Influence of addition of cobalamin to the extender on the post-thaw motility, viability, and DNA integrity of bovine ejaculate

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Summary

The present study was undertaken to investigate the effect of various concentrations of cobalamin (vitamin B₁₂) in Bioexcell® extender on the spermatozoa quality of Simmental bulls. The semen was collected from 12 bulls by means of an artificial vagina. Vitamin B₁₂ at concentrations of 1.50, 2.50, 3.50, and 5.00 mg/mL was added to bovine Bioexcell® extender. The semen aliquots were cooled and preserved at 4°C. Their quality was evaluated during pre-freezing, and then the cooled semen samples were packaged into 0.25 ml straws. The straws were frozen in the vapor of liquid nitrogen, and stored at -196°C in a container. The straws were thawed one day later, and the characteristics of spermatozoa were examined. The results showed that the effect of vitamin B₁₂ on the viability, DNA fragmentation and motility of spermatozoa was significant under both pre- and post-freezing conditions ($p < 0.05$), and revealed that supplementation of the extender with B₁₂ improved the post-thaw spermatozoa quality in bulls.

Keywords: bovine, DNA, semen freezing, spermatozoa, vitamin B12

The use of artificial insemination with cryopreserved semen in cattle greatly advances animal breeding and production, reducing its costs and facilitating the distribution of semen (3). A major cause of the poor quality of buffalo bull semen and the low fertility of frozen-thawed semen is cryocapacitation-inflicted damage to sperm during freezing and thawing (2). Spermatozoa are significantly affected by interactions of extracellular and intracellular fluid, sugars, proteins, salts of individual microelements, and other substances, whose concentration increases simultaneously with the osmotic pressure after ice crystal formation (33). Cold shock caused by the action of low temperatures on spermatozoa during the freezing process may damage mitochondria (28), plasma, and the acrosomal membrane of spermatozoa (24), inducing changes in the lipid composition or in the integrity and permeability of plasma and membranes (20).

Vitamin B₁₂ (*cobalamin*) is a large, complex molecule responsible for a range of functions within the body. In biochemical reactions, vitamin B₁₂ acts as a coenzyme (22). Moreover, it is a form usually used

in vitamin supplements because of its stability. It has been demonstrated in experiments with rats that atrophic changes and the arrest of spermatogenesis resulted from a vitamin B₁₂ deficiency. Watanabe et al. (32) observed that a deficiency of vitamin B₁₂ resulted in a decreased motility and velocity of sperm in male rats, as well as in an increased incidence of abnormal sperm. Research by Boxmeer et al. (5) showed that there was a positive correlation between the total cyanocobalamin concentration in seminal plasma and the spermatozoa concentration in semen. Cyanocobalamin is also known for its antioxidative potential, because, as a supplement, it decreases the amount of reactive oxygen species (ROS) produced during oxidative stress in human semen (8, 9). It has been suggested that vitamins B₁, B₆, and B₁₂ in rams play a key role in the thermoregulation of scrotal skin and rectal temperature and in maintaining libido, semen quality, and fertility during heat stress (10).

DNA sequences are involved in regulating the expression of genetic information. Sperm DNA integrity is important for the success of natural and assisted

fertilization, including normal development of the embryo, fetus, and offspring (25). Semen cryopreservation is a fundamental technique for the conservation of genetic resources in cryobanks used for artificial insemination. Gual-Frau et al. (13) and Ni et al. (26) reported that vitamin B₁₂ had a positive effect on DNA integrity in human semen.

The physiological role and biological significance of vitamin B₁₂ in the cryopreservation of spermatozoa have not been sufficiently clarified. Hitherto, little has been published on the effect of vitamin B₁₂ on the semen quality in bulls. Research on the effects of vitamin B₁₂ on bovine sperm has often been conducted using conventional methods, rather than modern semen quality evaluation techniques. Consequently, the results are still not sufficiently accurate. In the evaluation of the effects of vitamin B₁₂ on the quality of frozen spermatozoa, the role of vitamin B₁₂ during semen freezing-thawing is very important. Accordingly, the aim of this study was to evaluate the effects of supplementing the bovine sperm extender with vitamin B₁₂ at various concentrations on the quality of spermatozoa after the cooling and freezing-thawing process.

Material and method

The present experiments were performed as part of routine activities during semen production at a reproductive station and did not require the approval of the ethics committee. The experiments were performed at the breeding and insemination centre Małopolskie Centrum Biotechniki Sp. z o.o. (Krasne, Poland). In this study, twelve Simmental bulls with an average age of 3.5 ± 0.5 years were housed individually in pens. From each bull, seven ejaculates were collected using an artificial vagina at 7 a.m. The semen was held in a water bath at 37°C, while the sperm concentration and initial percentage of motile sperm were being estimated. The sperm concentration was assessed with a digital photometer (Dr Lange, LP 300 SDM; Minitube, Tiefenbach b. Landshut, Germany) at 560 nm.

Semen processing. Semen samples were transferred into graduated test tubes immediately after collection and placed in a water bath at 37°C. The fresh undiluted semen was then microscopically (Nikon E 200, China) evaluated for mass motility, percentage of motile sperm, progressive motility, viability, and abnormal spermatozoa. Semen samples that showed more than 80% motility and 60% viability were selected for the experiment. The semen was extended with the animal protein-free commercial BIOXcell® extender (IMV Technologies, L'Aigle, France) to a final concentration of 120×10^6 spermatozoa/mL.

After a positive evaluation, the semen samples were pooled to eliminate individual differences. The fresh semen was then divided into 5 equal fractions. One fraction was left for the control group (without vitamin B₁₂), and vitamin B₁₂ (ScanVet, Poland) at concentrations of 1.50, 2.50, 3.50, or 5.00 mg/ml was added to the other fractions. The semen was automatically packed (Bloc Machine FIN, IS 4, France) into polyvinyl chloride (PVC) straws (0.25 mL) (Biovet, France) filled and equilibrated for 1.5 h at 4°C. After equili-

bration, the straws were frozen in liquid nitrogen vapor using a computer-controlled automatic freezer at the rate of -3°C/min from 4°C to -15°C and at the rate of -10°C/min from -15°C to -80°C (IMV Technologies, France).

After reaching -80°C, the semen straws were plunged in liquid nitrogen and packaged in plastic goblets for 24 hours of storage in a liquid nitrogen container. After one day, the straws were thawed in a water bath at 38°C for 20 sec, and then the motility, progressive motility, viability and normality/abnormality of the semen were examined.

Volume. The volume of ejaculate was measured by reading graduated tubes (4).

Assessment of sperm DNA integrity. To further analyze the integrity of sperm DNA, chromatin susceptibility to acid-induced denaturation *in situ* was assessed. Chromatin instability was then quantified by flow cytometry (Cytot-Flex Beckman Coulter, B3-R1-V0, China) using the Sperm Chromatin Structure Assay (SCSA) test. The samples were thawed in a water bath (26°C for 30 sec). 13 µL of semen and 487 µL of NaCl (0.9%) were placed in a glass tube on ice. 50 µL of the mixture thus prepared was moved to another tube on ice, and 100 µL of acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% v/v Triton X-100, pH 1.2) was added. After exactly 30 sec (without light), 300 µL of acridine orange (AO) staining solution was added [6 µg (chromatographically purified) AO (Polysciences, Inc. – USA) per ml of citrate buffer (0.037 M citric acid, 0.126 M Na₂HPO₄, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0)]. Each sample was incubated for 3 minutes on ice (without light) and then examined by flow cytometry; 5000 spermatozoa were evaluated in each sample.

Sperm motility. Mass motility was examined in 20 µL of semen, which was placed on a pre-warmed slide without a coverslip and analyzed under a microscope equipped with phase-contrast optics (100×) (4). Mass motility was scored according to the following scale: + no motion, ++ free spermatozoa moving without forming any waves, +++ vigorous movement with moderately rapid waves, ++++ very rapidly moving waves. 15 µL of semen was placed on a pre-warmed slide and covered with a coverslip. Semen motility was determined at a higher magnification (400×) by an eye estimation of the percentage of spermatozoa moving progressively straight forward.

Computerized assessment. Sperm motility was examined using a Sperm Class Analyzer (SCA, version 5.1, Microptic, Barcelona, Spain), a light microscope (Nikon Eclipse E200) with a ×10 negative phase objective, a Basler camera (scA 780-54fc, Ahrensburg, Germany), a warm stage, and a computer to analyze and save data. The following sets were used: medium VAP – 50.0 µm s⁻¹, low VAP µ 10 µm s⁻¹, low LIN – 50.0%. Before analysis, semen was diluted 1 : 10 in a warm (25°C) physiological solution (sodium chlorate 0.9%). Then 2 µL of the prepared sample was placed in a Leja 4 analysis chamber (Leja Products B.V., Holland) 20.0 µm thick. The slide was mounted on a stage warmer set at 38°C. The following motility parameters were examined: percentage of motile sperm, curvilinear velocity (VCL), straight-line velocity (VSL), path velocity (VAP), linearity (LIN), and amplitude of lateral head displacement (ALH). A minimum of 500 cells were evaluated, and

depending on sperm concentration, two to four analyses were performed per sample.

Viability. The double stain SYBR-14 with propidium iodide (L-7011 LIVE/DEAD Sperm Viability Kit; Invitrogen, Molecular Probes, Barcelona, Spain) was used with a flow cytometer (CytoFlex Beckman Coulter, B3-R1-V0, China). To this end, 50 μ L of thawed semen was measured (37°C for 20 seconds), and 940 μ L NaCl (0.9%) and 5 μ L SYBR-14 were added. These were thoroughly mixed and incubated (36°C for 10 min) without light. Subsequently, 5 μ L of propidium iodide (PI) was remixed and incubated for 3 min without light, followed by a test.

Statistical analysis. Data are presented as mean $\pm$ standard error of the mean (SEM). Analysis of variance (ANOVA) was used to assess differences between concentrations of vitamin B₁₂ supplementation on all semen characteristics. When the F ratio was significant ($p < 0.05$), Duncan's multiple range test was used to compare treatment means. The statistical analysis of the results was performed with Statistica 12.0 (StatSoft, Poland).

Results and discussion

The influence of vitamin B₁₂ on the parameters of frozen/thawed semen from Simmental bulls is shown in Tables 1 and 2.

The highest motility ($72.71\% \pm 5.04$) was observed in the BIOXcell® extender containing 2.50 mg/mL of vitamin B₁₂. The motility and VCL, VSL, VAP, LIN, STR and WOB values of the frozen-thawed sperm supplemented with 2.50 mg/mL vitamin B₁₂ were significantly higher than those for other concentrations ($p < 0.05$). There was also a positive effect of vitamin B₁₂ at 3.50 mg/mL on VSL, LIN, WOB, and ALH ($p < 0.05$). On the other hand, when the concentration of vitamin B₁₂ in the extender was increased to 5.00 mg/mL, sperm motion characteristics deteriorated significantly. Compared with the control, sperm motility and motion characteristics were improved in the presence of vitamin B₁₂ at a concentration of 2.50 mg/mL.

The results of the tests of the viability and DNA integrity of frozen-thawed bovine sperm are shown in Table 2.

The percentages of DNA-intact spermatozoa were significantly improved ($p < 0.05$) by supplementing with 1.50 mg/mL, 2.50 mg/mL, and 3.50 mg/mL vitamin B₁₂. Higher concentrations (5.00 mg/mL) did not significantly

($p > 0.05$) affect DNA. In addition, sperm viability was not affected by the supplementation of 1.50, 3.50, and 5.00 mg/mL vitamin B₁₂, but was significantly increased by the addition of 2.50 mg/mL. A sperm viability analysis based on randomly selected graphs is shown in Figure 1. In part A of the figure, the graph presents the results of viability analysis for sperm without vitamin B₁₂, whereas in part B the semen containing 2.50 mg/mL of vitamin B₁₂ is analyzed.

We investigated the effect of vitamin B₁₂ on the characteristics of sperm from Simmental bulls during the freezing-thawing process. The results indicate that the addition of 2.50 mg/mL of vitamin B₁₂ to BIOXcell® extender increased the motility and viability of semen, as well as protected its DNA from defragmentation. Our observations indicate that vitamin B₁₂ protected spermatozoa under freezing conditions. Similar results were obtained by Ha and Zhao (14), who showed that extender supplemented with a vitamin B complex improved the quality of frozen-thawed semen during cryopreservation in rams. Asadpour et al. (1) reported that the addition of 2 mg/mL of vitamin B₁₂ to the extender increased sperm viability and motility, as well as the percentage of normal spermatozoa in the hypo-osmotic swelling test (HOST), in Ghezel $\times$ Baluchi and Ghezel $\times$ Arkharmerino rams during storage at 5°C. Jeyendran et al. (21) also noted that the HOST can be used to determine plasma membrane permeability and was shown to correlate with the numbers of spermatozoa undergoing capacitation. Hu et al. (18)

Tab. 1. Mean values of motility parameters of frozen-thawed bovine semen in the presence or absence of vitamin B₁₂ ($\bar{x} \pm$ SD; n = 12) obtained by CASA

Parameter	Control	Vitamin B ₁₂			
		1.5 mg/mL	2.5 mg/mL	3.5 mg/mL	5.0 mg/mL
Motility (%)	54.68 ^{cd} $\pm$ 1.70	57.23 ^c $\pm$ 4.97	72.71 ^a $\pm$ 5.04	61.41 ^b $\pm$ 4.17	51.94 ^d $\pm$ 6.70
VCL (μ m/s)	62.90 ^b $\pm$ 1.00	62.09 ^b $\pm$ 5.28	73.91 ^a $\pm$ 0.53	64.80 ^b $\pm$ 5.77	40.97 ^c $\pm$ 2.69
VSL (μ m/s)	42.02 ^c $\pm$ 0.95	44.26 ^{bc} $\pm$ 4.61	50.77 ^a $\pm$ 0.31	47.10 ^{ab} $\pm$ 7.05	25.61 ^d $\pm$ 4.15
VAP (μ m/s)	49.75 ^{bc} $\pm$ 0.97	53.13 ^{ab} $\pm$ 4.96	54.89 ^a $\pm$ 3.33	48.15 ^c $\pm$ 3.79	30.20 ^d $\pm$ 4.39
LIN (%)	62.93 ^b $\pm$ 0.97	62.97 ^b $\pm$ 1.09	66.80 ^a $\pm$ 0.58	65.40 ^a $\pm$ 2.39	58.53 ^c $\pm$ 4.57
STR (%)	82.74 ^b $\pm$ 0.66	82.76 ^b $\pm$ 1.82	85.36 ^a $\pm$ 0.29	82.94 ^b $\pm$ 1.72	80.59 ^c $\pm$ 1.46
WOB (%)	75.63 ^b $\pm$ 1.53	76.57 ^b $\pm$ 1.35	81.47 ^a $\pm$ 1.79	80.02 ^a $\pm$ 1.94	73.18 ^c $\pm$ 4.72
ALH (μ m)	2.91 ^a $\pm$ 0.03	2.89 ^a $\pm$ 0.15	2.92 ^a $\pm$ 0.08	2.71 ^b $\pm$ 0.11	2.55 ^c $\pm$ 0.10

Explanations: a, b, c, d – means with different superscript letters in the same row differ significantly at $p < 0.05$. VCL – Curvilinear Velocity; VSL – Straight Line Velocity; VAP – Average Path Velocity; LIN – Linearity; STR – Straightness; WOB – Wobble; ALH – Amplitude of Lateral Head Displacement

Tab. 2. Mean values of DNA integrity and viability for frozen-thawed bovine semen in the presence or absence of vitamin B₁₂ ($\bar{x} \pm$ SD; n = 12) obtained by flow cytometry

Parameter	Control	Vitamin B ₁₂			
		1.5 mg/mL	2.5 mg/mL	3.5 mg/mL	5.0 mg/mL
Viability (%)	34.30 ^{bc} $\pm$ 0.065	40.03 ^{ab} $\pm$ 0.050	44.09 ^a $\pm$ 0.034	40.45 ^{ab} $\pm$ 0.051	29.04 ^c $\pm$ 0.012
DNA Integrity (%)	89.35 ^b $\pm$ 0.022	92.19 ^a $\pm$ 0.028	92.22 ^a $\pm$ 0.034	92.30 ^a $\pm$ 0.025	91.29 ^{ab} $\pm$ 0.034

Explanation: a, b, c, d – means with different superscript letters in the same row differ significantly at $p < 0.05$

Fig. 1. Randomly selected charts obtained using a flow cytometer from frozen-thawed bovine semen: (A) semen without the addition of vitamin B₁₂, (B) semen containing 2.50 mg/mL of vitamin B₁₂

Explanations: LIVE – percentage of live spermatozoa; DEAD – percentage of dead spermatozoa; PI – propidium iodide; SYBR-14 – component

reported that semen frozen in extender supplemented with 2.50 mg/mL of vitamin B₁₂ showed a significant improvement in sperm quality during post-thawing. Cyanocobalamin (vitamin B₁₂) is active during cellular replication and DNA synthesis, and it is already used as a treatment for male infertility in humans (11). Some clinical observations have indicated vitamin B₁₂ as an important nutrient for maintaining normal fertility in men (16, 27). Cai et al. (7) showed that vitamin B₁₂ could improve the motility of bovine spermatozoa during the freezing-thawing process, which is consistent with the coenzyme A activity of vitamin B₁₂. Similar results were obtained by Hu et al. (18), who showed that addition of 2.50 mg/mL vitamin B₁₂ significantly improved sperm motility and movement characteristics, and the percentage of spermatozoa. This increase in motility is an important factor affecting fertilization because efficient transport of sperm from the cervix to the oviducts is required for high rates of ovum fertilization (15).

In our study, vitamin B₁₂ has probably protected sperm cells from morphological defects. The deleterious effects of cryopreservation were thus decreased and semen quality improved pre and post freezing. Based on the results of this study, it can be concluded that vitamin B₁₂ helps to improve the quality of bovine semen after thawing in terms of mobility, viability, and DNA damage. The optimum concentration of vitamin B₁₂ in bull semen freezing extender was determined as 2.50 mg/mL. Our results show that increasing the concentration of vitamin B₁₂ (to 5 mg/mL) could not improve the motility and viability of spermatozoa before and after freezing.

Hu et al. (17) reported that a higher concentration of vitamin B₁₂ (3.75 mg/mL) has a debilitating effect on bull spermatozoa. However, the exact mechanism of negative and positive effects of vitamin B₁₂ doses on sperm characteristics requires a detailed study to better understand its precise physiological role in reproduction.

In the present study, sperm viability was not affected by the supplementation of 1.50, 3.50, and 5.00 mg/mL vitamin B₁₂, but it was significantly increased by the addition of 2.50 mg/mL. Samplaski et al. (30) report that a reduced sperm viability is correlated with a high degree of sperm DNA fragmentation. One of the final steps before spermatozoa death is DNA de-fragmentation (19). DNA breaks are among primary factors inducing spermatozoa apoptosis (12, 31). The literature has demonstrated a clear link between DNA fragmentation and sperm viability (6).

DNA damage in spermatozoa induced by freezing/thawing needs to be clarified for efficient fertilization success. The conventional semen quality parameters do not provide information on DNA integrity, which is crucial for fertilization stage, embryo development, pregnancy and reproductive outcomes. Thus, the DNA integrity of sperm cells is of crucial importance. In a recent study by Gual-Frau et al. (13), infertile men with varicocele who were administered multivitamin including vitamin B₁₂ at 1 µg/day for 3 months showed about 22.1% lower sperm DNA fragmentation. Gual-Frau et al. (13) and Ni et al. (26) showed that men with varicocele had the highest percentage of sperm cells with damaged DNA compared with other infertile groups. In the present study, we found a significantly

lower percentage of DNA damage in the presence of vitamin B₁₂ in comparison with the control group.

Vitamin B₁₂ improves the motility and vitality of spermatozoa after thawing and protects DNA from damage. Thorough field research is still needed to determine how the addition of vitamin B₁₂ to semen affects the effectiveness of fertilization.

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