidly motile, sperm with straightness >70% and VAP >50 µm/s were classified as fast progressive whereas sperm with straightness >70% were considered progressively motile. The evaluation was performed and verified by the same persons who made the estimate.

The viability was assessed using the dual fluorescence staining with SYBR-14 and propidium iodide (PI) as described by Pukazhenthil *et al.* (2000), with minor changes. Spermatozoa were stained using the sperm viability kit (Live/Dead Sperm Viability Kit, Life Technologies Ltd, Carlsbad, CA, USA). An aliquot of 1000 µL of diluted samples (5 million sperm/ml in PBS) was pipetted into cytometric tubes, and 5 µL of SYBR-14 working solution was added. The working solution was obtained by diluting a commercial solution of SYBR-14 in distilled water with a ratio of 1:49. Samples were mixed and incubated at 37°C temperature in the dark for 10 minutes. Then, the cells were counterstained with 5 µL of PI 3 minutes before analysis. Measurements were carried out on a CytoFlex (Beckman Coulter, Brea, CA, USA) flow cytometer. The fluorescent probes were excited by blue laser (488 nm, 50mW). Detection of green fluorescence was set with an SYBR-14 band-pass filter (525/40 nm) and red fluorescence was measured using an PI band-pass filter (690/50 nm). Acquisitions were measured using the Cyt Expert 2.0 software (Beckman Coulter). The non-sperm events were gated out based on scatter and then PI/SYBR-14 dotplot and were not analyzed. A total of 10,000 events were analyzed for each sample. Four subpopulations of cells were observed on dot plots of PI/SYBR-14 fluorescence: dead spermatozoa (PI+/SYBR-14-), moribund spermatozoa (PI+/SYBR-14+), live spermatozoa (membrane-intact spermatozoa) (PI-/SYBR-14+), and a nonlabeled population (PI-/SYBR-14-) that was considered to be debris.

Statistical analysis

The results obtained are presented as mean ± SEM of measurements. Data were analyzed using ANOVA and differences between means of control and experimental groups were tested with the t-test. The level of significance was set at $P < 0.05$. An elaboration of tests was carried out using the Statistica 12 package (StatSoft Inc., Tulsa, OK, USA, 2015).

Results

The results of sperm motility and sperm membrane integrity of thawed semen are presented in Table 1.

Table 1. Assessment of sperm motility and membrane integrity of 8 young bull semen treated with HHP before freezing (mean $\pm$ SEM)

Group	Subjective motility (%) n=29	CASA			Membrane-intact spermatozoa (%) n=29
		rapid motility (%) n=29	fast progressive motility (%) n=29	progressive motility (%) n=29	
Control	54.5 $\pm$ 2.1 a	60.7 $\pm$ 2.4	38.4 $\pm$ 2.1 a	67.1 $\pm$ 2.3	50.7 $\pm$ 2.0
HHP after final dilution	55.2 $\pm$ 2.1 ab	59.3 $\pm$ 2.5	39.1 $\pm$ 1.8 ab	65.1 $\pm$ 2.5	45.5 $\pm$ 2.3
HHP after pre-dilution (1:1)	59.3 $\pm$ 1.9 b	64.3 $\pm$ 2.3	43.3 $\pm$ 2.1 b	70.4 $\pm$ 2.1	47.1 $\pm$ 1.9

a, b – values in columns with different letters differ significantly ($P \leq 0.05$).

The highest post-thaw sperm motility, as assessed by both the estimation method and computer-assisted analysis, was obtained by the semen that was prediluted 1:1, subjected to HHP, finally diluted, and frozen. Subjective motility and fast progressive motility were significantly higher for this group compared to control ($P < 0.05$). No significant differences were observed in percentage of membrane-intact spermatozoa between control and experimental groups. To determine if the applied hydrostatic pressure had the same effect on sperm from 8 bulls, the semen quality for each individual was compared (Table 2).

Table 2. Assessment of sperm motility and membrane integrity in the semen of 8 bulls treated with HHP before freezing (mean $\pm$ SEM)

Bull (no. of ejaculates)	Group	Subjective motility (%)	CASA			Membrane-intact spermatozoa (%)
			rapid motility (%)	fast progressive motility (%)	progressive motility (%)	
1	2	3	4	5	6	7
1 (n=3)	Control	48.3 $\pm$ 4.4	54.1 $\pm$ 12.8	32.0 $\pm$ 7.2	59.3 $\pm$ 12.2	55.8 $\pm$ 0.9
	HHP after final dilution	50.0 $\pm$ 7.6	46.5 $\pm$ 3.5	32.3 $\pm$ 1.4	53.4 $\pm$ 5.2	47.1 $\pm$ 3.6
	HHP after pre-dilution (1:1)	51.7 $\pm$ 1.7	48.5 $\pm$ 3.8	30.9 $\pm$ 6.3	59.0 $\pm$ 2.1	52.1 $\pm$ 4.7
2 (n=3)	Control	41.7 $\pm$ 6.0	48.8 $\pm$ 4.5	35.4 $\pm$ 9.0	54.1 $\pm$ 5.1 a	36.4 $\pm$ 2.5
	HHP after final dilution	50.0 $\pm$ 2.9	56.9 $\pm$ 5.2	41.4 $\pm$ 3.8	61.7 $\pm$ 5.5 ab	28.1 $\pm$ 4.8
	HHP after pre-dilution (1:1)	56.7 $\pm$ 3.3	58.7 $\pm$ 7.0	37.8 $\pm$ 10.0	63.4 $\pm$ 6.6 b	32.6 $\pm$ 3.3
3 (n=4)	Control	57.5 $\pm$ 6.0	59.4 $\pm$ 6.9	41.8 $\pm$ 6.8	66.5 $\pm$ 7.8	50.3 $\pm$ 3.3
	HHP after final dilution	53.8 $\pm$ 6.6	57.1 $\pm$ 7.8	37.5 $\pm$ 3.9	63.7 $\pm$ 7.9	45.0 $\pm$ 6.2
	HHP after pre-dilution (1:1)	51.3 $\pm$ 7.2	56.3 $\pm$ 3.3	37.5 $\pm$ 3.2	61.9 $\pm$ 4.1	44.6 $\pm$ 6.6

Table 2 – contd.

1	2	3	4	5	6	7
4 (n=4)	Control	51.3±5.2 a	53.5±7.0 a	35.0±5.8	62.2±4.7 a	55.9±5.5
	HHP after final dilution	60.0±8.7 ab	63.3±8.0 ab	46.0±4.2	68.4±7.3 ab	54.2±6.3
	HHP after pre-dilution (1:1)	63.8±3.3 b	70.3±9.3 b	42.9±7.0	78.5±6.7 b	52.0±6.3
5 (n=3)	Control	63.3±8.8	68.0±5.7	45.8±3.6	72.6±5.5	46.7±5.7
	HHP after final dilution	60.0±5.8	67.5±3.6	45.8±4.4	72.8±4.8	37.8±8.8
	HHP after pre-dilution (1:1)	63.0±2.9	67.3±6.3	53.2±2.1	70.2±6.3	44.7±6.4
6 (n=4)	Control	51.2±6.3	67.1±5.8	35.2±5.8	71.8±5.5	45.8±5.4
	HHP after final dilution	52.5±1.4	61.3±6.2	31.5±2.8	66.1±5.7	42.6±4.3
	HHP after pre-dilution (1:1)	62.5±4.3	66.8±5.7	46.9±1.8	71.2±6.0	42.2±2.9
7 (n=4)	Control	61.3±2.4	63.4±6.3	45.4±4.6	73.1±5.2	56.2±8.0
	HHP after final dilution	52.5±7.8	59.2±11.6	39.5±8.3	65.2±11.5	50.4±7.8
	HHP after pre-dilution (1:1)	68.8±1.3	73.5±2.0	54.8±2.4	80.0±1.1	53.8±2.2
8 (n=4)	Control	58.8±3.1	68.9±3.0	35.9±6.5	73.1±2.7	55.4±3.5
	HHP after final dilution	61.3±3.8	60.9±5.9	40.9±4.7	67.3±5.7	52.8±2.5
	HHP after pre-dilution (1:1)	61.3±4.3	68.8±5.1	39.9±3.8	74.6±5.1	51.6±3.2

a, b – values in columns within bull with different letters differ significantly ($P<0.05$).

In bull number 2, the HHP treatment after semen pre-dilution significantly improved progressive motility from 54.1 to 63.4 percent ($P<0.05$). In bull number 4, the HHP treatment after semen pre-dilution significantly improved subjective motility, rapid motility and progressive motility by 12.5, 16.8 and 16.3 percent, respectively ($P<0.05$). No effect was seen for 6 bulls.

Discussion

Research published to date has shown that the limited stress induced by high hydrostatic pressure may increase the cryotolerance, fertilizing ability, and developmental competence of gametes and embryos (Pribenszky *et al.*, 2005, 2007; Du *et al.*, 2008; Pribenszky *et al.*, 2008; Bogliolo *et al.*, 2010). The application of a pressure chamber for boar semen cryopreservation has improved its tolerance to freeze-thawing (Pribenszky *et al.*, 2006; Huang *et al.*, 2009). Huang *et al.* (2009) showed

higher expression of proteins involved in fertilization in HHP-treated semen before cryopreservation. The fact that the quality parameters of frozen boar semen can improve after HHP treatment was also reported by Bryła and Trzcińska (2020). The use of frozen semen treated with HHP to inseminate gilts, caused an increase in the number of piglets born per litter (Horváth et al., 2016; Kuo et al., 2008). In a study with bull semen, Pribenszky et al. (2007) reported improvements in post-thaw sperm motility and membrane integrity as well as improved fertility after insemination with the semen that had been finally diluted and treated with HHP (30 MPa for 90 min in ambient temperature) before freezing. Our study, performed with the semen of young bulls, failed to confirm the beneficial effect of the above procedure on post-thaw semen quality. However, modification of the procedure, in which HHP was used after pre-dilution of semen (1:1) significantly improved the post-thaw motility parameters. Analysis of the results showed significant differences between the bulls. For the semen of two bulls (2 and 4), motility parameters improved considerably after HHP treatment. Similar individual differences were reported by Pribenszky et al. (2007), who observed that HHP caused a considerable increase in post-thaw sperm motility in 3 out of 21 bulls, but had no effect in 6 of these bulls. For the semen of 4 out of 7 bulls, which exhibited very low post-thaw motility (3–19%) of spermatozoa in the control group, the motility was increased to 43–70% after the application of HHP (Pribenszky et al., 2007). These observations have relevance in the context of searching for methods to improve the freezing efficiency of semen from animals producing semen with low freezability, including young bulls that are valuable genetic material. This supports the earlier observations that there is no one optimal semen freezing protocol for all males. For some animals, low quality of semen subjected to cryopreservation using a given extender and protocol will improve after the use of another extender and/or a small modification of the protocol (Holt, 2000; Thurston et al., 2002; Ramón et al., 2013). Therefore, semen freezing procedures should be individualized rather than the same for the whole population. The proposed method broadens the possibilities of an individualized approach to the freezing of young bulls, as it increases the chance of obtaining early AI-suitable cryopreserved semen of young bulls. As mentioned before, it is important in the context of the functioning of the genomic assessment of cattle.

In conclusion, our study has shown that for some young bulls, the application of HHP before semen freezing may improve the cryopreservation outcome. However, this requires further research in this area, also to determine the fertilizing capacity of bull semen exposed to high hydrostatic pressure.

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