

## Effects of addition of vitamin B<sub>12</sub> to the extender on post-thaw motility, acrosome morphology, and plasma membrane integrity in bull semen

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**Abstract:** To investigate the effects of supplementation of vitamin B<sub>12</sub> on bovine sperm post-thaw quality, vitamin B<sub>12</sub> was added at the concentrations of 1.25, 2.50, 3.75, and 5.00 mg/mL to bovine semen cryoprotective medium. The results indicated that the motility and VSL, VCL, STR, and VAP values of sperm supplemented with 2.50 mg/mL vitamin B<sub>12</sub> were significantly higher than those of other concentrations ( $P < 0.05$ ). No significant difference was observed for LIN and ALH values and the percentage of grade a spermatozoa between the extenders containing 2.50 mg/mL and 3.75 mg/mL vitamin B<sub>12</sub> ( $P > 0.05$ ). The percentages of acrosome-intact and plasma membrane-intact spermatozoa were significantly improved ( $P < 0.05$ ) by supplementing with 2.50 mg/mL vitamin B<sub>12</sub>. With all parameters measured, the concentration of 2.50 mg/mL vitamin B<sub>12</sub> showed the best results on the quality of bovine semen in freezing-thawing. In conclusion, the addition of 2.50 mg/mL vitamin B<sub>12</sub> to the extender improved the bovine frozen semen quality.

**Key words:** Bovine semen, vitamin B<sub>12</sub>, cryopreservation, sperm motility, CASA

### Introduction

Vitamin B<sub>12</sub> is one of the water-soluble vitamins functioning as a coenzyme in a number of biochemical reactions, such as methionine synthesis and the metabolism of branched amino acids (1). Because of its stability, cyanocobalamin is the form typically used in vitamin supplements. As for reproductive performance, it was demonstrated that

spermatogenesis would be improved in humans when a large amount of vitamin B<sub>12</sub> was given to patients with oligospermia (2,3). In animal experiments, it was also shown that vitamin B<sub>12</sub> deficiency induced atrophic changes and the arrest of spermatogenesis in rats. Watanabe et al. (4) examined the effects of dietary vitamin B<sub>12</sub> deficiency on sperm maturation in developing fetuses and growing newborns in rats.

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Vitamin B<sub>12</sub> deficiency increased the incidence of abnormal sperm and decreased the motility and velocity of the sperm in male rats, suggesting that dietary vitamin B<sub>12</sub> deficiency during gestation might induce irreversible damage in the germ cells of embryos and affect the maturation of spermatozoa. Vitamin is essential for DNA, transfer RNA, and protein synthesis. Because DNA synthesis was a main part of spermatogenesis (5-7), vitamin B<sub>12</sub> was probably important to this process.

Recently, some reports have shown a positive effect of vitamin B<sub>12</sub> supplementation on spermatozoa motility and spermatozoa count. Ha and Zhao (8) indicated that vitamin B complex could improve post-thaw motility and protect the integrity of ram spermatozoa membrane during the freezing-thawing. Cai et al. (9) studied the effects of vitamin B<sub>12</sub> on configuration of bull's frozen-thawed sperm and indicated that 0.5% (v/v) vitamin B<sub>12</sub> supplementation in the extender significantly improved the sperm quality.

There has been little published work in recent years on the effects of supplemental vitamin B<sub>12</sub> in the extender on frozen semen quality in bovine. Despite the intrinsic roles of vitamin B<sub>12</sub> supplementation in sperm cryopreservation, the physiological role and biological significance of vitamin B<sub>12</sub> in cryopreservation of spermatozoa have not been well clarified. Therefore, in the evaluation of the effects of vitamin B<sub>12</sub> on frozen spermatozoa quality, the role of vitamin B<sub>12</sub> during semen freezing-thawing is very important.

Thus, the objective of this study was to investigate the effects of vitamin B<sub>12</sub> supplementation in semen extender on bovine sperm quality during the freezing-thawing process. This study will help us to understand and design better bovine frozen semen extender.

## Materials and methods

### Semen collection

Bovine ejaculated semen was collected by artificial vagina from 12 Holstein bulls at the Domestic Animal Improving Station (Shaanxi Province, China), and 2 ejaculates were obtained from each bull. The semen was held in a water bath at 35 °C while the sperm concentration and initial percentage of motile sperm

were being estimated. Sperm concentration was estimated by optical density using a calibrated spectrophotometer. The percentage of motile sperm was estimated at 37 °C by a light microscope. Totally, 24 ejaculates from 12 bulls were used; each ejaculate was apportioned between the treatments and control.

### Extender preparation

The cryoprotective extender for the treatments used in this study was composed as follows: 2.42 g of Tris, 1.48 g of citric acid, 1.00 g of fructose, 6.6 mL of glycerol, 20 mL of egg yolk, 25 mg of gentamicine, 50,000 UI of penicillin for 100 mL of sterile non-pyrogenic water, and was supplemented with different concentrations of vitamin B<sub>12</sub> (1.25, 2.50, 3.75, and 5.00 mg/mL). The cryoprotective extender for the control group was the same as that for the treatment groups except that it was not supplemented with vitamin B<sub>12</sub>. In this study, vitamin B<sub>12</sub> liquid supplement was used and the net content was 0.5 g/mL. It was made by China Ningxia Pharmaceutical Co., Ltd.

### Semen processing

After the evaluation of the quality, the fresh semen was then divided into 5 equal fractions. Whereas 1 fraction was diluted with the extender for the control group (no vitamin B<sub>12</sub>), the others were diluted with the extender for the treatments (1.25, 2.50, 3.75, and 5.00 mg/mL vitamin B<sub>12</sub>) to obtain  $120 \times 10^6$  sperm/mL. Semen was cooled from 37 °C to 4 °C for 1.5 h. Polyvinyl chloride (PVC) straws (0.25 mL) (Biovet, France) were filled and maintained at 4 °C for 2.5 h. The straws were cooled at approximately -15 °C/min from +4 °C to -120 °C. Then these straws were transferred into a liquid nitrogen tank (-196 °C) and stored at least 2 weeks before thawing.

### Automated analysis of sperm motility

The characteristics of sperm motion were assessed with WLJY-9000, a computer-aided sperm analysis (CASA) system (WeiLi Software Co. Ltd, Beijing, China). For each treatment, 3 straws were thawed separately by immersion in a water bath at 37 °C for 45 s. The thawed semen samples were immediately transferred into plastic tubes of 1 mL and incubated at 37 °C for 10 min. Five microliters of each straw were examined and 6 fields were randomly chosen. The median values of sperm motility (% of motile sperm), the straight line velocity (VSL,  $\mu\text{m/s}$ ), the curvilinear

velocity (VCL,  $\mu\text{m/s}$ ), velocity of the average path (VAP,  $\mu\text{m/s}$ ), amplitude of the lateral head displacement (ALH:  $\mu\text{m}$ ), and frequency of head displacement (BCF, Hz) were obtained from the video recording. The mean coefficient ( $\text{STR} = (\text{VSL}/\text{VAP}) \times 100$ ), linearity index ( $\text{LIN} = (\text{VSL}/\text{VCL}) \times 100$ ), and wobble coefficient ( $\text{WOB} = (\text{VAP}/\text{VCL}) \times 100$ ) were analyzed. At the same time, spermatozoa were classified by CASA as “grade a”, “grade b”, “grade c”, and “grade d”, which were respectively described as follows: rapidly progressive (grade a), slowly progressive (grade b), locally motile (grade c: flagellating but non-progressive motion), and immotile (grade d).

#### Assessment of acrosomal integrity

Acrosome status was evaluated by fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) according to Aboagla and Terada (10). In order to prepare smears on microscope slides, 30  $\mu\text{L}$  sperm was used. After air-drying, sperm smears were fixed with absolute methanol for 10 min at 20–22 °C and then allowed to dry. Afterwards, about 30  $\mu\text{L}$  FITC-labeled peanut agglutinin (FITC-PNA) solution (100  $\mu\text{g/mL}$ , Sigma) in PBS was spread over each slide. Subsequently, the slides were incubated in a dark and moist chamber for 30 min at 37 °C. After incubation, the slides were rinsed with PBS, air dried, and mounted with 10  $\mu\text{L}$  of antifade solution to preserve fluorescence. The slide smear was covered by a coverslip and sealed with colorless nail polish. The smears were examined and photographed using an epifluorescence microscope (LEIKA DM-IRB linked to a Nikon digital camera DXM). All samples were evaluated by one observer. The whole acrosome was visualized with strong green fluorescence under a fluorescence microscope and was scored as acrosome-intact sperm. The percentage of fluorescent acrosome-intact sperm was counted in at least 300 sperm cells per slide.

#### Assessment of membrane integrity

The plasma membrane integrity was evaluated using the hypotonic swelling test (HOST). The straws were thawed in a water bath at 37 °C for 45 s and 50  $\mu\text{L}$  of semen was added to 1 mL of a hypoosmotic solution prepared with 7.35 g of sodium citrate ( $2\text{H}_2\text{O}$ ) and 13.51 g of fructose in 1000 mL of distilled water. After incubation for 60 min at 37 °C, sperm

swelling was assessed by placing 15  $\mu\text{L}$  of well-mixed sample on a warm slide (37 °C) covered with a cover glass before being observed under light microscopy at 400 $\times$  magnification. Viable spermatozoa had coiled tails after HOST. At least 300 spermatozoa per slide were observed. The spermatozoa were classified as either positive or negative based on the presence or absence of coiled tail.

#### Statistical analysis

The CASA experiment was carried out in 24 replicates for each group (control and experimental groups for each bull). All results were expressed as mean values  $\pm$  S.D. The mean values of the percentages of motile sperm and the acrosome-intact and plasma membrane-intact spermatozoa were compared using the Duncan's multiple range test by ANOVA procedure, when the F-value was significant ( $P < 0.05$ ). All statistical analyses were performed using SPSS 11.5 for Windows (SPSS, Chicago, IL, USA).

## Results

#### Sperm motility and movement characteristics

The effects of the presence of vitamin  $\text{B}_{12}$  in the freezing extender on cryopreservation sperm motion characteristics are shown in Tables 1 and 2. Compared to the control, the sperm motility and motion characteristics were improved in the presence of vitamin  $\text{B}_{12}$  in the extender except at the concentration of 5.00 mg/mL. The motility and VSL, VCL, STR, and VAP values of frozen-thawed sperm supplemented with 2.50 mg/mL vitamin  $\text{B}_{12}$  were significantly higher than those of other concentrations ( $P < 0.05$ ), whereas the BCF value was significantly lower than that of other concentrations ( $P < 0.05$ ). No significant difference was observed for LIN or ALH values or the percentage of “grade a” spermatozoa after thawing between the extenders containing 2.50 and 3.75 mg/mL vitamin  $\text{B}_{12}$  ( $P > 0.05$ ). In contrast, when vitamin  $\text{B}_{12}$  concentration was increased to 5.00 mg/mL in the extender, sperm motion characteristics and the percentage of “grade a” spermatozoa significantly decreased. The results indicated that 2.50 mg/mL vitamin  $\text{B}_{12}$  supplementation significantly improved sperm motility, movement characteristics, and the percentage of “grade a” spermatozoa.

Table 1. CASA-obtained mean values of motility parameters from frozen-thawed bovine semen samples in the presence and the absence of vitamin B<sub>12</sub>.

Item	Control	VB <sub>12</sub> 1.25 mg/mL	VB <sub>12</sub> 2.50 mg/mL	VB <sub>12</sub> 3.75 mg/mL	VB <sub>12</sub> 5.00 mg/mL
Motility (%)	40.86 ± 1.53 <sup>bc</sup>	42.34 ± 1.49 <sup>bc</sup>	52.49 ± 1.70 <sup>a</sup>	43.49 ± 1.66 <sup>b</sup>	38.60 ± 1.95 <sup>c</sup>
VSL (µm/s)	20.32 ± 1.13 <sup>d</sup>	21.22 ± 1.31 <sup>d</sup>	33.65 ± 1.32 <sup>a</sup>	25.32 ± 1.24 <sup>b</sup>	17.13 ± 1.16 <sup>c</sup>
VCL (µm/s)	36.41 ± 1.36 <sup>c</sup>	36.37 ± 1.27 <sup>c</sup>	61.34 ± 1.12 <sup>a</sup>	47.41 ± 1.18 <sup>b</sup>	34.58 ± 1.23 <sup>c</sup>
LIN (%)	56.63 ± 1.22 <sup>ab</sup>	59.27 ± 1.78 <sup>a</sup>	56.41 ± 1.81 <sup>ab</sup>	54.67 ± 1.69 <sup>b</sup>	50.16 ± 1.62 <sup>c</sup>
STR (%)	76.49 ± 2.43 <sup>c</sup>	79.82 ± 2.38 <sup>c</sup>	93.72 ± 3.20 <sup>a</sup>	88.70 ± 2.77 <sup>b</sup>	80.28 ± 1.83 <sup>c</sup>
VAP (µm/s)	26.79 ± 1.44 <sup>b</sup>	26.70 ± 1.87 <sup>b</sup>	36.39 ± 1.46 <sup>a</sup>	28.78 ± 1.72 <sup>b</sup>	21.66 ± 1.99 <sup>c</sup>
WOB (%)	73.82 ± 2.53 <sup>a</sup>	74.11 ± 1.92 <sup>a</sup>	60.23 ± 2.13 <sup>b</sup>	61.82 ± 2.73 <sup>b</sup>	62.94 ± 2.42 <sup>b</sup>
ALH (µm)	2.16 ± 1.23 <sup>bc</sup>	2.08 ± 1.12 <sup>b</sup>	2.57 ± 1.17 <sup>a</sup>	2.41 ± 1.33 <sup>ac</sup>	2.10 ± 1.25 <sup>b</sup>
BCF (Hz)	13.33 ± 1.12 <sup>b</sup>	13.07 ± 1.26 <sup>b</sup>	6.13 ± 1.35 <sup>d</sup>	10.55 ± 1.16 <sup>c</sup>	16.32 ± 1.25 <sup>a</sup>

Values are mean ± S.D. of motility parameters from the CASA data set of thawing bovine spermatozoa in different treatments. Values in the same row with different letters mean significantly different (P < 0.05). (n = 24)

Table 2. CASA-obtained mean values of the percentage of spermatozoa grade from frozen-thawed bovine semen samples in the presence and the absence of vitamin B<sub>12</sub>.

Item	Control	VB <sub>12</sub> 1.25 mg/mL	VB <sub>12</sub> 2.50 mg/mL	VB <sub>12</sub> 3.75 mg/mL	VB <sub>12</sub> 5.00 mg/mL
Percentage of grade a sperm	16.56 ± 0.73 <sup>b</sup>	18.27 ± 0.55 <sup>b</sup>	30.52 ± 0.64 <sup>a</sup>	29.41 ± 0.79 <sup>a</sup>	11.45 ± 0.49 <sup>c</sup>
Percentage of grade b sperm	24.09 ± 0.91 <sup>a</sup>	23.85 ± 0.62 <sup>a</sup>	24.78 ± 0.44 <sup>a</sup>	26.17 ± 0.23 <sup>a</sup>	24.37 ± 0.92 <sup>a</sup>
Percentage of grade c sperm	48.03 ± 0.25 <sup>a</sup>	46.95 ± 0.52 <sup>a</sup>	40.84 ± 0.69 <sup>b</sup>	41.55 ± 0.71 <sup>b</sup>	49.31 ± 1.03 <sup>a</sup>
Percentage of grade d sperm	11.32 ± 0.39 <sup>a</sup>	10.93 ± 0.32 <sup>a</sup>	3.86 ± 0.58 <sup>b</sup>	2.87 ± 0.22 <sup>b</sup>	14.87 ± 0.57 <sup>a</sup>
Percentage of grade a+b sperm	40.65 ± 0.18 <sup>b</sup>	42.12 ± 0.31 <sup>b</sup>	55.30 ± 0.57 <sup>a</sup>	55.58 ± 0.57 <sup>a</sup>	35.82 ± 1.32 <sup>c</sup>

Values are mean ± S.D. of the percentage of sperm grade from the CASA data set of thawing bovine spermatozoa in different treatments. Values in the same row with different letters mean significantly different (P < 0.05). (n = 24)

#### Acrosome integrity and membrane integrity

The results of the tests of acrosome and membrane integrity on frozen-thawed bovine sperm are shown in Table 3. The percentages of acrosome-intact and plasma membrane-intact spermatozoa were significantly improved (P < 0.05) by supplementing with 2.50 mg/mL vitamin B<sub>12</sub>. However, when vitamin B<sub>12</sub> concentration in the

extender was increased at 3.75 mg/mL, the percentages of acrosome-intact and membrane-intact spermatozoa were significantly lower (P < 0.05) as compared to semen frozen in extender supplemented with 2.50 mg/mL vitamin B<sub>12</sub>. This result showed that the optimum vitamin B<sub>12</sub> concentration in bull freezing semen extender was 2.50 mg/mL.

Table 3. Microscope observation-obtained mean values of the acrosome integrity and membrane integrity from frozen-thawed bovine semen samples in the presence and the absence of vitamin B<sub>12</sub>.

Item	Control	VB <sub>12</sub> 1.25 mg/mL	VB <sub>12</sub> 2.50 mg/mL	VB <sub>12</sub> 3.75 mg/mL	VB <sub>12</sub> 5.00 mg/mL
Acrosome integrity (%)	54.92 ± 2.32 <sup>c</sup>	54.06 ± 2.27 <sup>c</sup>	67.25 ± 2.74 <sup>a</sup>	60.61 ± 2.85 <sup>b</sup>	48.59 ± 2.56 <sup>d</sup>
Membrane integrity (%)	39.04 ± 2.38 <sup>bc</sup>	39.45 ± 2.31 <sup>bc</sup>	50.69 ± 2.50 <sup>a</sup>	42.34 ± 2.36 <sup>b</sup>	37.46 ± 1.76 <sup>c</sup>

Values are mean ± S.D. of thawed bovine spermatozoa in all the concentration of vitamin B<sub>12</sub>. Values in the same row with different letters mean significantly different ( $P < 0.05$ ). (n = 24)

## Discussion

Sharp and Witts (11) indicated that vitamin B<sub>12</sub> played an important role in spermatogenesis. Since then, some clinical observations have implicated vitamin B<sub>12</sub> as an important nutrient for maintaining normal fertility in men (12-14). Furthermore, it was considered that vitamin B<sub>12</sub> could increase protection effects of freezing medium. In this study, we investigated the effects of vitamin B<sub>12</sub> on motility, movement characteristics and integrity of the acrosome and membrane of cryopreserved bovine spermatozoa. Our results showed that the addition of 2.50 mg/mL vitamin B<sub>12</sub> into freezing medium increased bovine sperm motility, movement characteristics, and the integrity of acrosome and membrane. Depending on their concentrations, positive effects were found as well as negative ones. Treatment with vitamin B<sub>12</sub> at the concentration of 2.50 mg/mL group caused a significant ( $P < 0.05$ ) increase in sperm motility and VSL, VCL, STR, and VAP values compared to the other groups (Tables 1 and 2). Similar results were reported elsewhere. Ha and Zhao (8) reported that vitamin B complex (3%, v/v) could improve post-thaw motility and protect the integrity of spermatozoa membrane of rams. Cai et al. (9) reported that vitamin B<sub>12</sub> supplementation (0.5%, v/v) improved acrosome integrity of frozen-thawed bull spermatozoa.

The HOST is an assay to determine plasma membrane permeability and was shown to correlate with the numbers of sperm undergoing capacitation (15). This mechanism explained the significant increases in motility, percentage live sperms, and the HOST in the treated bovines. The observed increases found in the semen quality may result in increased

fertility in the field; however, this needs to be verified with further studies. The results suggested that supplementation with vitamin B<sub>12</sub> might improve the semen quality in the field, even in pigs with the concentration of 2.50 mg/mL in the freezing extender. However, the mechanism of the negative effect of the higher doses of vitamin B<sub>12</sub> on spermatozoa movement characteristics needs to be probed.

As for the protection mechanism of vitamin B<sub>12</sub> in the extender, B vitamins are water-soluble vitamins required as coenzymes for enzymes essential for cell function. In the strictest sense, vitamin B<sub>12</sub> refers to cyanocobalamin. It assists the enzymes methylmalonyl-coenzyme A mutase in the formation of glucose (16). The only cobalamin-dependent enzyme that was found was methionine synthase (17, 18). Ha and Zhao (8) and Cai et al. (9) reported that vitamin B<sub>12</sub> could improve the sperm motility of pigs and sheep during the freezing-thawing process, which correlated with the coenzyme A activity of vitamin B<sub>12</sub>. The oxidized form (-S-S-) coenzyme A was reduced to reduced form (-SH-) coenzyme A by the coenzyme of vitamin B<sub>12</sub>. The increased motility, live sperm, and plasma membrane-intact sperm were probably due to the increased seminal plasma glutathione peroxidase. This was in agreement with the findings reported by Vézina et al. (19) in humans. The additional vitamin B<sub>12</sub> increased the antioxidant status as indicated by the increased glutathione peroxidase activities, which would give protection against spontaneous lipid peroxidation. Alvarez and Storey (20) showed spontaneous lipid peroxidation to cause the plasma membrane to lose its ability to act as a permeability barrier, leading to the loss of cytosolic enzymes and substrates and a decrease in sperm motility and survival.

Based on the results of the present study, it could be concluded that vitamin B<sub>12</sub> possessed remarkable properties for freezing-thawing bovine spermatozoa and improved spermatozoa quality, as higher motility percentage (>52%), "grade a" sperm percentage (>30%), and better movement characteristics were obtained compared to the extender lacking vitamin B<sub>12</sub>. The acrosome-intact and plasma membrane-

intact spermatozoa were also maintained (acrosome integrity: 67.25%; membranes integrity: 50.69%). The optimum concentration of vitamin B<sub>12</sub> in bull freezing semen extender was determined as 2.50 mg/mL. Vitamin B<sub>12</sub> might be an essential constituent for spermatogenesis in both animals and humans. More research is needed to evaluate and understand its precise physiological role in reproduction.

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