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Research article

THE INFLUENCE OF ZINC AND HEAVY METALS IN FEED AND WATER ON THE QUALITY OF CRYOPRESERVED BULL SEMEN

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This study aimed to assess the influence of different concentrations of zinc (Zn), lead (Pb), mercury (Hg) and cadmium (Cd) found in the feed and water provided to bulls on the concentrations of these metals in cryopreserved bull semen, and to determine their influence onto semen quality parameters. Correlations between heavy metal concentrations in the semen and the quality parameters of semen as estimated by computer-assisted sperm analysis (CASA) and flow cytometry (FC) methods were determined.

A total of 40 cryopreserved semen samples originating from bulls housed in 4 different centers for artificial insemination (A, B, C and D) were examined, making a total of 160 samples. The concentrations of metals and semen quality parameters were determined in cryopreserved semen of 10 bulls from each center, namely 4 samples from each bull. Concentrations of Zn, Pb, Hg and Cd in hay and concentrated feed were within the allowed limits as proposed by the National Research Council (NRC, 2000). A strong negative correlation was detected between curvilinear velocity (VCL) and Zn concentrations in the semen ($P < 0.01$; $r = -0.772$) in group D, and a positive correlation of VCL with Pb concentrations ($P < 0.05$ and $r = 0.718$) in group B. Mercury concentrations in cryopreserved semen correlated negatively to the percent of live sperm cells with intact acrosomes (V/IA: $P < 0.05$; $r = -0.640$) and positively with the percent of dead sperm cells with damaged acrosomes (D/DA: $P < 0.01$; $r = 0.766$) in group D. This finding confirms the hypothesis that Hg, even at low concentrations, may cause acrosome damage.

Key words: cadmium, CASA, cryopreserved bull semen, lead, mercury, zinc

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INTRODUCTION

We focused our attention on whether heavy metals might be present in mammalian seminal plasma, influencing important functions of sperm cells. To do this, we measured Zn, Hg, Pb and Cd concentrations in the water, feed and thawed bull semen in order to establish possible correlations. We also comprehensively evaluated the influence of these metals on semen quality parameters and sperm cell DNA integrity.

The reproductive epithelium is a sensitive tissue that reacts to changes in concentrations of certain chemicals, as well to changes in environmental parameters. According to this criterion, all chemical elements can be divided into three groups. The first comprises essential elements (K, Na, Ca, Mg and Fe), which constitute 2-5% of the animals' body weight and play key roles as structural elements and in the metabolic processes. The second group are microelements, which are found in traces (Zn, Cu, Mn, Co and Se). Their presence in the ejaculate is necessary for the majority of spermatozoa functions, but increased concentrations might be toxic. The third group of elements are heavy metals (Pb, Cd, Hg and Ni) which, even in small concentrations in the ejaculate, lead to sperm cell damage [1]. The National Research Council (NRC 2000) states that out of a total 17 minerals, 10 microelements are necessary in the diet of domestic animals: Cr, Co, Cu, J, Fe, Mn, Mo, Ni, Se and Zn [2]. Low levels of toxic elements, including aluminum (Al), arsenic (As), cadmium (Cd), lead (Pb), antimony (Sb), thallium (Tl) and uranium (U), have been demonstrated to exert detrimental effects on the reproductive health of rats [3,4].

Zinc (Zn) has a very important role in animal reproductive processes due to multiple effects on the metabolism of androgen hormones, estrogens and progesterone. In association with prostaglandins, it acts as an essential component or activator of enzymes necessary for the synthesis of steroid hormones. Zn plays an important role in the physiology of the prostate, epididymis and testes and contributes to the stability of sperm cells' plasma membrane. In male reproductive organs, Zn is present in high concentrations [5]. Further, the essential biological roles of Zn include signaling, enzymatic activities, regulation of normal growth and sexual maturation, digestion, homeostatic functions of the central nervous system, and mitochondrial oxidative stress [6,7].

It has been known for a while that Zn is concentrated in the tail of sperm cells and is involved in motility via the ATP system [8]. Using fluorescent dye specific for Zn detection, it was demonstrated that it accumulates in mitochondria of the spermatogonia and sperm cells [9]. In high doses, Zn is able to ameliorate the degree of testis tissue damage and spermatozoa quality in rats resulting from long-term administration of copper [4]. In addition, Zn^{2+} becomes incorporated into sperm cells during ejaculation [10], where it is believed to have a protective function in terms of sperm chromatin decondensation [11], sperm motility and metabolic inhibition, membrane stabilization and antioxidant activity [12-14].

In bull semen, toxic metals originating from feed and drinking water can be detected in traces. Lead (Pb) accumulates in different tissues and may lead to various neurological, hematological and reproductive disturbances [1]. Long-lasting exposure to Pb results in semen quality changes as well as changes in its chromatin structure [15]. As early as 1987, the International Agency for Research on Cancer classified Pb as a carcinogenic substance [2]. Interestingly, in humans it was not possible to detect the association between seminal plasma Pb levels and semen quality parameters [16].

The highly toxic effects of cadmium (Cd) result from its interactions with essential micro- and macroelements, especially with iron, calcium, copper and zinc. The elimination of Cd from the organism is slow and occurs through the kidneys, liver, milk and saliva. Cd is involved very little, if at all, in the direct metabolic exchange, but it bonds to various biological components, such as proteins, thiol (SH-) groups and anion groups of 25 various macromolecules exerting toxic effects [17]. Lead and cadmium influence selected motility parameters and the oxidant status of bull seminal plasma [18].

Methylmercury is a powerful toxin that primarily damages the central nervous system. Mercury (Hg) compounds disrupt steroid hormone synthesis, including sex hormones, and thus impair both male and female fertility. Hg also damages the hypothalamic-pituitary-thyroid and hypothalamic-pituitary-adrenal axes [9]. In humans, Hg levels in the hair were negatively correlated with sperm concentration, total sperm count and progressive motility [19].

The present work aimed to detect concentrations of zinc (Zn), lead (Pb), mercury (Hg) and cadmium (Cd) in the feed and water given to bulls, as well as to detect their concentrations in cryopreserved bull semen, and to determine their influence on semen quality parameters. In addition, one of the goals was to ascertain possible correlations between concentrations of these metals in semen and the semen quality parameters as determined by computer-assisted sperm analysis (CASA) and flow cytometry (FC). We firmly believe that investigations into the possible negative influence of selected pollution substances on semen quality of highly valuable breeding bull sires could contribute to a better understanding, enabling a successful follow-up of their reproductive characteristics.

Examining the available published data, we noticed that no studies had been conducted in which correlations between sperm mobility parameters, acrosomal damages and chromatin integrity along with the estimation of Zn, Hg, Pb and Cd concentrations in deep frozen-thawed bull semen were examined. Data regarding deep frozen-thawed semen are missing. These results are the first published data about the correlation between Hg concentrations in bull semen and acrosome damage.

MATERIALS AND METHODS

Ten Simmental bulls bred in each of four commercial centers for artificial insemination (A, B, C and D) were involved in the study. Two centers were located in Serbia, one in Croatia and one in Germany. From each bull, four straws of cryopreserved semen from the same batch were pooled and tested as 1mL samples, making a total of 40 doses of semen from each center for artificial insemination.

All animals were 6 to 8 years old and housed individually in a free-range system. Breeding bulls were fed daily on 8-12 kg of alfalfa hay from local meadows and on 4-6 kg of concentrated feed (DEWA-Kraftfutterwerk Georg Wagner GmbH & Co KG, Germany), with the addition of a mineral vitamin premix (Höveler Spezialfutterwerke GmbH & Co KG, Germany) in accordance with the manufacturers' instructions. The composition of the concentrated feed and vitamin mineral premix was in compliance with the normative proposed by the NRC 2000 for breeding bulls [2]. The premix contained zinc oxide in a concentration of 16.52 g/kg. The ejaculates were collected by means of an artificial vagina for bulls following mounting on a teaser animal (another bull). Ejaculates were diluted, depending on the sperm cell concentrations, in an AndroMed extender (Minitüb GmbH, Tiefenbach, Germany) and packed into previously marked 0.25-mL straws (Minitüb GmbH, Tiefenbach, Germany). After equilibration at +4°C in the duration of 240 min, the straws were frozen by suspending within liquid nitrogen vapor and stored in liquid nitrogen at -196°C. An assessment of Zn, Pb, Hg and Cd concentrations in feed and drinking water was performed at the beginning of the investigations. After a 6-month feeding period with the abovementioned diet, cryopreserved semen samples, originating from 10 bulls from each artificial insemination center, were examined.

The volume of each analyzed frozen semen sample was 1 mL. Thus, four straws of frozen semen per bull were thawed in a thermostatic water bath at 37°C for 30 s, and then pooled.

Semen samples, samples of feed and water provided to bulls were collected during the spring season.

Evaluation of deep frozen-thawed semen

Sperm motility assessment

Sperm motility was determined by means of the CASA system (ISAS Proiser, model V.1.2., Spain). For the analysis of structural and physiological characteristics of the sperm cells, a flow cytometer ("Guava EasyCyte", Guava-Millipore-IMV Technologies, USA) was used. Concentrations of Zn, Pb, Hg and Cd were determined in thawed semen samples.

For CASA analysis, 5 μL of semen was taken from each sample and placed into a 20-μm-deep Leja counting chamber (Proiser D4C20, Valencia, Spain), which was laid

on the heated stage of a microscope. The number of sperm cells analyzed per sample ranged from 1 500 to 5 000, which means 150-250 sperm cells per footage in 7 defined areas of the chamber. The program was set to analyze 25 footages per second, and for a exposure of 2 s (50 sequences in total).

On processing the photos, the following parameters were obtained:

– Sperm concentrations ($\times 10^6$ in 1 mL and per dose);
– The percentage of sperm cells was classified according to motility (progressively motile, non-progressively motile and non-motile), and according to velocity (rapid, medium rapid, slow and static sperm cells);
– Total motility of spermatozoa (TM), progressive motility (PM), the average velocity (VAP) and curvilinear velocity (VCL) of the sperm cells.

Flow cytometry

The following quality parameters of cryopreserved semen were determined by flow cytometry (FC): integrity of the sperm membrane and of the acrosome, sperm viability and DNA damages. After decondensation, the histones that are within the DNA chains are being colored. The "Guava EasyCyte" flow cytometer (Guava Technologies Inc., Hayward, CA, USA) was equipped with software adjusted for analyzing and data processing (IMV Technologies, L'Aigle, France).

FC detected the percentage of live sperm cells (L), both those with intact acrosome (V/IA) and those with broken chromatin status (SC), the percentage of live sperm cells with acrosome damage (V/AD), dead sperms with acrosome damage (D/AD) and dead sperm cells with intact acrosomes (D/IA).

Test for sperm and acrosome membrane integrity (propidium iodide/ fluorescein isothiocyanate-labeled peanut agglutinin – PI/FITC-PNA test)

In this test, the following chemicals were used: propidium iodide (PI) 2.4 mM (LIVE/ DEAD Sperm Viability Kit, Invitrogen, Thermo Phisher Scientific Inc, Waltham, MA, USA), PNA-FITC (Lectin FITC from *Arachis hypogaea*, Invitrogen, Thermo Phisher Scientific Inc, Waltham, MA, USA) and EasyBuffer (IMV Technologies, Saint-Ouen-sur-Iton, France).

Peanut (*Arachis hypogaea*) lectin, labelled with FITC, stains acrosomes with damaged membranes or if an acrosome has reacted by bonding with the carbohydrate group of glycoproteins. If an acrosome is damaged, it stains more intensively than when intact: PI enters the sperm cells and stains those with damaged membranes. Thus, sperm cells with both intact membranes and acrosomes are not stained with either green or red. Intact cell membranes with damaged acrosomes are green, and those with damaged membranes but intact acrosomes are red. Sperm cells with both membrane and acrosome damage are stained with both dyes.

Eppendorf tubes were filled with 2 μL of PI, 1 μl of PNA-FITC, 97 μL EasyBuffer and 4 μL of semen. The mixture was diluted with 296 μL EasyBuffer, bringing the total volume of the tested solution to 400 μL. The samples were incubated in the dark for 10 min at 37°C. A total of 5 000 particles were analyzed with Viability Acrosome Setup software (CytoSoft, Data Acquisition, and Analysis Software, version 4.4 beta1, Guava-Millipore-IMV, France).

Viability testing – the ratio of live vs. dead spermatozoa (SYBR14/PI test)

To test membrane permeability, SYBR14 dye and PI were used. Live spermatozoa stain green: SYBR-14 penetrates through undamaged membranes, causing these cells to fluoresce green. In contrast, PI penetrates through damaged sperm membranes and they fluoresce red. For these analyses, the LIVE/DEAD Sperm Viability Kit (Invitrogen, USA) was used: SYBR 14 was dissolved up to the concentration of 1 mM in DMSO, and PI to 2.4 mM in deionized water. Further dilutions were prepared with EasyBuffer extender (IMV, France).

In the Eppendorf tubes, 2 μL of PI and 2μL of SYBR-14 were placed and diluted with 96 μL of EasyBuffer extender. Finally, 4 μL of bull semen were added. The samples were additionally diluted by 296 μL of EasyBuffer, bringing the total volume of the tested solution to 400 μL. This was incubated for 10 min in the dark at 37°C. A total of 5 000 particles were analyzed with viability setup software (Cyto Soft, Data Acquisition, and Analysis Software, version 4.4 beta1, Guava-Millipore-IMV Technologies, France).

DNA damage assessment – chromatin condensation

The sperm chromatin structure assay (SCSA) implies the use of acridine orange (AO) dye. The stability of nuclear chromatin was assessed following exposure to the acid. The color of AO changes to red if it bonds to torn fragments of DNA or to green if it bonds to intact DNA.

For this test, the following chemicals were used: TNE solvent (a mixture of 0.15M NaCl, 0.01M Tris, and 1mM EDTA, pH 7.39), an acidic detergent (0.1% Triton X-100 and 0.08N HCl, pH 1.21) and AO in the final 6 μg/mL marker solution (a mixture of NaCl, EDTA, citric acid and Na_2HPO_4 in deionized water).

In the first step, 4 μl of bull semen were mixed with 196 μL of TNE extender and 0.4 mL of the acidic reagent. After 30 s, 1.2 mL of AO solution was added. Exactly 3 min after the addition of the acidic detergent, the result was read. A total of 2 000 particles in triplicate were analyzed using CDNA setup software (Cyto Soft, Data Acquisition, and Analysis Software, version 4.4beta1, Guava-Millipore-IMV Technologies, France). The means, calculated from the values obtained in triplicate, were further processed.

Determination of Zn, Pb, Cd and Hg concentrations in feed, water and cryopreserved bull semen

All the chemicals used for heavy metal and zinc content determination were of analytical grade (J. T. Baker Inc., Phillipsburg, NJ, USA), and the water was deionized (specific electrical resistance 18 MΩ). For sample preparation, the analytical scale KERN ABS-220-4 (Kern & Sohn GmbH, Balingen, Germany) and a microwave digestion system Anton Paar MW 3000 (with cuvettes MF 100) (Graz, Austria) were used.

The samples of feed, water and cryopreserved bull semen were prepared by microwave-assisted digestion. On the analytical scale, 1 ± 0.0001 g of pulverized and homogenized samples were measured in cuvettes. In the process of digestion, a mixture of 65% HNO_3 (6 mL) and 30% H_2O_2 (1 mL) was used. The digestion was performed using a program for food sample digestion at the power ranging from 250 to 630 W in four steps: 250 W (3 min), 630 W (8 min), 500 W (22 min) and 0 W (30 min). Alongside the samples, a blank was prepared. After cooling to room temperature, the samples were transferred into calibrated flasks. To determine the content of Zn, Cd, Pb and Hg, the calibrated flasks were filled up to the designated level with 0.1 mol/L of HNO_3. The content (mg/L) of heavy metals and zinc in the samples was determined using atomic absorption spectrometry (AAS), the technique of flame atomization (Zn), electrothermal atomization in a graphite cuvette (Pb and Cd) and the cold vapor method (Hg).

The content of heavy metals and zinc was determined by atomic absorption spectrometry (AAS) – Zn, Pb and Cd according to SRPS EN 14084 Standard (2008) and Hg in accordance with SRPS EN 13806 (2008). All metal detections were performed on the atomic absorption spectrometer Perkin Elmer Aanalyst 700 with the MHS system (Waltham, MA, USA), using lamps of the same manufacturer.

Approval of this study was obtained from the Ethics Commission for Experimental Animal Welfare Protection of the Faculty of Veterinary Medicine, University of Belgrade, Serbia (Opinion N° 14/2019). The research protocol and animal management were in compliance with the Directive 2010/63/ EU of the European Parliament (2010) on the protection of animals used for scientific purposes.

Statistical analysis

Descriptive statistical features were used for statistical analysis of the results. The normalcy of data distribution was tested by the Kolmogorov-Smirnov test. To determine the significance of differences between the experimental groups, parametric one-way analysis of variance (ANOVA) or non-parametric Kruskal-Wallis analysis of variance on ranks were used. The pairs of groups were compared using the parametric Tukey test and the non-parametric Dunn's multiple comparison test, respectively. The significance of differences was determined at significance levels of 5% and 1%. The correlation between the tested parameters was expressed via Pearson's coefficient of correlation (r). All obtained results are presented in tables. Statistical analysis was

performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com) and MS Excel.

RESULTS

Heavy metal and zinc concentrations in the feed and water

The concentrations of Zn, Hg, Pb and Cd in the hay, the concentrated feed and in water are presented in Table 1.

Table 1. Concentrations of Zn, Hg, Pb and Cd (mg/kg) in hay, concentrated feed and drinking water (mg/L) provided for the bulls in the study

Metals	Assessed matrix	AI Center A (X ± SD)	AI Center B (X ± SD)	AI Center C (X ± SD)	AI Center D (X ± SD)
Zn	Hay	12.00 ± 2.00^{abc}	16.00 ± 2.00^{a}	17.00 ± 3.00^{b}	16.00 ± 2.00^{c}
	Feed	50.00 ± 8.00^{ab}	78.00 ± 12.00^{ac}	84.00 ± 12.00^{bd}	51.00 ± 8.00^{cd}
	Water	0.18 ± 0.03^{a}	0.03 ± 0.01^{b}	0.04 ± 0.01^{c}	0.74 ± 0.11^{abc}
Hg	Hay	0.12 ± 0.02^{a}	0.52 ± 0.10^{a}	0.11 ± 0.02^{b}	0.41 ± 0.08^{c}
	Feed	0.08 ± 0.02^{a}	0.65 ± 0.13^{b}	0.14 ± 0.03^{c}	0.62 ± 0.12^{a}
	Water	$< 0.001^{a}$	$< 0.001^{b}$	$< 0.001^{c}$	$< 0.001^{d}$
Pb	Hay	5.40 ± 1.10^{abc}	2.80 ± 0.60^{a}	3.08 ± 0.80^{b}	2.20 ± 0.40^{c}
	Feed	1.30 ± 0.30^{a}	1.80 ± 0.40^{b}	1.60 ± 0.30^{c}	2.50 ± 0.50^{d}
	Water	0.012 ± 0003^{a}	0.020 ± 0.004^{b}	0.022 ± 0.004^{c}	0.020 ± 0.004^{d}
Cd	Hay	0.16 ± 0.03^{a}	0.16 ± 0.03^{b}	0.19 ± 0.04^{c}	0.11 ± 0.02^{d}
	Feed	0.22 ± 0.04^{a}	0.18 ± 0.04^{b}	0.14 ± 0.03^{c}	0.15 ± 0.03^{d}
	Water	$< 0.001^{a}$	0.0013 ± 0.0004^{b}	0.0014 ± 0.0004^{c}	0.0010 ± 0.0003^{d}

Mean ± standard deviation (X ± SD). All samples were assessed in triplicate. Means within the same row that share a common superscript are significantly different $^{a–d}$ $P < 0.05$.

The highest average Zn concentration in the hay was in group C, 29.41% higher than in group A and 5.88% higher than in groups B and D. The highest Zn concentrations in concentrated feed were also detected in group C. On average, they were 39.29%, 7.14% and 40.48% higher than in groups D, B and A, respectively. Significantly higher Zn concentrations in drinking water in group D were measured than in groups A, B and C (40.48%, 95.95% and 94.59%, respectively).

Hg concentrations in hay provided to bulls in group B were higher by 76.92% than in group A, 78.85% higher than in group C and 21.15% higher than in group D. Similarly, Hg concentration analysis in the concentrated feed revealed maximum concentrations in group B, which were higher by 87.69%, 78.46% and 4.62% than in the groups A, C and D, respectively. In water given to bulls of all groups, the average Hg concentration was below 0.001 mg/kg.

Analysis of Pb concentration in the hay detected the highest values in group A, which were 48.15%, 42.96% and 57.41% higher than in the hay given to bulls in groups B, C, and D, respectively. In the concentrated feed, maximum values for Pb concentration were obtained for group D, higher by 48.00% in comparison with group A, by 28.00% compared to group B, and by 36.00% in comparison with group C. In the drinking water given to the bulls during the trial, the highest average Pb concentration was recorded in group C, which was 45.45% higher in comparison with group A and 9.09% in comparison with groups B and D.

The highest Cd concentrations in the hay were detected in group C, being by 9.09% higher in comparison with groups A and B, and 42.11% in comparison with group D. The analysis of Cd content in concentrated feed pointed to its maximum levels in group A, which were higher by 18.18%, 36.35% and 31.82% than in the feed given to bulls in groups B, C and D, respectively. In the water consumed by the bulls throughout the study, Cd concentrations were roughly the same: 0.001 mg/kg.

The results of cryopreserved bull semen quality obtained by the CASA system are given in Table 2, and the results of flow cytometry are presented in Table 3.

Table 2. Quality parameters of cryopreserved bull semen after thawing (mean ± SD) as assessed by CASA

n = semen samples	CON ($x10^6$)	TM (%)	PM (%)	RM sperm (%)	VAP (mm/s)	VCL (mm/s)
A (n = 40)	24.03 ± 11.04	42.95 ± 12.3	24.70 ± 2.43	29.46 ± 3.21	49.14 ± 10.71	79.80 ± 14.31
B (n = 40)	23.08 ± 4.22	49.10 ± 17,86	29.18 ± 4.05	33.92 ± 5.04	51.46 ± 14.44	73.12 ± 17.51
C (n = 40)	26.80 ± 4.20	54.14 ± 10,95	32.88 ± 2.74	39.27 ± 3.72	60.32 ± 10.55[a]	89.28 ± 16.49[a]
D (n = 40)	17.08 ± 3.38	51.66 ± 19,87	28.89 ± 3.71	35.47 ± 5.39	46.86 ± 8.01[a]	69.46 ± 10.64[a]

A, B, C, D: AI Center, CON – concentration of spermatozoa, TM – total sperm motility, PM – progressive sperm motility, RM – rapid spermatozoa, VAP – average sperm motility, VCL – curvilinear velocity
Statistically significant differences in means are indicated with the same letter: a, $P < 0.05$

Analysis of the results obtained in this investigation related to the semen samples from bulls from different artificial insemination centers did not reveal significant differences ($P > 0.05$) in the following parameters of semen quality: the percentages of TM and number of TM sperm cells per dose, the percentages and numbers of PM per dose, and the percentages of rapid spermatozoa per dose. VAP and VCL were significantly lower ($P < 0.05$) in group D in comparison to group C.

Table 3. Quality parameters of cryopreserved bull semen after thawing (mean ± SD) as assessed by flow cytometry (FC)

n = semen samples	V/AD (%)	V (%)	V/IA (%)	D/AD (%)	SC (%)
A (n = 40)	1.51 ± 0.74^{AB}	37.79 ± 16.58^{aA}	36.27 ± 16.31^{ab}	37.91 ± 10.19^{abA}	9.93 ± 6.47^{A}
B (n = 40)	4.39 ± 2.95^{AC}	62.47 ± 19.43^{a}	58.08 ± 18.18^{a}	20.23 ± 14.96^{a}	35.15 ± 21.30^{A}
C (n = 40)	1.31 ± 0.57^{CD}	43.86 ± 8.78^{b}	42.55 ± 8.83	22.45 ± 10.12^{b}	19.94 ± 12.95
D (n = 40)	5.12 ± 1.17^{BD}	64.23 ± 16.36^{Ab}	59.11 ± 17.11^{b}	16.61 ± 7.92^{A}	22.34 ± 10.52

A, B, C, D: AI Center, V/AD – live sperm with acrosome damage, V – live sperm, V/IA – live sperm with intact acrosomes, D/AD – dead sperm with damaged acrosomes, SC – sperm with damaged chromatin.
Statistically significant differences in means are indicated with the same letters: A, B, C, D: $P < 0.01$; a, b: $P < 0.05$.

Analyses of the mean values of viable sperm cell percentages (V) in thawed semen revealed the lowest values in group A (37.79 ± 16.58%), which were significantly lower than the values obtained for group D (64.23 ± 16.36%; $P < 0.01$). Moreover, V was significantly lower in group C (43.86 ± 8.78%) than in group D (64.23 ± 16.36%; $P < 0.05$). Values recorded in group A were significantly lower than in group B (62.47 ± 19.43%; $P < 0.05$). Among other studied groups of bulls no statistically significant differences in mean values for this parameter were documented.

The mean values of live sperm cells with intact acrosomes (V/IA) were lowest in group A (36.27 ±1 6.31%) and they were statistically lower in comparison to the highest values recorded in group D (59.11 ± 17.11%; $P < 0.05$). This value was significantly lower in bulls from group A (36.27 ± 16.31) when compared to group B (58.08 ± 18.18%; $P < 0.05$).

The mean percentage values of live sperm cells with damaged acrosomes (V/AD) were lowest in group C (1.31 ± 0.57%) and significantly lower in comparison to group D ($P < 0.01$) in which the highest values were recorded (5.12 ± 1.17%). This parameter was significantly lower in group C (1.31 ± 0,57%) when compared to group B (4.39 ± 2.95%; $P < 0.01$). Group A had a significantly lower ($P < 0.01$) V/AD percentage (1.51 ± 0.74%) than group D (5.12 ± 1.17%). Finally, the V/AD percentage was significantly lower in group A (1.51 ± 0.74%) in comparison to group B (4.39 ± 2.95%; $P < 0.01$).

The mean percentage values of dead sperm cells with damaged acrosomes (D/AD) were lowest in group D (16.61±7.92%) and the statistical difference was significant ($P < 0.01$) in comparison to the highest values of this parameter recorded in group A (37.91 ± 10.19%). In group B, D/AD values (20.23 ± 14.96%) were significantly lower ($P < 0.05$) when compared to group A (37.91 ± 10.19%). Also, the D/AD value was significantly lower ($P < 0.05$) in group C (22.45 ± 10.12%) in comparison to group A.

Analyses of the percentage of sperm cells with damaged or disrupted chromatin (SC) revealed the highest mean values for bulls in group B (35.14 ± 21.30%), while the lowest SC was recorded in group A (9.93 ± 6.47%). Only between these two groups statistically significant differences were noted ($P < 0.01$).

Determination of heavy metal and zinc concentrations in cryopreserved bull semen

The results of the analysis of Zn, Hg and Pb contents in cryopreserved bull semen are given in Table 4.

Table 4. Concentrations of Zn, Hg and Pb (X ± SD) in cryopreserved bull semen

n = semen samples	Zn (µg/mL) X ± SD	Hg (µg/mL) X ± SD	Pb (µg/mL) X ± SD
A (n = 40)	$6.44^{A} \pm 0.57$	$0.07^{AB} \pm 0.01$	$0.02^{AB} \pm 0.01$
B (n = 40)	$3.15^{Aa} \pm 2.74$	$0.04^{AC} \pm 0.01$	$0.04^{AD} \pm 001$
C (n = 40)	$6.08^{a} \pm 1.25$	$0.06^{CD} \pm 0.01$	$0.07^{BCD} \pm 0.02$
D (n = 40)	3.84 ± 3.12	$0.03^{BD} \pm 0.01$	$0.03^{D} \pm 0.02$

A, B, C, D: AI Center. Statistically significant differences in means are indicated with the same letters: a, b: $P < 0.05$; $P < 0.01$, A, B, C, D: $P < 0.01$

Analysis of the Zn content in bull semen revealed the lowest Zn concentration in group B (3.15 ± 2.74 mg/mL), which was significantly lower ($P < 0.01$) than in group A (6.44 ± 0.57 mg/mL) and group C (6.08 ± 1.25 mg/mL; $P < 0.05$). The differences between the other pairs of groups were not statistically significant ($P > 0.05$).

The results of Hg concentration analysis in bull semen revealed significantly ($P < 0.01$) lower concentrations in groups D (0.03 ± 0.01 mg/mL) and B (0.04 ± 0.01 mg/mL) in comparison to groups A (0.07 ± 0.01 mg/mL) and C (0.06 ± 0.01 mg/mL). No significant differences were detected between other groups ($P > 0.05$).

Chemical analysis of bull semen revealed the highest Pb concentrations in group C (0.07 ± 0.017 mg/mL), which were significantly higher ($P < 0.01$) in comparison to all other groups. In addition, analysis showed significantly higher ($P < 0.01$) values in group A (0.02 ± 0.005 mg/mL) when compared to those obtained for group B (0.04±0.009 mg/mL). All other differences were considered to be insignificant ($P > 0.05$).

Cadmium content in all samples of cryopreserved bull semen was below the sensitivity level of the method applied.

Correlation analysis

The results of correlation analysis between the parameters of semen quality and the concentrations of Zn, Hg and Pb are presented in Table 5.

Table 5. Correlations between sperm quality parameters and the concentrations of Zn, Hg and Pb in cryopreserved bull semen

		CASA					FC			
		TM	**PM**	**VAP**	**VCL**	**V/AD**	**V**	**V/IA**	**D/AD**	**SC**
Zn	A	-0.047	-0.005	-0.047	-0.309	-0.220	0.056	0.044	0.066	0.5705
	B	0.405	0.428	0.302	0.144	0.610	-0.506	0.495	0.469	0.4200
	C	0.117	0.390	0.276	-0.102	-0.032	-0.288	0.491	0.304	0.6169
	D	0.390	0.452	0.521	-0.772**	0.156	-0.230	0.330	0.361	0.6002
Hg	A	0.329	0.408	0.229	0.004	-0.168	0.126	0.335	0.425	0.4135
	B	-0.135	-0.130	-0.178	-0.310	-0.102	0.008	-0.063	-0.028	0.7334
	C	0.509	0.250	0.546	-0.244	0.508	-0.170	-0.059	0.001	0.4345
	D	-0.590	-0.562	-0.572	0.615	-0.363	0.444	-0.640*	0.766**	0.8711
Pb	A	-0.143	-0.176	0.325	0.000	-0.069	-0.639	-0.177	0.565	0.3603
	B	-0.288	-0.412	-0.220	0.718*	0.095	-0.079	-0.499	0.163	0.3014
	C	0.081	-0.051	-0.169	0.075	0.440	0.408	-0.137	0.208	0.7719
	D	0.156	0.224	0.318	0.077	-0.021	-0.035	-0.142	0.775	0.4898

Computer sperm analysis system (CASA), flow cytometry (FC), total sperm motility (TM), progressive motility (PM), average sperm motility (VAP), curvilinear velocity (VCL), percentages of live sperm with acrosome damage (V/AD), live sperm (V), live sperm with intact acrosomes (V/IA), dead sperm with damaged acrosomes (D/AD), sperm with damaged chromatin (SC)
Statistical significance*$P < 0.05$, **$P < 0.01$

The relationship between the parameters of semen quality and Zn content proved the existence of a negative correlation between the concentration of this heavy metal and the curvilinear sperm motility ($P < 0.01$ and $r = -0.772$) in semen samples from group D bulls. The corresponding correlations between Zn content and semen quality parameters in all the other groups were considered to be insignificant.

The results of the analysis of the relationship between concentrations of Hg and V/IA and D/AD pointed to statistically significant negative correlations between these parameters in group D for V/IA ($P < 0.05$ and $r = - 0.640$) and a significant positive correlation for D/AD ($P < 0.01$ and $r = 0.766$).

However, a positive statistically significant correlation was detected between the concentrations of Pb and VCL ($P < 0.05$ and $r = 0.718$).

DISCUSSION

In general, our results regarding bull semen characteristics obtained either by CASA or FC were similar to those reported for Simmental bulls by several other authors [20-22]. Despite the numeric and statistically significant differences in the values obtained for concentrations of Zn, Pb, Hg and Cd in hay (Table 1), concentrated feed and drinking water were within the allowed limits as proposed by the NRC 2000; therefore, we may consider the noted differences of little biological importance.

The NRC defines the maximum tolerable concentrations of minerals in feed intended for animals that are fed for a limited period as those that "will not impair animal performance and should not produce unsafe residues in human food derived from the animal" [23]. The maximum tolerable dietary mineral concentrations are 0.5 mg/kg for Cd, 30 mg/kg for Pb and 2 mg/kg for Hg. It is considered that the maximum allowed level of Zn in the feed intended for cattle is 500 mg/kg of a meal [2]. The levels obtained in our study for Zn, Hg, Pb and Cd were lower than the maximal allowed concentrations determined by the NRC.

It is well known that Zn is one of the highly abundant elements on Earth, being an essential micronutrient to all living organisms and typically occurring as a divalent metal cation with moderate reactivity and reducing properties. Zinc ions are associated with sperm membranes, where they interact with lipoproteins and membrane-bound metal-proteins. They react with sulfhydryl groups of cysteine and therefore fulfil a membrane-stabilizing function [7]. They are considered to be essential for spermatogenesis [24]**.** However, high concentrations of Zn (100 μM) in seminal plasma decreased sperm motility [25]. According to the literature data, Zn concentrations in bull semen ranged from 25 to 36 μg/g [26]**.** However, it was stated that the average Zn levels in bull semen were 83.15 ± 61.61 mg/kg, and it was suggested that the Zn concentration in the feed should be from 35 to 40 mg/kg [6], the amount necessary for the normal physiology of the male reproductive organs. This value was provided in the feed during our study. The maximal concentrations of Zn in our research were detected in bulls from the AI center A (6.44 ± 0.57 mg/mL), and they were significantly lower than the values obtained by others [27] which, in bull semen from 30 breeding animals, measured average concentrations of 235.9 ± 20.5 mg/L, and in sperm cells 94.0 ± 11.0 mg/L. These differences may be explained by the fact that these authors measured Zn concentrations in the whole ejaculate, unlike in our research, where cryopreserved bull semen was assessed. Zn levels in the concentrated feed and the cryopreservedsemen in group D were lower than in the other groups, which may be related to the strong negative correlation to the sperm VCL detected only for this group.

It is well known that Hg is a very toxic element for both humans and animals. Its effects on sperm were assessed by authors [28] who suggested that alongside an increase in Hg chloride in the samples there was a statistically significant decrease in the percentages of live sperm cells. This is also in accordance with our results, which pointed to a negative correlation between Hg concentrations and V/IA spermatozoa

and a positive correlation to the D/AD value. Acrosome damages due to higher Hg concentrations are most likely connected to oxidative stress. It was proven that $HgCl_2$ increases the concentrations of malonyl dialdehyde and of reactive oxygen species (ROS), thereby reducing the total antioxidative capacity and the activity of superoxide dismutase (SOD) in spermatozoa after only 15 min of exposure [29]. It is worth noting that, apart from toxic substances, there is a significant influence of other factors such as age, cold and warm climates on antioxidative and oxidative stress variables in bull spermatozoa [30]. Hg is classified as an environmental pollution factor and a potential oxidant that leads to male infertility [31]. Chronic exposure of rats to low doses of Hg significantly decreased their ejaculate quality and negatively influenced their reproductive traits [32].

In addition to this, in the current work a positive, statistically significant correlation between Pb concentrations and VCL was detected. This finding may be explained by the fact that, on a molecular level, Pb increases ROS production [33], which stimulates lipid peroxidation [13] and damages the sperm cell membrane. In one study [34], the authors analyzed Pb influence on sperm motility and the activity of the antioxidative system in bull seminal plasma. The recorded Pb concentration was 0.57 ± 0.01 μg/mL, which was much higher than the corresponding values obtained in the cryopreserved semen in our study, where the highest levels were noted in bulls from the AI center B (0.07 ± 0.017 μg/mL). The differences in the obtained values may be explained by the fact that we assessed the concentrations of Pb in cryopreserved bull semen that was diluted prior to freezing. Our results, obtained by the assessment of Pb concentrations in thawed bull semen, were in line with values given by other researchers, who reported Pb concentrations of 0.06 mg/L [1], 0.057 mg/L [34] and 1 mg/L [35]. In the current work, the contents of heavy metals and zinc in drinking water, in water used for the production of extenders, feed and bull semen prepared for artificial insemination varied among the samples but were within the standard ranges. The results of our study suggest that increased Pb concentrations in the cryopreserved semen increased the percentage of sperm cells showing curvilinear motility in group B, where the concentration of Zn was also the lowest due to the competitive relationship with Pb. In the semen of bulls from group D, low concentrations of Zn and Hg were detected, and by flow cytometry it was proven that in tandem with increased Hg concentrations there was an increase in the levels of acrosome damage. In the same group, a low Zn concentration led to an increase in the percentage of sperm cells expressing curvilinear motility.

CONCLUSION

Exposure of breeding bulls to Hg from feed and drinking water, even in amounts below proposed limits, may exert detrimental effects on their reproductive health (including impaired semen quality) and especially on the integrity of the acrosome membrane. This is confirmed by the strong negative correlation and statistically

significant difference between Hg concentration in semen and V/IA and positive correlation to D/AD values. Low concentrations of the investigated Zn, Hg and Pb in bull semen do not affect the total and progressive motility of sperm cells, neither their DNA integrity.

This investigation pointed to the presence of Zn, Hg and Pb in various concentrations in feed originating from local areas without a significant influence on their concentrations in semen or the quality of commercially available semen of the tested bulls. Further research should be aimed at continual sampling and control of feed and its influence on semen quality, whichshould be performed over a longer period with shorter time intervals between samplings.

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Authors' contributions

JG designed the study and drafted the manuscript. MA, NS, AJ, ŠD performed laboratory analyses. MM performed statistical analyses. VS and LM have contributed to the interpretation of data, revision of the manuscript and supervision of the experiment, as well.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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UTICAJ CINKA I TEŠKIH METALA IZ HRANE I VODE NA KVALITET DUBOKO ZAMRZNUTOG SEMENA BIKOVA

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Cilj rada bio je da se ispita uticaj različitih koncentracija cinka (Zn), olova (Pb), žive (Hg) i kadmijuma (Cd) iz hrane i vode na koncentracije ovih metala u duboko zamrznutom semenu nakon otapanja i da se ispita njihov uticaj na parametre kvaliteta semena. Korelacije između koncentracije teških metala u semenu i parametra kvaliteta

semena su određivane nakon ispitivanja semena kompjuterski asistiranom analizom semena (eng. computer-assisted sperm analysis - CASA) i protočnom citometrijom (eng. flow cytometry - FC).

Iz 4 različita centra za veštačko osemenjavanje (A, B, C i D) ispitivano je po 40 uzoraka duboko zamrznutog semena bikova, što čini ukupno 160 uzoraka. Koncentracije metala i parametri kvaliteta semena su određivani u duboko zamrznutom semenu od 10 bikova iz svakog centra, odnosno 4 uzorka od svakog bika. Koncentracije Zn, Pb, Hg i Cd u senu i koncentrovanim hranivima bile su ispod propisanih dozvoljenih vrednosti Nacionalnog Istraživačkog Veća (eng. National Research Council - NRC, 2000). Jaka negativna korelacija uočena je između krivolinijske brzine spermatozoida (eng. curvilinear velocity - VCL) i koncentracije Zn u semenu ($P < 0.01$; $r = -0.772$) u grupi D, i pozitivna korelacija između VCL i koncentracije Pb ($P < 0.05$ and $r = 0.718$) u grupi B. Koncentracija žive u duboko zamrznutom semenu negativno je korelirala sa procentom živih spermatozoida sa neoštećenim akrozomom (eng. live sperm cells with intact acrosomes - V/IA: $P < 0.05$; $r = -0.640$) i pozitivno sa procentom mrtvih spermatozoida sa oštećenim akrozomom (eng. dead sperm cells with damaged acrosomes - D/DA: $P < 0.01$; $r = 0.766$) u grupi D. Ovi rezultati potvrđuju hipotezu da Hg, čak i u niskim koncenracijama može da prouzrokuje oštećenje akrozoma.