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Injection of Vitamin B_{12} and Phosphorus around Mating: Effect on Metabolic Status and Reproductive Performance of Afshari Ewes

Research Article

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ABSTRACT

The effects of vitamin B_{12} (B_{12}) and phosphorus (P) around mating on reproductive performance of ewes have not been investigated previously. Therefore, this study aimed to investigate the effect of injection of a solution (Catosin 10%) contained B_{12} and P on metabolic status and different aspects of reproductive performance of ewes. A total of sixty multiparous Afshari ewes were divided into two groups (n=30 ewes/group) based on age, body condition score (BCS) and body weight (BW). Animals were received either 5 mL of Catosin (B_{12}+P) or 5 mL of sterile water (Control) subcutaneously every 7 days during 28 days before mating and until 7 days after mating. Each mL of Catosin contained 0.05 mg of cyanocobalamin and 100 mg of butaphosphan, which provided 17.3 mg of P in the form of [1-(butylamino)-1-methylethyl]-phosphonic acid. Two weeks after the start of the experiment, the estrus cycles of all ewes were synchronized using intravaginal 12-day CIDRs. Ewes receiving B_{12} + P had higher BW and BCS at day of CIDR removal and BCS at day 30 after mating ($P<0.05$). Plasma E_2 and testosterone to P_4 ratio at the day of CIDR removal tended to be lower in ewes receiving B_{12} + P ($P=0.06$). Injection of B_{12} + P did not influence plasma concentrations of minerals and metabolites ($P>0.05$), whereas, ewes receiving B_{12}+P had higher plasma concentration of B_{12} at the day of CIDR removal and the day of estrus and mating ($P<0.05$). The results suggest that B_{12} + P injection around mating may be an effective strategy for improving reproductive performance of ewe. However, further researches need to re-evaluate and confirm these findings.

KEY WORDS Afshari ewe, metabolic status, phosphorus, reproductive response, vitamin B_{12}.

INTRODUCTION

Nutrition around mating affects various aspects of ewe reproduction, mainly through altering hormone and metabolite concentrations in blood circulation (Robinson *et al.* 2006). Energy is the main dietary component affecting ewe ovulation and lambing rates (Daghighkia *et al.* 2015). Energy intake increases blood glucose, insulin, and IGF-1, which may promote LH-receptor development in granulosa cells and ovulation of follicles in conjunction with FSH (Rassu *et al.* 2004). Pregnancy, early embryonic survival, and fetal development depend on oocyte quality (Krisher *et al.* 2004). Glycolysis, the pentose phosphate pathway (PPP), the hexosamine biosynthesis pathway (HBP), and the polyol pathway metabolize glucose as a vital metabolite for the oocyte. The glycolytic pathway metabolizes most glucose during oocyte development to produce pyruvate as an energy-containing substrates. Glucose regulates nuclear maturation and redox state via the PPP. It synthesizes extracellular matrices (cumulus expansion) and O-linked glycosylation (cell signaling) via the HBP during oocyte maturation (Sutton-McDowall *et al.* 2010). Glucose also affects

circulatory insulin and IGF-1 levels. Insulin increases glucose movement into extrahepatic organs such as ovaries (Kawashima *et al*. 2012). High plasma concentrations of glucose, insulin, and IGF-1 are key regulators of the reproductive axis (Kawashima *et al*. 2012). Corn, barley, and wheat grains comprise the most portion of flushing diets for sheep and goats (Scaramuzzi *et al*. 2006). In general, cereal grains starch increases rumen organic acid production, notably propionate (Huntington *et al*. 2006). In ruminants, 20% of circulatory glucose comes via glucose absorption into the hepatic portal vein after starch digestion, whereas the rest originates from liver gluconeogenesis (Galindo *et al*. 2011). Propionate is the primary precursor of glucose in fed ruminants, and liver clearance of propionate accounts for 60% of hepatic glucose release into the plasma (Larsen and Kristensen, 2013). Propionate must first convert to propionyl-CoA, then methyl malonyl-CoA and ultimately succinyl-CoA before entering the Krebs cycle. Conversion of methyl malonyl-CoA to succinyl-CoA by methylmalonyl - CoA mutase requires vitamin B_{12} as a cofactor (Mann *et al*. 2001). Besides energy metabolism and glucose production, dairy cows given B_{12} had better tissues responsiveness to insulin (Girard *et al*. 2019), which may improve reproductive performance. Previous *in vitro* (Zacchini *et al*. 2017) and *in vivo* (Mitchell *et al*. 2007) studies showed that supplemental B_{12} as a methyl donor improved oocyte growth and development in ewes. Phosphorus (P), the second most abundant mineral within the animal body, is essential for gluconeogenesis, fatty acid transport, amino acid and protein syntheses, and sodium/potassium ion pump activity (Grunberg, 2014). Furthermore, P involves in phosphorylation of many intermediates in gluconeogenesis pathway. So, it regulates gluconeogenesis and glycolysis in several organs (Berg *et al*. 2006). Deficiency of minerals are often associated with reproductive failure in ruminant animals. Low P intake causes inactive ovaries, quiet or irregular estrus, delayed sexual development, poor conception rates, and impaired fertility (Velazquez, 2011). Based on blood glucose, non-esterified fatty acids (NEFA), beta hydroxybutyrate (BHB), and cholesterol levels, B_{12} and P injection enhanced dairy cow energy status during peripartum (Furll *et al*. 2010). To our knowledge, the influence of supplemental B_{12} and P around mating on ewe reproductive performance has not previously been examined previously. We hypothesized that injections of B_{12} and P around mating would improve ewe reproductive outcomes through improving energy status. Therefore, the objective of this study was to assess the effect of subcutaneous (SC) injection of B_{12} and P around mating on metabolic status and reproductive performance of Iranian Afshari ewes.

MATERIALS AND METHODS

Animal care

The Animal Care and Use Committee accepted this study's experimental and management methods (Iranian Council of Animal Care (1995), Guide to the Care and Use of Experimental Animals, vol. 1. Isfahan University of Technology).

Experiment design and animal management

This study was performed in Ilam, Iran, during the non-breeding season (May–June). Sixty multiparous Afshari ewes were randomly assigned into two groups (n=30 ewes/per group) according to age, body condition score (BCS), and body weight (BW). One month before flushing, all ewes were vaccinated against foot and mouth disease (ARRIAH FMD Vaccine, Rooyan Daroo Pharmaceutical Co., Tehran, Iran), enterotoxaemia (Syva-Bax, Syva, Spain), and agalactia (Agalaksivac-Oil, Vetal, Turkey) and treated with anthelmintic drugs against external and internal parasites (Ivermectin, subcutaneous injection, 0.2 mg/kg BW; Levamisole+Triclabendazole 8.75%, suspension, 1 mL/5 kg BW; Rooyan Daroo). Animals received 5 mL of Catosin (Erfan Daroo, Alboorz, Iran) or sterile water subcutaneously once a week from 28 days before mating and until 7 days after mating. Each mL of Catosin contained 0.05 mg of cyanocobalamin and 100 mg of butaphosphan, which provided 17.3 mg of P in the form of [1-(butylamino)-1-methylethyl]-phosphonic acid. From 28 days before mating and until 21 days after mating, all animals were given the same flushing diet (Table 1) as total mixed ration (TMR) twice daily (09:00 a.m. and 18:00 p.m.). Flushing diet was prepared according to recommendations of NRC (NRC, 2007). Water was available throughout the study. All ewes were maintained in single group and kept in same feeding and management condition from 21 d after mating till lambing.

Estrus synchronization program and pregnancy diagnosis

Two weeks after the beginning of feeding the flushing diet, the estrus cycle of all ewes was synchronized using CIDRs (Eazi-Breed CIDR® Sheep and Goat Inseet, New Zealand) intra-vaginally for 12 days, followed by intramuscular 500 IU/ewe of PMSG (Gonaser 5000 IU, Hipra, Spain) injection immediately after CIDR removal. Animals also received an intramuscular injection of 5 mL of a solution containing vitamin A (50000 IU/mL), vitamin D_3 (10000 IU/mL) and vitamin E (20 mg/mL; 3VitADE, Nasr Fariman Co., Fariman, Iran) and 5 mL of a solution containing vitamin E (50 mg/mL) and Se (0.5 mg/mL; SelojectE, Razak

Co., Tehran, Iran) at the day of CIDR insertion and removal, respectively. All ewes were kept with Afshari rams for three consecutive days. Pregnancy diagnosis was performed at d 30 post-mating using plasma progesterone assay and transabdominal ultrasonography.

Table 1 Ingredients and chemical composition of flushing diet of ewes studied

Ingredients	Amount (% of DM)
Corn silage	17.5
Alfalfa hay	27.5
Wheat straw	14.0
Barley grain	24.0
Corn grain	8.0
Wheat bran	2.0
Calcium salts of n-3 fatty acids[1]	3.0
Fish meal	3.0
Minerals and vitamins[2]	1.0
Chemical composition[3]	
ME (Mcal/kg DM)	2.4
CP (% of DM)	13.1
RUP (% of DM)	5.3
RDP (% of DM)	7.8
NDF (% of DM)	42.3
NFC (% of DM)	34.2
Ash (% of DM)	7.5
EE (% of DM)	5.4
Ca (% of DM)	0.92
P (% of DM)	0.71
Mg (% of DM)	0.43
K (% of DM)	2.07
Na (% of DM)	0.18
Na + K/Ca + Mg ratio	1.38

[1] Persian Fat Omega-3.
[2] Per kilogram of mineral and vitamin supplement, vitamin A: 250000 IU; vitamin D3: 100000 IU; vitamin E: 100 mg; Calcium: 50 g; Phosphorus: 32 g; Magnesium: 11 g; Sodium: 6 g; Manganese: 2 g; Iron: 3 g; Copper: 1 g; Zinc: 2 g; Cobalt: 60 mg; Iodine: 60 mg; Selenium: 1 mg; vitamin B1: 20 mg and vitamin B2: 40 mg.
[3] ME: metabolizable energy; CP: crude protein; RUP: rumen undegradable protein; RDP: rumen degradable protein; NDF: neutral detergent fiber; NFC: non-fibrous carbohydrate; EE: ether extract; Ca: calcium; P: phosphorus; Mg: magnesium; K: potassium and Na: sodium.

Data recording, sample collection and analysis

The BW and BCS of all ewes were recorded one day before starting the flushing diet (day 0), at the day of CIDR removal, and at 30 days after mating. The BCS of ewe are assessed by the palpation of the lumbar region, specifically on and around the backbone (spinous and transverse processes) in the loin area, immediately behind the last rib and above the kidneys to examine the degree of sharpness or roundness. In simple terms, it is a means of subjectively assessing the degree of fatness or condition of a live animal. The technique was based on 5 scale units (Kenyon *et al.* 2014). Flushing diet samples were collected once a week, composited after the end of study, dried at 55 ˚C in a forced air oven for 72 h and ground to pass through a 1-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Crude protein (CP), ether extract (EE), ash (AOAC, 2000), and neutral detergent fiber (Van Soest *et al.* 1991) of flushing diet were measured. Non-fibrous carbohydrate (NFC) was calculated as: NFC (% of DM)= 100 – (NDF%+CP%+ash%+EE%) according to National Research Council recommendation (NRC, 2007). Blood samples were taken from the jugular vein (n=10 ewes/group) into a 10-mL evacuated tube containing sodium heparin one day before the experiment, at CIDR removal, at estrus and mating and at 30 days after mating. Samples were immediately placed on ice immediately, centrifuged (3000 rpm at 4 ˚C for 15 min) and separated plasma was kept at -20 ˚C until analysis. Plasma were analyzed for glucose, total protein (TP), total cholesterol (TC), and urea nitrogen (UN) concentrations by a commercial analyzer (BT1500, Biotecnica, SRL, Italy) based on the manufacturer's procedure. An ELISA reader (STAT-FAX 3200, USA) and a commercial kit (Monobind Inc. USA, ELISA kit) were used to evaluate plasma insulin, progesterone, estrogen, and testosterone levels. The concentrations of sodium (Na), potassium (K), calcium (Ca) and magnesium (Mg) of diet and plasma samples were measured by a flame atomic absorption spectrometer (Analytik Jena AG-novAA® 400p, Germany). All lambs were ear-tagged after lambing. Lambs were counted, sexed, and weighed. The reproductive performance in terms of estrus response (number of ewes showing estrus/total treated ewes in each group×100), fertility rate (number of ewes lambing/total number of ewes mated×100), lambing rate (total number of lambs/number of ewes lambing × 100), twining rate (number of ewes lambing twin/total number of ewes lambing in each group×100), male lamb rate (number of male lamb/total number of lambs×100) and female lamb rate (number of female lamb/total number of lambs×100) were recorded.

Statistical analyses

The MIXED procedure of SAS (2003) was used for analyzing data from BW, BCS and plasma hormones, and metabolites data. The comparison of means was carried out by the least square mean (LSM) method. Tukey test was used to compare difference between treatments. Ewe BW and age were used as covariate in the model. The reproductive parameters (%) were analyzed using the Chi-square test. Data on birth weight of lambs were analyzed using the MIXED procedure. Effects of ewe weight and type of birth were added as covariate factors to the model. Significance and tendency differences between treatments were determined at $P < 0.05$ and $P < 0.1$ levels, respectively. Data were expressed as LSM ± SEM unless otherwise stated otherwise.

RESULTS AND DISCUSSION

Body weight and body condition score

Effect of B_{12}+P injection around mating on BW and BCS changes during the experiment in Afshari ewes was presented in Table 2. Ewes receiving B_{12} + P injection had higher BW on the day of CIDR removal and higher BCS at the day of CIDR removal and at day 30 after mating (P<0.05). Whereas, BW tended to be higher on day 30 after mating in ewes that received B_{12} + P than those in the control group (P= 0.07).

Plasma hormone concentration

Effects of experimental treatments on plasma hormone concentrations are shown in Table 3. Plasma concentrations of progesterone (P_4) and insulin on the days of CIDR removal and of estrus and mating were not affected by experimental treatments (P>0.05). Injection of B_{12} + P was not affect plasma concentrations of estradiol (E_2) at the day of estrus and mating and testosterone on the day of CIDR removal (P>0.05). However, plasma E_2 concentration at the day of CIDR removal decreased and testosterone at the day of estrus and mating increased in ewes receiving B_{12}+P, respectively (P<0.05). Plasma E_2 and testosterone to P_4 ratio at the day of CIDR removal tended to be lower in ewes receiving B_{12} + P (P=0.06). This parameter on the day of estrus and mating was not influenced by experimental treatments (P>0.05).

Plasma mineral concentration

Effects of experimental treatments on plasma B_{12} and mineral concentrations are shown in Table 4. Ewes receiving B_{12} + P had higher plasma concentration of B_{12} on the day of CIDR removal and the day of estrus and mating compared to the control group (P<0.05). Plasma concentrations of Ca, Mg, Na and K were not influenced by experimental treatments on the day of CIDR removal and the day of estrus and mating (P>0.05).

Plasma metabolites concentration

Effects of B_{12} + P injection around mating on plasma metabolites concentrations are shown in Table 5. Experimental treatments did not affect plasma concentrations of TP, glucose, TC and UN at the day of CIDR removal and the day of estrous and mating (P>0.05).

Reproductive outcomes

Effects of B_{12} + P injection around mating on reproductive outcomes of Afshari ewes and their lamb gender and birth BW are shown in Table 6. Estrus response, lambing rate, and twining rate were similar between the two groups (P>0.05).

Ewes receiving B_{12} + P had higher fertility rate compared to those in the control group (P<0.05). Injection of vitamin B_{12}+P did not affect lamb birth BW and gender (P>0.05).

The current study examined the effects of B_{12}+P injection around mating on BW, BCS, circulatory concentrations of metabolites, B_{12}, minerals and hormones with known links to improving reproductive performance in ewe. The most significant findings were alterations to several indices including BW, BCS, E_2, B_{12} and fertility rate. The difference in several reproductive parameters was not statistically significant between treatment groups. Nevertheless, B_{12} + P injection numerically decreased the female lamb rate. These results confirmed previous findings that reproductive health and performance of farm animals are influenced by nutrition (Smith and Akinbamijo, 2000). Nutrition during mating affects various aspects of ewe reproduction, mostly through circulatory hormone and metabolite concentrations (Scaramuzzi *et al*. 2006).

Antioxidants, minerals and vitamins affect many aspects of reproduction in ruminants (Awawdeh *et al*. 2019). Effect of vitamin B_{12} and P injection around mating on BW and BCS ruminant animals are poorly understood. Considering the previous reports, in line with the results of the current study, vitamin B_{12} injection increased goat weight gain compared to control group (Kadim *et al*. 2006). Furthermore, cobalt supplementation through improving vitamin B_{12} synthesis by rumen microorganisms increased goat weight gain (Mgongo *et al*. 1985). The BCS is an indicator of nutritional status and body fat stores with pronounced effects on reproductive performance in ruminant animals. The recommended BCS around mating for ewe is 2.5-3.5 (Kleemann *et al*. 2006; Kenyon *et al*. 2014). The rates of fertility, pregnancy, and lambing are positively correlated with BCS (Molina *et al*. 1991). In one hand, the P requires to activate all intermediated energy-producing metabolic pathways.

On other hand, vitamin B_{12} is an essential coenzyme for methylmalonyl coenzyme A mutase during conversion of propionate to glucose through gluconeogenesis and Krebs cycle pathways in ruminants (McDowell, 2000). Thus, the greater BW and BCS in ewes receiving B_{12} + P compared to the control group in this experiment may be due to improved energy metabolism (McDowell, 2000). Our knowledge about the effect of B_{12} on steroid hormones synthesis in ruminant animals is limited. Kim *et al*. (2020), found that higher circulatory B_{12} levels decreased E_2 and increased testosterone in women's plasma. Increased energy intake raises blood glucose, insulin, and IGF-1, which may work with FSH and LH receptors in granulosa cell to cause follicle maturation and ovulation (Rassu *et al*. 2004). High levels of glucose, insulin, and IGF-1 regulate the reproductive axis (Kawashima *et al*. 2012).

Table 2 Effect of B_{12} + P injection around mating on BW and BCS of Afshari ewes

Parameters studied	Experimental treatment		SEM	P-value
	No B_{12} + P injection	B_{12} + P injection		
Body weight (BW, kg)				
Beginning of experiment	46.55	44.72	2.02	0.52
Day of CIDR removal	48.48[b]	49.92[a]	0.46	0.03
Day 30 after mating	51.26	52.87	0.60	0.07
Body condition score (BCS)				
Beginning of experiment	3.09	2.92	0.07	0.11
Day of CIDR removal	3.20[b]	3.36[a]	0.04	0.01
Day 30 after mating	3.25[b]	3.52[a]	0.06	0.01

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 3 Effect of B_{12} + P injection around mating on plasma hormone concentrations of Afshari ewes

Parameters studied	Experimental treatment		SEM	P-value
	No B_{12} + P injection	B_{12} + P injection		
Progesterone (ng/mL)				
Beginning of experiment	0.70	0.84	0.09	0.28
Day of CIDR removal	1.55	1.42	0.25	0.73
Day of estrus and mating	0.59	0.51	0.12	0.64
Day 30 after mating	3.15	4.64	0.64	0.11
Estradiol (pg/mL)				
Beginning of experiment	70.04	83.39	7.50	0.24
Day of CIDR removal	79.20[a]	58.20[b]	7.12	0.05
Day of estrus and mating	88.39	73.51	7.52	0.19
Testosterone (ng/mL)				
Beginning of experiment	0.16	0.20	0.03	0.44
Day of CIDR removal	0.35	0.40	0.08	0.66
Day of estrus and mating	0.36[b]	0.64[a]	0.06	0.01
Insulin (µIU/mL)				
Beginning of experiment	11.71	10.95	0.77	0.50
Day of CIDR removal	11.47	11.46	0.52	0.98
Day of estrus and mating	10.47	9.85	0.96	0.66
Estradiol + testosterone : progesterone ratio				
Beginning of experiment	107.13	105.25	10.62	0.90
Day of CIDR removal	78.90	62.20	5.80	0.06
Day of estrus and mating	224.15	168.17	25.90	0.15

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

In the last stage of the estrus cycle, decreased P_4 and increased E_2 output increase gonadotropin secretion. The P_4 and E_2 influence LH pulse frequency and amplitude, and pulsatile LH release is inversely related to P_4 release by corpus luteum (CL; Barrett *et al.* 2007). In ewes with an active estrus cycle, P_4 is an endocrine signal that regulates both the frequency of follicular waves and the periodic rise in FSH concentration (Bartlewski *et al.* 2011). The P_4 plays an effective role in increasing the sensitivity of gonadotrope cells to GnRH to produce FSH. By enhancing energy status, B_{12} may influence the expression of genes related to ovarian follicle development (Gagnon *et al.* 2015). Vitamin B_{12} increases tissue sensitivity to insulin, glucose synthesis and energy metabolism, as well as reproductive outcomes (Girard *et al.* 2019). The lack of effect of experimental treatments on plasma insulin concentration in the current study may be due to similar plasma glucose concentration. The ratio of E_2 and testosterone to P_4 is used as an indicator of offspring gender ratio in mammals, which a rise in this ratio is often correlated with a decrease in the birth of female offspring (Navara, 2018).

Higher numerical, but not significant, proportion of female lambs in ewes of the control treatment in the present study may be related to higher E_2 and testosterone to P_4 ratio in these animals. Methionine synthase, which converts homocysteine to methionine, uses B_{12} as a cofactor (McFadden *et al.* 2020). According to Kanakkaparambil *et al.* (2009), ewes with higher granulosa cell homocysteine concentrations (not studied herein) have higher concentration of E_2 in their follicular fluid, which may explain higher plasma E_2 level at the day of CIDR removal in ewes of the control group in the present trials.

Table 4 Effect of B_{12} + P injection around mating on plasma B_{12} and mineral concentrations of Afshari ewes

Parameters studied	Experimental treatment		SEM	P-value
	No B_{12} + P injection	B_{12} + P injection		
Vitamin B_{12} (pg/mL)				
Beginning of experiment	1974.21	2051.23	96.32	0.35
Day of CIDR removal	1461.17^{b}	2260.33^{a}	119.94	0.01
Day of estrus and mating	1664.19^{b}	2191.73^{a}	87.52	0.01
Calcium (mg/dL)				
Beginning of experiment	7.86	8.22	0.38	0.53
Day of CIDR removal	7.66	7.84	0.27	0.65
Day of estrus and mating	7.48	7.28	0.24	0.58
Magnesium (mg/dL)				
Beginning of experiment	3.17	3.79	0.42	0.32
Day of CIDR removal	2.07	2.98	0.15	0.72
Day of estrus and mating	3.01	2.94	0.10	0.58
Sodium (mEq/L)				
Beginning of experiment	188.27	192.95	4.40	0.46
Day of CIDR removal	185.27	175.25	5.20	0.19
Day of estrus and mating	168.01	180.48	5.02	0.10
Potassium (mEq/L)				
Beginning of experiment	3.96	4.02	0.12	0.77
Day of CIDR removal	4.10	4.07	0.18	0.91
Day of estrus and mating	4.10	3.86	0.12	0.19

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 5 Effect of B_{12} + P injection around mating on plasma metabolites concentrations of Afshari ewes

Parameters studied	Experimental treatment		SEM	P-value
	No B_{12} + P injection	B_{12} + P injection		
Total protein (g/dL)				
Beginning of experiment	5.96	6.00	0.20	0.89
Day of CIDR removal	6.06	5.42	0.63	0.49
Day of estrus and mating	5.97	6.16	0.17	0.43
Urea nitrogen (mg/dL)				
Beginning of experiment	14.43^{a}	12.12^{b}	0.70	0.03
Day of CIDR removal	11.93	12.56	1.75	0.82
Day of estrus and mating	12.61	12.59	1.13	0.99
Glucose (mg/dL)				
Beginning of experiment	66.86	64.75	2.20	0.51
Day of CIDR removal	60.43	59.12	7.15	0.90
Day of estrus and mating	73.14	64.87	3.90	0.16
Total cholesterol (mg/dL)				
Beginning of experiment	47.43	45.37	4.15	0.74
Day of CIDR removal	49.00	46.37	6.60	0.78
Day of estrus and mating	52.00	53.87	3.25	0.69

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 6 Effect of B_{12} + P injection around mating on reproductive parameters of Afshari ewes

Parameters studied	Experimental treatment		SEM	P-value
	No B_{12} + P injection	B_{12} + P injection		
Estrus response (%)	85.00	94.12	-	0.36
Fertility rate (%)	82.35^{b}	100.00^{a}	-	0.04
Lambing rate (%)	107.14	107.69	-	0.96
Twining rate (%)	7.14	7.69	-	0.95
Lamb birth weight (kg/lambed ewe)	4.74	4.81	0.28	0.86
Proportion of female lambs (%)	53.33	42.85	-	0.57
Proportion of male lambs (%)	46.67	57.15	-	0.57

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

The embryo's viability is negatively influenced by high plasma UN levels because it is linked to a lower progesterone levels during the luteal phase (Butler, 1998). According to the results of the current study (Table 4), the experimental treatments had no significant effect on plasma UN concentration, which helps to partially explain why they had no effect on plasma progesterone concentration. Similar to the results of the current study in ewes, injection of a vitamin B_{12}-containing solution increased plasma B_{12} concentration in dairy cows during early lactation (Duplessis *et al*. 2017) and transition period (Wang *et al*. 2018), as well as in pigs (Matte *et al*. 2006), and goats (Kadim *et al*. 2006). Pereira *et al.* (2013) examined the impact of vitamin B_{12} and P injection in dairy cows during early lactation and found no differences in plasma Ca and P concentrations. According to Wilde (2006), minerals have a pronounced impact on animal reproduction. The initiation of estrus and ovulation may be delayed due to changes in the ratio of Ca to P on the pituitary gland, which may disrupt ovarian activity. Additionally, the absence of estrus symptoms has been linked to decreased plasma Ca level. Reproduction of ruminants is indirectly influenced by Na and K. For example, Na deficiency disrupts normal reproductive physiology by reducing protein and energy metabolism. On the other hand, it seems that consuming large amounts of K can interfere with forming CL and ovulation. Additionally, lower fertility of ruminant animals has been linked to an improper Na and K balance (Yasothai, 2014). Similar to the results of the current study, Wang *et al*. (2018) found that vitamin B_{12} injection had no effect on plasma concentrations of glucose, UN and TC of dairy cows during the transition period. Pereira *et al*. (2013) observed that vitamin B_{12} and P injection had no impact on plasma concentrations of glucose and UN in Holstein dairy cows throughout the early lactation. In ruminant animals, most of blood glucose originates from glucogenic precursors sources such as propionate, lactate, glycerol, and glucogenic amino acids (Lucy, 2016). According to De Koster and Opsomer (2013), fermentation of quickly fermentable carbohydrates like starch in the rumen is the major source of propionate production. Nitrogen (N) source, as used for urea synthesis in the liver, is produced during the deamination of tissue amino acids (AA) and ammonia (NH_3) absorbed through the rumen wall (Lapierre and Lobley, 2001). Although, ruminal volatile fatty acids (VFA) and NH_3 concentrations were not measured in the present experiment, the lack of effect of experimental treatments on plasma glucose and UN concentrations may be related probably to similar concentrations of propionate and NH_3 in the rumen. The formation of ovarian follicles and reproductive performance in ruminants are positively influenced by circulatory glucose. It is one of the most important regulators of reproductive events in ruminants, both directly by providing energy and indirectly by increasing insulin secretion (Lucy, 2016). Ruminant reproductive performance decreases with increasing plasma UN levels (Rhoads *et al*. 2004). It has been shown that ewes fed with diet containing higher rumen undegradable protein (RUP) during 3 weeks before to 2 weeks after mating have higher rates of twinning, fertility, and lambing, as well as lower plasma UN level (Daghighkia *et al*. 2015). On the other hand, increased CP or rumen degradable protein (RDP) intake and consequently higher plasma UN levels are associated with lower pregnancy rate (Butler, 2001). Reproductive tissues require cholesterol for production of steroid hormones like E_2, P_4, and testosterone (Sèdes *et al*. 2018). In the study of Pereira *et al*. (2013), injection of vitamin B_{12} and P decreased plasma concentrations of non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB) and cholesterol of Holstein cows in early lactation. These findings suggest that the energy status of animals has improved and that the liver no longer needs to produce as much lipoproteins. Triglycerides and very low-density lipoprotein (VLDL), which include cholesterol, exit the liver and enter into the bloodstream (Grummer, 1993). Herein, it may be spacuulated that the lack of effect of the experimental treatments on plasma cholesterol concentration would be due to similar plasma concentrations of NEFA and BHB (but this presumpsion needs experimental confirmation). Energy, protein, minerals, and vitamins are some of the most significant nutritional factors influencing reproduction in ruminant animals (Vázquez-Armijo *et al*. 2011). These nutrients influence reproduction by altering the concentrations of plasma parameters such as glucose, cholesterol, AA, fatty acid (FA), UN, Ca, P, magnesium (Mg), other minerals, insulin, leptin, LH, FSH, IGF-1, E_2, and P_4 (Ferguson, 2005). All these factors affect various aspects of reproductive processes in ruminant animals including follicle number and development, number of embryos, duration of gestation period and embryo survival (Fleming *et al*. 2012). The P is one of the key factors affecting reproductive success in ruminant animals (Kumar, 2003). Important clinical symptoms of phosphorus deficiency reported in the literature (Chaudhary and Singh, 2004) include delayed puberty, irregularity or lack of estrus, lower fertility or even fetal mortality. Nevertheless, increasing P percentage in the diet of ewe had no effect on pregnancy rate, offspring sex ratio and reproductive performance (Lopez *et al*. 2004). In several mammalian systems, maternal nutrition around mating is one of the key determinants of the gender ratio of offspring. Numerous data show that the gender ratio of mammalian offspring may be more influenced by the type of diet and its nutrients content than food availability (Navara, 2018). Additionally, there are evidences that offspring gender can be influenced by plasma Na and K to Ca

and Mg ratio around mating. The receptors on the egg wall that interacts sperm can alter depending on Na and K to Ca and Mg ratio. Sperm with the X chromosome can be absorb better by egg wall when there is a high ratio of Ca and Mg to Na and K (Vahidi and Sheikhha, 2007).

CONCLUSION

The present results suggest that B_{12} + P injection around mating can alter some factors including BW, BCS, and plasma B_{12} and E_2 concentrations affecting reproductive outcomes such that ewes receiving these micronutrients had higher fertility rate. Hence, it seems that the injection of B_{12} + P around mating may be a practical strategy to improve energy and metabolic statues and reproductive performance of ewes. However, further studies are needed to confirm these findings.

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