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Investigating the Effect of Using Vitamin AD_3E on the Reproductive Performance of Afshari Sheep

Research Article

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ABSTRACT

Nutrition during mating is one of the most important factors affecting the reproductive performance of ewes. The aim of this experiment was to investigate the effect of vitamin AD_3E injection around mating on body weight (BW), body condition score (BCS), plasma hormones, minerals, metabolites, vitamins, antioxidant enzymes and reproductive performance of Afshari ewes. Eighty-eight Afshari ewes were divided into 2 groups (n=44) based on age (2-4 years), BW (average 40 kg) and BCS (mean 2.75) and assigned randomly to one of the two experimental treatments. Experimental treatments consisted of control (C; no vitamin AD_3E injection) and AD_3E injection (AD_3E) groups. Ewes in AD_3E group received 5 mL of vitamin AD_3E solution subcutaneously at the beginning of the experiment, the day of CIDR insertion, the day of CIDR removal, and one week after mating. Two weeks after the start of the experiment, the estrous cycles of all ewes were synchronized using intravaginal 12-day CIDRs. The BW, BCS and plasma minerals and metabolites concentrations of ewes did not influence by experimental treatments ($P>0.05$). Ewes received AD_3E had lower estrogen (E_2) and progesterone (P_4) and higher testosterone concentrations at the day of CIDR removal ($P<0.05$). The AD_3E injection increased plasma vitamin D_3 and glutathione peroxidase (GPX) at the day of CIDR removal and at the day of estrous and decreased plasma malondialdehyde (MDA) concentration at the day of CIDR removal ($P<0.05$). The results obtained indicated that there was no significant difference ($P>0.05$) between the treatment groups in reproductive outcomes including estrous response, fertility rate, lambing rate, twining rate and lamb sex and birth weight. These results indicated that vitamin AD_3E injection around mating improved antioxidant enzyme status but had no significant effect on reproductive performance of Afshari ewes. Further researches are needed to evaluate the effect of different levels of vitamin AD_3E injection around mating on reproductive performance of Afshari ewes.

KEY WORDS Afshari ewe, antioxidant enzyme, fertility rate, plasma metabolite.

INTRODUCTION

Meat production is the main purpose of raising sheep in Iran. Efficiency of reproductive performance and the number of lambs born are main factors affecting meat production in sheep flocks. Most Iranian sheep breeds have seasonal reproduction, they usually give birth to one lamb at each birth, and therefore their profitability is low. Reproductive performance in sheep is affected by the length of the reproductive season, ovulation rate, pregnancy rate, number of lamb and offspring survival, which are influenced by the interaction of genetics and environmental factors (Gordon, 2004; Greyling, 2010). Nutrition is one of the most important environmental factors affecting reproduc-

tion efficiency in ruminant animals, which affects them from the beginning of puberty to the total number of offspring born during their lifetime (Ferguson, 2005; Robinson *et al*. 2006). Energy, protein, minerals and vitamins are among the most important nutritional factors affecting reproduction in ruminant animals (Smith and Akinbamijo, 2000; Martin *et al*. 2004; Rassu *et al*. 2004; Vazquez-Armijo, 2011). These nutrients affect reproduction through changes in plasma concentration of metabolites, hormones, growth factors, enzymes, minerals and vitamins (Ferguson, 2005). Although vitamins are present in relatively minute quantities in the body and are required in low amounts in the diet, they play an essential role in mammalian reproduction, including ovarian folliculogenesis and embryo development (Smith and Akinbamijo, 2000; Ikeda *et al*. 2005; Gómez *et al*. 2006; Kanakkaparambil *et al*. 2009; Velazquez, 2011). Fat-soluble vitamins including A, D_3 and E have important roles in reproductive outcomes of ruminant animals (Smith and Akinbamijo, 2000; Velazquez, 2011). Oxidative stress is the result of an imbalance between free radicals and antioxidants. Under normal physiological conditions, free radicals are involved in reproductive events such as cell cycle activation, ovulation and luteolysis (Aitken, 2020). However, when an overproduction of free radicals surpasses antioxidant capacity, oxidative damage, reproductive anomalies and diminished fertility occur (Almansa-Ordonez *et al*. 2020). Supplementation with antioxidants prevents oxidative damage and can be incorporated into reproductive management to improve fertility in females (Smith and Akinbamijo, 2000; Spears and Weiss, 2008). The most known exogenous antioxidants are the vitamins, among which the vitamins A, C, E and the provitamin beta-carotene stand out in this role (Spears and Weiss, 2008). Despite the well-established and important role of vitamin D_3 in Ca metabolism, discussions have been ongoing regarding the importance of this vitamin as an antioxidant (Sepidarkish *et al*. 2019; Alamir *et al*. 2021). In other hand, the presence of vitamin D_3 receptors in the reproductive tract of women (Lerchbaum and Obermayer-Pietsch, 2012), and females of other species, such as sheep (Cleal *et al*. 2017), goat (Yao *et al*. 2017), mouse (Shahbazi, 2011) and rat (Johnson *et al*. 1996) indicates that vitamin D_3 may influence reproductive performance. Vitamin A is well known to regulate the development, cellular growth and differentiation, and tissue function. Its metabolites affect ovarian follicular growth, uterine environments and oocyte maturation. Vitamin A is required for maintaining healthy tissue in the reproductive tract. A deficiency of vitamin A has a direct effect on the structure and function of pituitary gland, gonads and uterus (Ikeda *et al*. 2005; Clagett-Dame and Knutson, 2011). Ikeda *et al*. (2005) reported that vitamin A may promote cytoplasmic maturation of bovine oocytes via its modulatory effects on the gene expression of gonadotrophin receptors, midkine, cyclooxygenase-2, and nitric oxide synthase in cumulus-granulosa cells. Dairy cows and heifers consuming diets deficient in β-carotene suffered with following problems like delayed uterine involution, delayed first estrus after calving, delayed ovulation, increased incidence of cystic ovaries, more early embryonic death and abortion (Amin, 2014; Yasothai, 2014). Vitamin E, chain-breaking antioxidant, is one of the primary components of the antioxidant system. It plays an important role in the management of oxidative stress. Oxidative stress may also compromise follicular development and ovarian activity. It is particularly important in protecting cells against oxidative damage. Therefore, vitamin E status is important for reproductive efficiency in females and in the survival of lambs and weaners (Liu *et al*. 2014). In addition, vitamin E is also important for oocyte quality and maturation in female reproduction (Tao *et al*. 2004). To our knowledge, the effects of vitamin AD_3E injection around mating on reproductive performance and metabolic status of ewes have not been well investigated. We hypothesized that injection of a solution containing vitamin AD_3E around mating would increase reproductive performance of ewes. Therefore, the objective of the present study was to evaluate the effect of subcutaneous injection of AD_3E solution around mating on plasma hormone levels, blood metabolites, fertility, lambing rate and proportion of female lambs in Afahari ewes.

MATERIALS AND METHODS

The Iranian Council of Animal Care (1995) gave their stamp of approval to the study's experimental and management methods. The authors swear they did everything by the book to make sure the animals in their study were safe.

Animals and experimental design

This research was conducted between May and June (2022) on a commercial farm in Ilam, Iran. A total of 88 ewes with average body weight of 40 kg/head and at an age of 2-4 years were randomly divided in to two groups of 44 ewes each. One month before the beginning of the experiment, all the ewes were vaccinated against clostridial diseases (Razi Institute, Hesarak, karaj, Iran) and treated with anthelmintic drugs against external and internal parasites (Ivermectin, subcutaneous injection, 0.2 mg/kg BW; Hepatec 500, bolus, 5 mg/kg BW; and Niclosam 1250, bolus, 62 mg/kg BW; Damloran Pharmaceutical Co., Borujerd, Iran). Animals in the control and treatment groups received subcutaneous injections either of 5 mL of sterile water or vitamin AD_3E solution at the beginning of the experiment, on the day the CIDR was placed, on the day the

CIDR was withdrawn, and seven days following mating. The vitamin AD_3E solution (Razak Co., Tehran, Iran) included 20 milligrams of vitamin E per milliliter and 50000 international units of vitamin A. Animals were fed a total mixed ration (TMR; ME=2.38 Mcal/kg and CP=13.5% of DM) consisting of the same flushing diet (Table 1) twice daily. The NRC claims that the Flushing diet was created (NRC, 2007).

Table 1 Ingredients and chemical composition of flushing diet

Item	Amount
Ingredients (% of DM)	
Alfalfa hay	46.70
Wheat straw	20.00
Barley grain	15.00
Corn grain	9.40
Wheat bran	1.60
Calcium salts of n-3 fatty acids[1]	3.30
Fish meal	3.30
Minerals and vitamins premix[2]	0.70
Chemical composition[3]	
ME (Mcal/kg)	2.38
CP (% of DM)	13.50
NDF (% of DM)	39.00
NFC (% of DM)	33.70
Ash (% of DM)	7.80
EE (% of DM)	6.00
Na (% of DM)	0.12
K (% of DM)	2.06
Ca (% of DM)	0.99
Mg (% of DM)	0.35
Na + K / Ca + Mg	1.63

[1] Persia Lin omega-3.
[2] Mineral and vitamin premix contained Ca: 196 g/kg; P: 96 g/kg; Mg: 19 g/kg; Na: 46 g/kg; Mn: 2 g/kg; Fe: 3 g/kg; Zn: 2 g/kg; Cu: 3 g/kg; I: 100 mg/kg; Co: 100 mg/kg; Se: 1 mg/kg; Antioxidant: 400 mg/kg; vitamin A: 500000 IU/kg; vitamin D_3: 100000 IU/kg and vitamin E: 100 IU/kg.
[3] ME: metabolisable energy; CP: crude protein; NDF: neutral detergent fibre; NFC: non fibre carbohydrate [100 - (CP+CF+EE+Ash)]; EE: ether extract; Na: sodium; K: potassium; Ca: calcium and Mg: magnesium.

The animals of each group kept in separate pen had access to water at all times during the study. After the last day of the flushing diet (day 14 post-mating), all ewes were managed and fed in the same manner until after they had their lambs.

Estrous synchronization program and pregnancy diagnosis

All ewes had their estrous cycles synchronized 14 days after the start of the flushing diet by inserting CIDRs (Eazi-Breed CIDR® Sheep and Goat Inseet, New Zealand) intravaginally for 12 days, followed by injecting 400 IU of pregnant mare serum gonadotropin (PMSG; Gonaser 5000 IU, Hipra, Spain) intramuscularly immediately after the CIDRs were removed. All ewes were impregnated by Afshari males for three days straight, with one ram for every five ewes. 30 days after mating, an ultrasound was used to confirm the pregnancy.

Data recording and sample collection

At the outset of the study, the day the CIDR was removed, and 30 days following mating, all ewes had their BCS and BW recorded. After the experiment, samples of the flushing diet were dried at 55 ˚C in a forced air oven for 72 hours, composited them per diet, and ground with a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to pass through a 1-mm screen. To establish the flushing diet's chemical composition, crude protein, ether extract, ash, and neutral detergent fiber were analyzed according to AOAC (2007). The non-fibrous carbohydrate (NFC) was determined by following the advice provided by the National Research Council (NRC, 2007). At the beginning of the experiment, on the day of CIDR removal, and on the day of estrous and mating, samples were obtained through jugular vein using 10-mL evacuated tubes containing sodium heparin to determine blood metabolic tests. After collecting blood samples, they were placed on ice and centrifuged at 3000 rpm and 4°C for 15 minutes to separate the plasma, which was then frozen at -20 ˚C until analysis.

Laboratory analysis and calculations

Total cholesterol, total protein, urea nitrogen (UN), and glucose were measured by a commercial analyzer (BT1500, Biotecnica, SRL, Italy) according to the manufacturer's protocol. Plasma insulin, progesterone (P_4), estradiol (E_2) and testosterone concentrations were measured using an ELISA reader (STAT-FAX 3200, USA) and commercial kits (Monobind Inc. USA, ELISA kit). The inter- and intra-assay coefficients of variation were 4.5% and 3.8%, 5.2 and 3.7, 6.1 and 4.2, and 3.8 and 4.2 for insulin, progesterone, estradiol and testosterone, respectively. Plasma vitamin A, D3, and E levels were measured using an ELISA reader (Bio-Red, USA) and commercial kits (Zellbio, Germany). Plasma concentrations of glutathione peroxidase (GPX), superoxide dismutase (SOD), and malondialdehyde (MDA) were measured using an ELISA reader (Bio-Red, USA) and commercial kits (Nalondi, USA). The levels of Na, K, Ca, and Mg in the plasma and food samples taken during the flush were determined using a Flame Atomic Absorption Spectrometer (Analytik Jena AG-novAA® 400p, Germany). During lambing, all of the mothers and lambs were identified by ear tags. Counted, weighed, and sorted lambs by sex. The reproductive performance outcomes were recorded as follow (Mirzaei Alamouti *et al.* 2018):

Estrous response= (number of ewes showing estrous/total treated ewes in each group×100)

Fertility rate= (number of ewes lambing/total number of ewes×100)
Lambing rate= (number of ewes lambing/total number of ewes in each group×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)
Female lamb rate= (number of female lamb/total number of lambs×100)

Statistical analysis

Using SAS (9.1)'s MIXED approach, data for BCS, BW, and plasma hormones, vitamins, enzymes, minerals, and metabolites were examined (SAS, 2003). Pre-feeding values were co-variates. The Tukey test was used to compare the differences. Chi-square tests assessed reproductive factors (%). The MIXED method was used to evaluate data on the number of lambs born per ewe and birth weight. Covariates included sheep body weight and age. P 0.05 and P 0.1 thresholds, respectively, were used to determine if there were significant and substantial differences between treatments. Except as otherwise stated, data were provided as LSM SEM.

RESULTS AND DISCUSSION

Body condition score and body weight

Effect of vitamin AD_3E injection around mating on BCS and BW of Afshari ewes were shown in Table 2. The BCS and BW of animals at the day of CIDR removal and 30 days post-mating were not affected by vitamin AD_3E injection ($P<0.05$).

Plasma hormones concentration

Effect of vitamin AD_3E injection around mating on plasma hormones concentration of Afshari ewes were shown in Table 3. Injection of vitamin AD_3E decreased plasma concentrations of P_4 and E_2 at the day of CIDR removal ($P<0.05$). However, ewes received vitamin AD_3E solution had higher plasma E_2 level at the day estrous and mating ($P<0.05$). Experimental treatments had no effect on plasma insulin concentration at the day of CIDR removal and at the day estrous and mating ($P>0.05$). Plasma testosterone concentration at the day of CIDR removal was higher in ewes received vitamin AD_3E solution compared to the C group ($P<0.05$).

Plasma minerals concentration

Effect of vitamin AD_3E injection around mating on plasma minerals concentration of Afshari ewes were shown in Table 4.

Vitamin AD_3E injection increased plasma Ca concentration at the day of CIDR removal and at the day of estrous and mating and Mg concentration at the day of CIDR removal ($P<0.05$). Whereas, plasma concentrations of K and Na at the day of CIDR removal and at the day of estrous and mating did not influence by vitamin AD_3E injection ($P>0.05$).

Plasma metabolites concentration

Plasma concentrations of total protein, glucose, total cholesterol, and UN are presented in Table 5. Experimental treatments had no effect on plasma levels of total protein, glucose, total cholesterol, and UN at the day of CIDR removal and at the day of estrous and mating ($P>0.05$).

Plasma vitamins, antioxidant enzymes and malondialdehyde concentration (MDA)

Effect of vitamin AD_3E injection around mating on plasma vitamin, antioxidant enzyme and MDA concentrations of Afshari ewes were shown in Table 6. Injection of vitamin AD_3E had no effect on plasma levels of vitamin A and E were at the day of CIDR removal and at the day of estrous and mating ($P>0.05$). Plasma concentrations of vitamin D_3 at the day of CIDR removal and at the day of estrous and mating were higher in ewes received vitamin AD_3E than the C group ($P<0.05$). Ewes received vitamin AD_3E had lower plasma MDA concentration at the day of CIDR removal ($P<0.05$). Experimental treatments had no effect on plasma concentrations of SOD at the day of CIDR removal ($P>0.05$), whereas, its plasma level at the day of estrous and mating tended to be higher in ewes received vitamin AD_3E than those in the C group ($P=0.08$). Injection of vitamin AD_3E increased plasma GPX level at the day of CIDR removal and at the day of estrous and mating ($P<0.05$).

Reproductive outcomes

Effect of vitamin AD_3E injection around mating on reproductive outcomes and lamb sex and birth weight of Afshari ewes were shown in Table 7. Estrous response, fertility rate, lambing rate and twining rate were similar between two groups ($P>0.05$). Injection of vitamin AD_3E had no effect on lamb birth weight and sex ratio ($P>0.05$). However, the proportion of female lambs was numerically, but not significant, higher in ewes received vitamin AD_3E compared to the C group.

The current study examined the effects of vitamin AD_3E injection around mating on circulating concentrations of metabolites, antioxidant enzymes and hormones with known links to improving reproductive performance in ewes. The most significant findings were alterations to several of these markers, including E_2, testosterone, Ca, Mg, D_3, GPX and MDA.

Table 2 Effect of vitamin AD_3E injection around mating on body condition score and body weight of Afshari ewes

Parameters	Experimental treatments		SEM	P-value
	No AD_3E injection	AD_3E injection		
Body condition score				
Beginning of experiment	2.93	2.85	0.09	0.59
Day of CIDR removal	3.13	3.00	0.09	0.32
Day 30 after mating	3.9	3.62	0.13	0.15
Body weight				
Beginning of experiment	49.37	48.69	1.84	0.79
Day of CIDR removal	50.99	50.11	1.88	0.75
Day 30 after mating	53.92	53.62	2.83	0.94

SEM: standard error of the means.

Table 3 Effect of vitamin AD_3E injection around mating on plasma hormone concentrations of Afshari ewes

Parameters	Experimental treatments		SEM	P-value
	No AD_3E injection	AD_3E injection		
Progesterone (ng/mL)				
Beginning of experiment	2.61	2.31	0.18	0.26
Day of CIDR removal	9.00[a]	7.96[b]	0.36	0.05
Day of estrous and mating	5.81	5.47	0.44	0.59
Day 30 after mating	3.90	3.52	0.25	0.27
Estradiol (pg/mL)				
Beginning of experiment	60.18	57.66	1.18	0.15
Day of CIDR removal	73.32[a]	67.16[b]	1.83	0.03
Day of estrous and mating	52.60[b]	62.18[a]	2.09	0.01
Insulin (µIU/mL)				
Beginning of experiment	1.37	1.41	0.04	0.52
Day of CIDR removal	2.33	2.51	0.10	0.23
Day of estrous and mating	2.73	2.61	0.14	0.56
Testosterone (ng/mL)				
Beginning of experiment	0.28	0.27	0.12	0.59
Day of CIDR removal	0.46[b]	0.64[a]	0.03	0.01
Day of estrous and mating	0.78	0.71	0.02	0.09

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 4 Effect of vitamin AD_3E injection around mating on plasma mineral concentrations of Afshari ewes.

Parameters	Experimental treatments		SEM	P-value
	No AD_3E injection	AD_3E injection		
Calcium (mg/dL)				
Beginning of experiment	9.76	10.32	0.38	0.32
Day of CIDR removal	10.29[b]	12.29[a]	0.44	0.01
Day of estrous and mating	10.39[b]	12.99[a]	0.53	0.02
Magnesium (mg/dL)				
Beginning of experiment	1.72	1.85	0.14	0.53
Day of CIDR removal	2.34[b]	3.51[a]	0.17	0.01
Day of estrous and mating	2.92	2.99	0.24	0.83
Sodium (mEq/L)				
Beginning of experiment	139.81	139.65	3.71	0.97
Day of CIDR removal	171.56	170.82	3.65	0.89
Day of estrous and mating	173.92	163.96	4.19	0.11
Potassium (mEq/L)				
Beginning of experiment	3.21	3.31	0.05	0.15
Day of CIDR removal	3.60	3.76	0.11	0.30
Day of estrous and mating	3.91	3.68	0.17	0.34

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 vitamin AD_3E injection around mating on plasma metabolites concentrations of Afshari ewes

Parameters	Experimental treatments		SEM	P-value
	No AD_3E injection	AD_3E injection		
Total protein (g/dL)				
Beginning of experiment	4.10	3.58	0.20	0.09
Day of CIDR removal	9.05	8.99	0.33	0.90
Day of estrous and mating	9.05	8.27	0.37	0.15
Blood urea nitrogen (mg/dL)				
Beginning of experiment	15.18	15.03	0.37	0.78
Day of CIDR removal	17.37	17.74	0.48	0.59
Day of estrous and mating	17.17	19.02	0.74	0.09
Glucose (mg/dL)				
Beginning of experiment	85.92	84.99	0.79	0.41
Day of CIDR removal	93.72	95.30	0.94	0.25
Day of estrous and mating	100.98	100.23	2.31	0.82
Total cholesterol (mg/dL)				
Beginning of experiment	108.15	105.66	1.04	0.11
Day of CIDR removal	118.76	118.98	1.31	0.91
Day of estrous and mating	120.81	121.21	2.31	0.90

SEM: standard error of the means.

Table 6 Effect of vitamin AD_3E injection around mating on plasma vitamins, malondialdehyde and antioxidant enzyme concentrations of Afshari ewes

Parameters	Experimental treatments		SEM	P-value
	No AD_3E injection	AD_3E injection		
Vitamin A (µg/dL)				
Beginning of experiment	62.13	61.54	1.05	0.69
Day of CIDR removal	78.28	80.07	0.75	0.11
Day of estrous and mating	78.69	79.22	2.10	0.86
Vitamin D_3 (ng/dL)				
Beginning of experiment	51.79	51.07	1.58	0.67
Day of CIDR removal	68.37^{b}	72.48^{a}	0.87	0.02
Day of estrous and mating	68.56^{b}	72.04^{a}	1.03	0.03
Vitamin E (ng/dL)				
Beginning of experiment	53.75	54.09	0.86	0.78
Day of CIDR removal	55.56	56.12	1.05	0.71
Day of estrous and mating	69.05	70.92	1.66	0.43
MDA (nmol/L)				
Beginning of experiment	15.18	15.03	0.37	0.78
Day of CIDR removal	9.26^{a}	6.61^{b}	0.40	0.01
Day of estrous and mating	6.53	6.29	0.37	0.65
SOD (IU/mL)				
Beginning of experiment	36.37	35.74	0.47	0.35
Day of CIDR removal	36.89	38.12	0.62	0.18
Day of estrous and mating	39.63	41.50	0.72	0.08
GPX (IU/mL)				
Beginning of experiment	14.68	14.33	0.49	0.63
Day of CIDR removal	15.98^{b}	20.04^{a}	0.76	0.01
Day of estrous and mating	21.37^{b}	24.91^{a}	0.63	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 7 Effect of vitamin AD_3E injection around mating on reproductive parameters of Afshari ewes

Parameters	Experimental treatments		SEM	P-value
	No AD_3E injection	AD_3E injection		
Estrous response (%)	85.70	93.30	-	0.34
Fertility rate (%)	67.86	76.67	-	0.45
Lambing rate (%)	115.79	118.75	-	0.82
Twining rate (%)	15.79	18.75	-	0.82
Lamb birth weight (kg/lambed ewe)	4.64	4.31	0.27	0.39
Proportion of female lambs (%)	54.54	63.16	-	0.58
Proportion of male lambs (%)	45.46	36.84	-	0.58

SEM: standard error of the means.

Although the difference in reproductive performance was not statistically significant between treatment groups. Nevertheless, AD_3E injection numerically increased female lamb rate. The reproductive well-being and performance of farm animals largely depend on their nutritional status (Smith and Akinbamijo, 2000). Nutrition during mating is known to affect many aspects of the reproductive process in sheep (Martin *et al*. 2004; Scaramuzzi *et al*. 2006), largely through the impact on circulating concentrations of hormones and metabolites (Robinson *et al*. 2006). There is substantial evidence that the antioxidant minerals (Aliarabi *et al*. 2019; Van Emon *et al*. 2020; Studer *et al*. 2022) and vitamins (Nayyar and Jindal, 2010; Liu *et al*. 2014; Yasothai, 2014; Awawdeh *et al*. 2019) can influence many aspects of reproductive events of ruminant animals.

There is no enough information regarding the effect of concurrent vitamin A, D_3 and E injection during breeding season on BW and BCS of ruminant animals. Nevertheless, the lack of effect of vitamin AD_3E injection around mating on BW and BCS of ewes in the present study was in line with a previous study (Awawdeh *et al*. 2019), who reported no difference in BW change of Awassi ewes when they received intramuscular injections of vitamin E/Se on three occasions at the time of sponge insertion, sponge withdrawal, and 19 days after sponge removal. Similar BW and BCS in ewes received AD_3E in the current study may be in part related to the same nutrients level of their diets (Table 1). This may explain no difference in reproductive performance of ewes in the current study, as increasing BCS leading up to mating is associated with improved ovulation rate (Kenyon *et al*. 2014). Previous studies have reported direct impacts of ewe BCS on reproductive performance (Yılmaz *et al*. 2011), and survival and growth rates of lambs (Corner-Thomas *et al*. 2015).

In the current study, vitamin AD_3E injection resulted in higher plasma level of E_2 at the day of estrous and mating compared to the C group. These results confirmed previous reports regarding the importance of antioxidant vitamins on cell proliferation and steroidogenesis in goat ovarian granulosa cells (Yao *et al*. 2017), who reported that vitamin D_3 receptor (VDR) was prominently localized in granulosa cells (GCs), with expression increasing with follicle diameter. Addition of vitamin D_3 to GCs caused an increase in VDR and in steroidogenic acute regulator (StAR) and 3b-hydroxysteroid dehydrogenase (3b-HSD) mRNA expression. Additionally, vitamin D_3 increased the cyclic adenosine monophosphate (cAMP) and E2 level. Meanwhile, vitamin D_3 enhances the E_2 output of GCs by regulating the expression of 3b-HSD and StAR and the level of cAMP, which regulate steroidogenesis, supporting a potential role for vitamin D_3 in follicular development. As shown in Table 6, ewes received vitamin AD_3E in the current study significantly had higher plasma vitamin D_3 level, which may, in part, be a reason for increased E_2 level in this animals. In other hand, injection of vitamin AD_3E in the current study resulted to higher plasma Ca level (Table 4), which may be another factor influencing E_2 secretion by ovary. Calcium ions positively involved in steroidogenesis by ovary cells (Van der Kraak, 1991). Higher plasma concentration of testosterone at the day of CIDR removal in ewes received AD_3E was in line to Al-Asadi *et al*. (2020). Testosterone has an active role in several biological events of female reproduction including health of reproductive organs, vaginal lubrication and arousal, preventing vaginal atrophy and offspring sex ratio (Ketterson *et al*. 2005; Navara, 2018).

Increased plasma Ca level in ewes received AD_3E can be attributed to higher plasma vitamin D_3 level (Table 6) in these animals. Active transcellular transport of Ca is a predominant route for Ca absorption in mature ruminants fed typical diets. This process is controlled by active form of vitamin D_3. The active vitamin D_3 circulating in the blood enters the enterocytes and binds to vitamin D receptors, initiating transcription and translation of several proteins necessary for active transport of Ca (Goff, 2018). Additionally, higher plasma Mg level (Table 4) in ewes received AD_3E may be another factor influencing plasma Ca level in these animals. Nutrients usually act in a coordinated manner in the body. Intestinal absorption and subsequent metabolism of a particular nutrient, to a certain extent, is dependent on the availability of other nutrients. Mg and vitamin D are two essential nutrients that are necessary for the physiologic functions of various organs. Mg in the activation of vitamin D, which helps regulate Ca homeostasis. All of the enzymes that metabolize vitamin D seem to require Mg, which acts as a cofactor in the enzymatic reactions in the liver and kidneys. In addition, Mg is required for the binding of vitamin D to its transporter protein and the expression of vitamin D receptors for cellular effects. On the other hand, vitamin D can affect the state of Mg in the body. In this way, activated vitamin D in turn can increase the intestinal absorption of Mg (Uwitonze and Razzaque, 2018; Shahsavani *et al*. 2021).

The lack of effect of vitamin AD_3E injection around mating on plasma metabolites including glucose, total protein, total cholesterol and UN in the current study may be related to the same nutrients level of their diets (Table 1) and probably similar ruminal fermentation. These results were in contrast to Al-Asadi *et al*. (2020), who reported higher plasma total protein in Arabi rams revived vitamin AD_3E. Additionally, Araripe Sucupira *et al*. (2019) did not observe any difference in plasma glucose level ewes received vitamin AD_3E in prepartum. Similar plasma glucose level in ewes of both experimental treatments may be attributed to

the same insulin level in their plasma (Table 3). Insulin is the first hormone that comes to mind when thinking about glucose regulation (Sliwowska *et al*. 2014; Lucy, 2016).

Similar to results of the current study, vitamin E injection around mating had no significant effect on plasma vitamin E level in goats following estrous synchronization using intravaginal sponges (Sönmez *et al*. 2009). Vitamin E is also present in the pre-ovulatory follicle. The plasma vitamin E concentration may affect its follicular concentration, and it was found to be significantly higher in the follicular fluid than in the plasma. Therefore, the increase in vitamin E concentration causes to reduce level of ROS in the follicular fluid (Agarwal *et al*. 2005). Vitamin E is important for oocyte quality and maturation, implantation and fetal growth in female reproduction. It protects denuded oocytes from degeneration and facilitates meiotic maturation of cumulus-free oocytes (Tao *et al*. 2004). Therefore, the vitamin E treatment may increase fertilization rate of oocytes. In addition, vitamin E decreases the portion of follicles undergoing follicular atresia by counteracting potential generation of oxidative stress in oocytes. Nevertheless, in this study, the vitamin AD_3E injection around mating had no effect on twinning rate and the mean number of lambs born per ewe.

It was observed that vitamin AD_3E injection resulted in higher plasma GPX and SOD activity and lower MDA concentration. These results were in contrast to Araripe Sucupira *et al*. (2019), who reported no effect of vitamin AD_3E on antioxidant enzyme (SOD) and MDA level in ewes during transition period. Furthermore, vitamin E injection around mating had no effect on plasma MDA level in goats (Sönmez *et al*. 2009). Although, Yao *et al*. (2017) reported that vitamin D_3 significantly decreased reactive oxygen species (ROS) production and increased mRNA and protein expression of SOD and catalase in goat GCs cultured *in vitro*. They concluded that VDR is expressed in GCs of the goat ovaries and vitamin D_3 might play an important role in GCs proliferation by regulating cellular oxidative stress and cell cycle related genes. Free radicals are normally involved in reproductive events such as follicular development, ovulation, corpus luteum development, luteolysis and early embryo development (Rizzo *et al*. 2012; Almansa-Ordonez *et al*. 2020). However, an imbalance between free radicals and antioxidants can cause failure to conceive. The ROS are part of the mechanism controlling ovulation; failure to achieve enough intra-follicular concentrations to produce preovulatory follicle rupture causes follicular cyst in cattle (Talukder *et al*. 2014). Oocyte and embryo well-being can be compromised by oxidative damage generated as the result of common reproductive practices around the periconceptional period (Almansa-Ordonez *et al*. 2020). Furthermore, estrus synchronizations using progesterone releasing devices is common in small ruminants. Several studies have reported inflammatory responses and changes in normal flora and vaginal histology of ewes after using estrus synchronization devices (Suárez *et al*. 2006; Sonmez *et al*. 2009; Manes *et al*. 2015). Therefore, the evidence suggests that antioxidant supplementation may be beneficial, by improving fertility during the periconceptional period. The presence of antioxidants such as catalase, GPX, SOD, ascorbic acid, vitamin E, and β-carotene has been demonstrated in ruminant follicle compartments (Combelles *et al*. 2010; Gupta *et al*. 2011; Hennet *et al*. 2013; Hozyen *et al*. 2014). Antioxidants prevent oxidative damage by preventing ROS concentrations from reaching levels that could harm the follicle and oocyte (Liu *et al*. 2014). However, situations leading to increased ROS production or to a reduction in antioxidant concentrations are likely to affect fertility by causing oxidative damage (Almansa-Ordonez *et al*. 2020).

In the current experiment, we did not observe any significant effect of vitamin AD_3E injection on estrus response, fertility, lambing rate and twining rate. These result were in line to previous researches (Maldonado *et al*. 2017), who reported no effect of antioxidant vitamins (E and C) on reproductive outcomes in ruminant animals. Furthermore, vitamin E injection around mating had no effect on estrous response, conception rate and kidding rate, whereas, increased twinning rate and prolificacy rate in goats (Sonmez *et al*. 2009). The positive effects of antioxidant vitamins on reproductive events including growth of follicle (Yao *et al*. 2017; Xu *et al*. 2018) oocyte (Ikeda *et al*. 2005; Clagett-Dame and Knutson, 2011) and placenta (Ganguly *et al*. 2018), trophoblast function and implantation (Ganguly *et al*. 2018) and embryonic development (Clagett-Dame and Knutson, 2011) has been reported previously.

Vitamin AD_3E injection resulted in a numerically higher (not significant) proportion of female lambs in the current study compared to the C group. Several factors including BCS of mother and her plasma levels of metabolites such as Ca, Mg, sodium, potassium and glucose around mating can affect offspring sex ratio in mammals (Navara, 2018). As shown in Table 4, ewes received AD_3E had higher plasma Ca and Mg level, which was in line to result of Gharibi *et al*. (2023), who reported higher proportion of female lambs in ewes with higher plasma Ca and Mg concentrations. It has been found that feeding mineral nutrients to animals may especially modify the secretion of the exocrine glands in the oviduct. This modification may provide an environment that likely favors one of two sperm (X or Y) to live or race to fertilize the ovum in the oviduct. Additionally, the change in nutrients might modify or change in the composition of the sperm receptors on the surface of the oocytes (zona pellucida) that may have a role in the sex preselection

concerning the association with the X or Y sperm (Navara, 2018). This finding confirmed that the sex ratio of lambs may be affected primarily by events at or before conception rather than events post-conception.

CONCLUSION

The results of the present study showed that injection of vitamin AD_3E around mating increased plasma levels of some hormones (E_2 and testosterone), minerals (Ca and Mg), vitamins (D_3) and antioxidant enzymes (GPX) and decreased plasma MDA level in Afshari ewes. These changes were not associated with improvement in reproductive outcomes including estrous response, fertility rate, twining rate and lambing rate. Nevertheless, ewes received AD_3E around mating had numerically higher female lambs rate. Further researches are required to investigate the effect of antioxidant vitamins on ewe reproductive performance.

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