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Full Length Research Paper

Effect of vitamin B12 addition to extenders on some physicochemical parameters of semen in crossbred rams

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The aim of this study was to examine the effect of different doses of vitamin B12 on some physicochemical parameters and antioxidtive enzyme activities in crossbreed rams semen during storage at 5°C. Semen samples were collected from eight crossbreed rams, evaluated and pooled at 33°C. Fresh semen was diluted with a Tris-based extender containing 0, 1, 2 and 3 mg/mL vitamin B12 and was cooled at 5°C. In both genetic group, the extender supplemented with vitamin B12 (1, 2 and 3 mg/mL) led to higher motility percentages than control group. While, the addition of 2 mg/mL vitamin B12 into the semen extenders led to higher viability sperm, in comparison to control group. In Ghezel × Baluchi genotype, the percentage of spermatozoa abnormality was reduced with vitamin B12 when compared with control group. Supplementation with vitamin B12 improved significantly sperm membrane integrity in both genotypes. Addition of vitamin B12 did not cause significant differences in the levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) when compared with the control group in both genotypes. In the current study, the addition of 2 mg/mL of vitamin B12 (as an antioxidant) to extender had higher SOD activities than the other groups in both genetic groups. In conclusion, vitamin B12 supplementation in semen extender benefit the motility and viability of crossbreed ram sperm.

Key words: Antioxidant, crossbreed ram, semen parameters, sperm, crossbreed ram.

INTRODUCTION

Oxidative stress is defined as an imbalance between the cellular antioxidant defence systems and the production of reactive oxygen species (ROS) (Sies, 1986). Due to the high percentage of polyunsaturated fatty acids (PUFA) in the plasma membrane, mammalian sperm are highly susceptible to ROS-induced damage (Alvarez and Storey, 1995). An unbalanced, excessive production of ROS and decreased level of antioxidant enzymes causes decreased sperm motility and viability, and increased sperm defects by initiating an oxidation chain reaction damaging proteins, lipids and DNA (Aitken and Baker, 2004). The addition of various antioxidants to sheep semen diluents extend the period of storage of semen, improve the motility, reduce the degree of cellular damage, improve the acrosomal integrity, and increase the viability and fertilization capacity of sperm *in vitro* (Maxwell and Stojanov, 1996; Sarlos et al., 2002). Vitamin B12 is one of the water-soluble vitamins that functions as a coenzyme in a number of biochemical reactions, such as methionine synthesis and the metabolism of branched amino acids (Juanchi et al., 2000); because of its stability, cyanocobalamin is the form that is typically used in vitamin supplements.

In animal experiments, it had also been demonstrated that vitamin B12 deficiency induced atrophic changes and the arrest of spermatogenesis in rats. Watanabe et al.

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Abbreviations: SOD, Superoxide dismutase; **GPx,** glutathione peroxidase; **HOST,** hypoosmotic swelling test.

(2003) reported that vitamin B12 deficiency increased the incidence of abnormal sperm and decreased the motility and velocity of the sperm in male rats. Recently, some reports had shown a positive effect of vitamin B12 supplementation to the extender on sperm motility and sperm count. Ha and Zhao (2003) indicated that vitamin B complex (3%, v/v) could improve post-thaw motility and protect the integrity of ram spermatozoa membrane during cryopreservation. Hu et al. (2011) indicated that the motility and straight line velocity, curvilinear velocity, mean coefficient, velocity of the average path values of bull sperm supplemented with 2.50 mg/mL vitamin B12 were significantly higher than other concentrations. There was no adequate information on the effects of supplemental vitamin B12 to the extender on semen quality and antioxidant activity especially in rams. Therefore, the aim of the present study was to evaluate effects of vitamin B12 supplementation in semen extender on semen quality and antioxidant activities in seminal plasma when ram semen is stored at refrigeration temperature (5°C).

MATERIALS AND METHODS

Animals and management

This trial was performed at the sheep breeding Station, located in Tabriz, Iran. Eight crossbreed rams (four Ghezel × Baluchi; four Ghezel × Arkharmerino), two to three years old and a live weight of 60 to 65 kg was used in this study. The animals were maintained under natural photoperiod, and during the trial, the rams were housed separately from the ewes. Levels of nutrition remained equal as each ram was fed a daily with *ad libitum* diet according to the National Research Council (NRC) containing a 20% concentrate (75% barley, 25% corn, soya and bran) and 80% alfalfa hay.

Microscopic evaluation of sperms

A total of eight ejaculates per rams were collected by artificial vagina twice a week during the breeding season. Semen was collected in the mornings, and transported to the laboratory (at 37°C), within 3 to 4 min, and were placed in a water bath at 37°C and then evaluated. Spermatic assessment was performed in the fresh and cool semen. The volume of the ejaculates was measured in a conical tube graduated at 0.1 ml intervals. The sperm concentration was determined by means of a haemocytometer (Smith and Mayer, 1955), and sperm motility was estimated using phase contrast microscopy (×400). Progressive sperm motility was subjectively evaluated using the standard method (Bearden and Fuquay, 2000). Motility estimations were performed from six different microscopic fields in each sample at 37°C. The mean of the six estimations was used as the final motility score. The viability of spermatozoa in samples was assessed by means of the nigrosin-eosin stain method (Evans and Maxwell, 1987). The final composition of the stain was: eosin-Y 1.67 g, nigrosin 10 g, and sodium citrate 2.9 g, dissolved in 100 ml distilled water. Sperm suspension smears were prepared by mixing a drop of sperm sample with two drops of stain on a warm slide and spreading the stain with a second slide; viability was assessed by counting 400 sperm cells under phase-contrast at ×400 magnifications. Sperm displaying partial or complete purple staining were considered non-viable; only sperm showing strict exclusion of stain were counted as viable. For abnormal sperm assessment, at least three drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution (62.5 ml formalin (37%), 150 ml sodium saline solution, 150 ml buffer solution and 500 ml of double-distilled water) (Schafer and Holzmann, 2000). One drop of this mixture was put on a slide and covered with a cover slip. The percentage of spermatozoa abnormality was determined by counting a total of 200 spermatozoa under phase contrast microscopy (magnification × 1000, using immersion oil).

Assessment of spermatozoon membrane integrity

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails, and was performed by incubating 30 ml of semen with 300 ml of a 100 mOsM hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) (Revell and Mrode, 1994) at 37°C for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide. 400 sperm were evaluated under magnification ×400 with bright-field microscopy. Sperm with swollen or coiled tails were recorded (Buckett et al., 1997).

Processing of semen

Only ejaculates containing a semen volume varying between 1 and 2 ml, spermatozoa with >80% forward progressive motility and concentrations higher than 2.5×10^9 spermatozoa/mL were mixed in a pool, balancing the sperm contribution of each male to eliminate individual differences in crossbreed rams (Gil et al., 2003). Tris-citrate modified solution (Tris-hydroxymethyl-aminomethane 30.28 g/L, citric acid 17.0 g/L, fructose 12.50 g/L, egg yolk 20% (v/v) was used as the base cooling extender (Paulenz et al., 2002). After the evaluation of quality, the pool semen from four Ghezel × Baluchi and four Ghezel × Arkharmerino rams separately were divided into four equal fractions. One of the fractions was diluted with the extender for the control group (no vitamin B12) and the others with extender for the treatments (1, 2 and 3 mg/mL vitamin B12) to obtain final concentration of approximately 4×10^8 spermatozoa per milliliter. Extended semen was placed in cooling chamber at 5°C for 12 h.

Biochemical assays of semen

Biochemical assays were performed in semen samples immediately after cooling.

Preparation of seminal plasma

The seminal plasma was separated immediately after cooling. Fresh semen was centrifuged at 1500 × g for 15 min at 5°C. The supernatants were transferred into 1.5 ml Eppendorf tubes and recentrifuged at 14000 × g for 10 min at 5°C to eliminate the remaining sperm.

Determination of glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was measured by following the assay by Paglia and Valentine (1967). The assay was based on a nicotinamide adenine dinucleotide phosphate (NADPH)-coupled reaction where oxidized glutathione, produced upon reduction of organic peroxide (tert-butyl hydroperoxide) by GPx, was recycled to its reduced state by utilizing the enzymes GR and NADPH. The

Table 1. Spermatozoa motility, viability, abnormality and integrity of plasma membrane in the semen of Ghezel × Baluchi rams, stored at 5°C in diluent supplemented with vitamin B12.

Vitamin B12	Progressive motility (%)	Viability (%)	Abnormality (%)	HOST (%)
Control	55.00±1.24^{c}	69.33±1.20^{b}	16.33±0.57^{a}	32.33±0.67^{c}
Vitamin B12 (1 mg/mL)	63.33±1.67^{b}	60.00±1.45^{c}	11.00±0.57^{b}	39.67±0.67^{b}
Vitamin B12 (2 mg/mL)	68.33±1.67^{a}	73.67±1.00^{a}	10.00±0.33^{b}	43.67±1.52^{a}
Vitamin B12 (3 mg/mL)	65.00±1.24^{a,b}	61.00±1.00^{b,c}	12.33±0.33^{b}	39.00±1.20^{b}

Different superscript letters (a to c) within the same column showed significant differences among the groups ($P < 0.05$).

Table 2. Spermatozoa motility, viability, total abnormality and integrity of plasma membrane in the semen of Ghezel × Arkharmerino rams, during storage at 5°C in diluent supplemented with vitamin B12.

Vitamin B12	Progressive motility (%)	Viability (%)	Abnormality (%)	HOST (%)
Control	56.67±3.33^{c}	72.00±3.92^{b}	11.33±1.52^{b}	32.67±2.18^{c}
Vitamin B12 (1 mg/ml)	66.67±5.00^{b}	75.33±4.84^{b}	10.00±1.67^{b}	40.00±1.85^{b}
Vitamin B12 (2 mg/mL)	75.00±1.67^{a}	79.33±1.52^{a}	8.33±0.88^{b}	44.33±1.52^{a}
Vitamin B12 (3 mg/mL)	68.33±1.67^{a,b}	62.67±1.20^{c}	15.33±0.67^{a}	43.67±1.45^{a,b}

Different superscript letters (a to c) within the same column showed significant differences among the groups ($P < 0.05$).

oxidation of NADPH to $NADP^{+}$ was associated with a decrease in absorbance at 340 nm, providing a spectrophotometric means for monitoring GPx activity. The rate of decline in absorbance at 340 nm was directly proportional to the GPx activity.

Determination of superoxide dismutase activity

The superoxide dismutase (SOD) activity in spermatozoa was determined using a method proposed by Nishikimi et al. (1972) based on the principle that the reduction of nitro blue tetrazolium (NBT) with NADH mediated by phenazine methosulfate (PMS) is inhibited upon addition of SOD. The PMS acts as a generator of superoxide anion radical. The enzyme assay solution consisted of 2.6 ml of phosphate buffer (0.017 M, 8.3 pH) at 20°C in a cuvette, to which was added 0.1 ml each of PMS (0.093 mM), NBT (1.5 mM) and supernatant prepared as above. The reaction was started by adding 0.1 ml of NADH (2.34 mM) and an increase in absorbance was recorded at 560 nm for 2 min at 30 s interval. A control was also run. A unit of SOD was defined as the activity of enzyme required for suppressing the increase in absorbance by 50%.

Statistical analysis

The study was replicated five times. The results were expressed as the mean ± standard error (S.E.). Means were analyzed by one-way analysis of variance, followed by the Tukey's post hoc test to determine significant differences in all the parameters among all groups, using the SPSS/PC computer program (Version 16.0; SPSS, Chicago, IL). Differences with values of $P < 0.05$ were considered to be statistically significant.

RESULTS

The influence of vitamin B12 on the standard semen parameters of Ghezel × Baluchi ram semen were evaluated in three independent experiments as shown in Table 1. Extender supplemented with1, 2 and 3 mg/mL vitamin B12 led to higher motility percentages, in comparison to control group ($P < 0.01$). Also, the addition of 2 mg/mL vitamin B12 to semen extender led to higher viability sperm, in comparison to control group. The percentage of abnormal sperm was significantly lower in vitamin B12 (1, 2 and 3 mg/mL) treatments, when compared to the control ($P < 0.05$). The vitamin B12 supplementation also improved significantly sperm membrane integrity. The effects of different concentrations of vitamin B12 on the semen parameters of Ghezel × Arkharmerino rams were evaluated in three independent experiments as shown in Table 2. The supplementation of extender with 2 mg/mL vitamin B12 had significant effect on sperm motility, viability and morphology when compared to the control ($P < 0.05$).

Biochemical parameters of sperm samples

The effect of the various concentrations of vitamin B12 on the activities of SOD and GPx in fresh semen in Ghezel × Baluchi and Ghezel × Arkharmerino rams are shown in Table 3. The addition of vitamin B12 did not cause significant effect in levels of SOD and GPx in the semen of Ghezel × Baluchi or Ghezel × Arkharmerino rams when compared to the control.

DISCUSSION

The effect of vitamin B12 on parameters of the semen and SOD and GPx activity in Ghezel × Baluchi and Ghezel × Arkharmerino crossbred rams during storage at 5°C was investigated in this study. The capacity of

Table 3. The SOD and GPx activities in the semen of Ghezel × Baluchi and Ghezel × Arkharmerino rams during storage at 5°C in diluents supplemented with vitamin B12.

Parameter	Crossbred ram			
	Ghezel × Baluchi		Ghezel × Arkharmerino	
	SOD (U/ml)	GPx (mU/ml)	SOD (U/ml)	GPx (mU/ml)
Control	353.96±90.54	0.43±0.32	469.00±107.54	0.16±0.01
Vitamin B12 (1 mg/mL)	238.71±16.46	0.38±0.25	294.00±84.00	0.20±0.01
Vitamin B12 (2 mg/mL)	359.30±97.78	0.31±0.19	613.00±45.00	0.20±0.01
Vitamin B12 (3 mg/mL)	350.89±75.56	0.71±0.24	283.50±111.50	0.11±0.03

Values in the same column without different letters not differ significantly from each other ($P > 0.05$). Means were significantly different at $P = 0.05$.

vitamin B12 to protect spermatozoa of the stress induced by cold may vary widely with the concentration used. The addition of 2 mg/ml vitamin B12 into extender increased sperm motility and viability in Ghezel × Baluchi and Ghezel × Arkharmerino, indicating that the antioxidant was able to protect sperm membranes during storage at 5°C. Similar results were obtained by Hu et al. (2011), who showed that addition of 2.5 mg/mL vitamin B12 into freezing medium increased the bovine sperm motility. Our results are also in agreement with the findings of Ha and Zhoa (2003) and Cai et al. (2004). The results indicate that vitamin B12 could protect sperm membranes during storage at 5°C.

Probably, the protection mechanism of vitamin B12 in the extender is due to the fact that the B vitamins are water-soluble vitamins required as coenzymes for enzymes essential for cell function. In the strictest sense, vitamin B12 refers to cyanocobalamin. Coenzymes B12 assist the enzymes methylmalonyl coenzyme A mutase in the formation of glucose (Bergman et al., 1966). Methionine synthase is the only enzyme cobalamin-dependent known found (Kennedy et al., 1990; Shangari et al., 2003). Cai et al. (2004) reported that vitamin B12 could improve the sperm motility of bovine and sheep during the freezing-thawing process, which is consistent with coenzyme A activity of vitamin B12. Under the action of the coenzyme of vitamin B12, the oxidized form (-S-S-) of coenzyme A is reduced to form (-SH-), required for enzyme reaction. Moreover, the oxidized (-S-S-) glutathione and homocysteine were reduced to reduced (-SH-) glutathione and homocysteine by the coenzyme of vitamin B12, respectively (Hu et al., 2011). Especially, the reduced glutathione has very important biological activities for sperm cells metabolism. In addition, Ha and Zhao (2003) had indicated that glutamic oxaloacetic transaminase (GOT) emission of ram seminal plasma was significantly decreased when vitamin B12 was added to the semen extender, and this might be an important factor in improving sperm motility during cryo-preservation. Neild et al. (2003) had demonstrated that GOT was released from seminal plasma during the freezing-thawing process and it resulted in the damage of sperm acrosome. Previous research showed that the highest concentration (3.75 mg/mL) of vitamin B12 had a toxic effect on bull spermatozoa (Hu et al., 2011). An excessive addition of vitamin B12 to the semen extender can neutralize the oxidative stress induced by excessive ROS formation, but it can also stop the normal sperm functions associated with ROS. Therefore, it is important to select the appropriate antioxidant concentration to maintain the natural balance that exists between ROS generation and scavenging activities. The exact mechanism, by which higher vitamin B12 concentrations adversely affected the liquid sperm characteristics, remains unclear. The interaction between extender components and antioxidants is important factors to be considered when evaluating the effectiveness of any antioxidant in the protection of spermatozoa against the cold shock. The HOST is an assay to determine plasma membrane permeability and was shown to be correlated with the numbers of sperm undergoing capacitation (Jeyendran et al., 1984). In the present study, addition of 2 mg/mL vitamin B12 in the extender before cooling increased the plasma membrane integrity in the sperm of both genotypes. This is in contrast to the finding of Bucak et al. (2007) who demonstrated that supplementation with additives of other antioxidants did not give significant positive effect on plasma membrane integrity in ram semen.

The sperm plasma membrane is rich in poly-unsaturated fatty acids and is therefore susceptible to peroxidative damage with consequent loss of membrane integrity, decreased sperm motility, and eventually loss in fertility, resulting from reactive oxygen species during aerobic incubation (Alvarez et al., 1987). Therefore, free radicals must be eliminated by supplementation with antioxidants. In this study, although SOD value was higher in the extender supplemented with 2 mg/mL vitamin B12, in both breed, differences in SOD levels between the vitamin B12 groups were not statistically important. Current finding on the effect of vitamin B12 are in agreement with those reported by Hu et al. (2011) who demonstrated that the extender for bull semen supplemented with vitamin B12 at 2.50 mg/mL got the

highest value of SOD. The GPx plays a role in the elimination of hydrogen peroxide (Meister and Anderson, 1983). In the present study, GPx value was higher in the extender supplemented with 3 and 2 mg/mL vitamin B12 in Ghezel × Baluchi and Ghezel × Arkharmerino rams, respectively. This finding is in contrast with Hu et al. (2011) who reported that the extender for bull supplemented semen with vitamin B12 significantly decreased GPx activity. Our finding indicates that the vitamin B12 could reduce the oxidative stress during storage at 5°C.

Conclusion

The present study shows that the addition of vitamin B12 in the extender can protect the semen of both sheep genotypes during storage at 5°C and improve the semen quality. Our finding shows still that 2 mg/mL vitamin B12 is the optimal supplementation level in Tris based extender for ram semen during cooling.

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