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Effect of Calcium and Magnesium Supplementation of Ewes during Pre- and Post-Mating on Lamb Sex Ratio

Research Article

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ABSTRACT

The aim of this study was to investigate the effect of calcium (Ca) and magnesium (Mg) injection around mating on skewing lamb sex ratio in ewes. Thirty cycling multiparous Kurdish × Romanov ewes were divided into two groups (15 per group) based on age and body weight (BW). Animals were received either 20 mL of solution (CaMg) or 20 mL of sterile water (Control) subcutaneously every 7 days from 28 days pre- to 7 days post-mating. Each mL of CaMg solution contained 400 mg of Ca borogluconate and 22 mg of Mg hypophosphate, which provided 30.4 mg of Ca and 2.04 mg of Mg. Ewes in CaMg group tended to had lower body condition score (BCD) at the day of controlled internal drug release (CIDR) removal (2.79 *vs.* 2.69; P=0.09) and its change from beginning of experiment to CIDR removal (0.21 *vs.* 0.04; P=0.06) than control group. The CaMg injection increased plasma insulin (13.2 *vs.* 11.33 µIU/mL), Ca (9.13 *vs.* 8.30 mg/dL), Mg (3.66 *vs.* 3.33 mg/dL), Na (188.83 *vs.* 175.73 mEq/L) and glucose (56.94 *vs.* 50.07) concentrations at the day of CIDR removal and Ca (8.87 *vs.* 8.03 mg/dL) at the day of estrous and mating compared to control group (P<0.05). Lower estradiol (E_2) + testosterone to progesterone (P_4) ratio at the day of estrous and mating (102.87 *vs.* 135.67) was observed in ewes received CaMg injection compared to other group (P<0.05). Injection of CaMg solution had no significant effect on lamb sex ratio (P>0.05), although proportion of female lambs (68.18 *vs.* 56.52%) was numerically higher in ewes of the CaMg group than control group. These results indicated that there was a potential for skewing the sex ratio of lambs toward females when CaMg solution was injected subcutaneously during mating.

KEY WORDS hormones, metabolites, minerals, offspring gender, sheep.

INTRODUCTION

Sheep enterprises would benefit from the opportunity to skew the sex ratio of offspring towards their preferred gender (Clayton *et al.* 2016). For instance, faster growth rates are reported in male compared with female lambs (Tatum *et al.* 1998), thereby male lambs reach a higher market weight over a set time period. However, prime lamb enterprises require breeding females, which may lead to a higher sale price of first-cross ewes at weaning (Clayton *et al.* 2016). In mammals, offspring sex is strictly determined by genetic input from the parents. Sex allocation would occur either through a mechanism within one or both parents that influences the genetic makeup of the offspring prior to fertilization or through mechanisms that trigger sex-biased mortality either during gestation or beyond (Rosenfeld and Roberts, 2004). For these species, the term primary sex ratio adjustment is used to describe sex allocation processes that occur prior to fertilization and result in biases in the initial production of males and females. Though, secondary sex ratio adjustment is used to describe sex allocation that occurs after fertilization and results in biased survival of

males and females. This could occur either during gestation or, in cases where parents care for offspring, during the period of parental care (Navara, 2018). There are many factors influencing offspring sex ratio in mammals including parental age, maternal body condition, the season of the mother's birth, maternal emotional stress, previous year offspring sex, the season of offspring birth, litter size, coat color, population history, changing the pH of the female reproductive tract, and time of mating before or after ovulation (Rosenfeld and Roberts, 2004; Grant and Chamley, 2010; Abecia *et al.* 2017; Navara, 2018). Moreover, maternal nutrition around mating is one of the most important factors affecting offspring sex ratio in many mammalian species (Rosenfeld and Roberts, 2004). Reports suggested that diets varied in nutrient composition such as total energy intake (Mathews *et al.* 2008), fatty acid type (Clayton *et al.* 2016; Mirzaei Alamouti *et al.* 2018), the intake of glucose (Kimura *et al.* 2005), and blood mineral concentration such as sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg), (Arangasamy *et al.* 2015; Alhimaidi *et al.* 2021) could skew the offspring sex ratio in mammals.

Several investigators considered the hypothesis that the mineral intake could skew the sex ratio of mammalian offspring. Both Chandraju *et al.* (2012) and Oun *et al.* (2016) have studied mothers who fed a pre-conception diet specific in its amounts of Ca, Mg, Na and K and most of them were successful in delivering an offspring of the desired sex. In these experiment, mothers received more Ca and Mg during mating had more female offspring and *vice versa.* It has been shown to results that higher concentrations of K and Na and lower concentrations of Mg and Ca in diet of sows (Bolet *et al.* 1981), dairy cattle (Stolkowski and Lorrain, 1982), rats (Vahidi and Sheikhha, 2007), mice (Alfageeh and Alhimaidi, 2013), chicken (Saleh and Iriyanti, 2011) and sheep (Alhimaidi *et al.* 2021) increased the sex ratio of males to females.

Minerals are required in small amounts. Although, the variation in dry matter intake (DMI) can be high, making precise and accurate measurements of minerals intake difficult (Bicalho *et al.* 2014). Although, NRC (2007) set requirements of minerals by diet for sheep, while limitations such as variation in feed intake, interactions between dietary minerals in diet and stress may reduce the amount of nutrient absorption and their bioavailability. For example, adverse effects of excess dietary Ca on absorption of other divalent elements such as zinc, copper and manganese has been reported (Goff, 2018). On the other hand, inclusion of minerals in the diet may not ensure proper intake or availability (Machado *et al.* 2013). Minerals in drinking water can also have a negative effect on mineral absorption from the digestive tract (Spears, 2003). Many water supplies contain high concentrations of Na and Ca, which have adverse effects on absorption of other minerals including zinc and copper (Kononoff *et al.* 2017). Therefore, administration of minerals via injection could potentially provide an alternative way of dietary mineral intake. To our knowledge, the influence of an injectable solution containing Ca Mg around mating of ewe on lamb sex ratio has not previously been examined. We hypothesized that injection of a solution containing Ca Mg around mating would skew offspring sex ratio toward female in ewe. Therefore, the objective of the present study was to evaluate the effect of subcutaneous injection of CaMg administration on Kurdish × Romanov ewes during mating.

MATERIALS AND METHODS

The experimental and management protocols of this study was approved by the Animal Care and Use Committee (Iranian Council of Animal Care, 1995). The authors confirm that they have followed these standards for the protection of animals used in this experiment.

Experiment design and animal management

This study was conducted in a commercial farm (Ilam, Iran) during the breeding season period (May to Jun 2021). Thirty Kurdish × Romanov ewes were divided and randomly allocated into two groups (15 per group) based on age and body weight (BW). One month before the beginning of experiment, all ewes were vaccinated. Animals in treatment groups were injected 20 mL of Ca Mg solution (CaMg; Calcimaphor 40, Makidam, Tehran, Iran), while animals in control group were injected with 20 mL of sterile water subcutaneously every 7 days from 28 days pre- to 7 days post-mating. Each mL of Ca Mg solution contained 400 mg of Ca borogluconate and 22 mg of Mg hypophosphate, which provided 30.4 mg of Ca and 2.04 mg of Mg. All animals were fed the same flushing diet (Table 1) twice daily (09:00 and 18:00) as total mixed ration (TMR) from 28 days before to 21 days after mating. Flushing diet was formulated according to NRC (NRC, 2007). Animals had free access to water. At the end of feeding flushing diet (d 21 after mating), all ewes managed as a single group and kept in similar management and feeding condition throughout pregnancy and lambing.

Estrous synchronization program and pregnancy diagnosis

Sixteen days after the beginning of feeding the flushing diet and injection of Ca and Mg solution, the estrous cycle of all ewes were synchronized using CIDRs (Eazi-Breed CIDR® Sheep and Goat Inseet, New Zealand) intra-vaginally for 12 days, followed by 350 IU intramuscular injection of eCG (Gonaser 5000 IU, Hipra, Spain) immediately after CIDR removal. Animals also received an intramuscular injection

of 5 mL of a solution contained 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 contained 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 mated with Romanov rams for 3 consecutive days (1 ram per 5 ewes). Pregnancy diagnosis was performed 30 days post-mating using plasma P_4 assay by an ELISA reader (STAT-FAX 3200, USA) and a commercial kit (Monobind Inc. USA, ELISA kit).

Table 1 Ingredients and chemical composition of flushing diet

Item	Amount
Ingredients (% of DM)	
Alfalfa hay	33.80
Wheat straw	29.60
Barley grain	14.80
Corn grain	5.70
Soybean meal	3.00
Wheat bran	7.20
Calcium salts of n-3 fatty acids[1]	2.60
Fish meal	2.60
Minerals and vitamins[2]	0.30
Calcium carbonate	0.20
Sodium chloride	0.20
Chemical composition[3]	
CP (% of DM)	14.30
NDF (% of DM)	44.50
NFC (% of DM)	30.00
Ash (% of DM)	8.40
EE (% of DM)	5.30
ME (MJ/kg of DM)	9.49
Na (% of DM)	0.27
K (% of DM)	2.43
Ca (% of DM)	1.25
Mg (% of DM)	0.82

[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.
ME: metabolisable energy; CP: crude protein; NDF: neutral detergent fibre; NFC: non fibre carbohydrate; EE: ether extract; Na: sodium; K: potassium; Ca: calcium and Mg: Magnesium.
[3] NFC: [100 - (CP+CF+EE+Ash)].

Data recording, sample collection and analysis

Body condition score (BCS) of all ewes was recorded at one day before the beginning of Ca Mg injection and introduction to flushing diet (day 0), on day of CIDR removal and 30 days post- mating. Samples of flushing diet was collected weekly, dried at 55 ˚C in a forced air oven for 72 h, composited per diet at the end of the experiment and ground to pass through a 1-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Chemical composition of the flushing diet was measured for crude protein, ether extract, ash (AOAC, 2005) and neutral detergent fiber (Van Soest *et al.* 1991).

Non-fibrous carbohydrate (NFC) was calculated based on National Research Council recommendation (NRC, 2007). For blood metabolic assays, samples were collected via jugular vein and put into a 10-mL evacuated tube containing sodium heparin a day before the beginning of experiment, on day of CIDR removal and at day of estrous and mating. Blood samples were immediately placed on ice and after centrifugation (3000 rpm with rotor radius of 10 cm at 4 ˚C for 15 min), plasma was harvested and stored at -20 ˚C until the analysis.

Total cholesterol, total protein, urea nitrogen and glucose concentrations were determined using a commercial analyzer (BT1500, Biotecnica, SRL, Italy) according to the manufacturer's protocol. Plasma insulin, P_4, estradiol (E_2) and testosterone concentrations were measured using an ELISA reader (STAT-FAX 3200, USA) and a commercial kit (Monobind Inc. USA, ELISA kit). The inter- and intra-assay coefficients of variation were 5.4% and 2.5%, 6.3 and 2.2, 6.6 and 2.1, and 5.8 and 2.7 for insulin, P_4, E_2 and testosterone, respectively. Flame Atomic Absorption Spectrometer (Analytik Jena AG-novAA® 400p, Germany) was used for determination of Na, K, Ca and Mg contents in flushing diet and plasma. Ewes were observed during the lambing time and all lambs born were ear-tagged and their dam identified. Total number, sex and birth weight of lambs were recorded. The reproductive performance in terms of estrous response (number of ewes showing estrus/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) and female lamb rate (number of female lamb/total number of lambs×100) were recorded (Mirzaei Alamouti *et al.* 2018).

Statistical analyses

Data for body condition score (BCS), hormones and blood metabolites were analyzed using the MIXED procedure of SAS (2003). Pre-feeding plasma concentrations of total cholesterol, glucose, Na and Na + K: Ca + Mg ratio were used as co-variates. The differences were compared using Tukey test. The reproductive parameters (%) were analyzed using the chi-square test. Data on the number of lambs born per ewe and birth weight were analyzed using the MIXED procedure.

Effects of ewe body weight and age were added as co-variate 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.

RESULTS AND DISCUSSION

The BCS at the day of CIDR removal (2.69 *vs*. 2.79; P=0.09) and its change from the beginning of experiment to day of CIDR removal (0.04 *vs*. 0.21; P=0.06) tended to be lower in ewes of CaMg group than other group (Table 2). However, BCS at day 30 after mating (2.91 *vs*. 2.79 for control and CaMg groups, respectively) and its change from mating to day 30 after mating (0.12 *vs*. 0.10 for control and CaMg groups, respectively) were similar between experimental treatments (P>0.05).

Plasma concentrations of P_4, E_2 and testosterone at the day of CIDR removal (1.72 *vs*. 1.98; 68.33 *vs*. 70.58 and 0.44 *vs*. 0.54, respectively) and day of estrous and mating (0.57 *vs*. 0.83; 77.15 *vs*. 85.22 and 0.18 *vs*. 0.16, respectively) were similar for ewes in control and CaMg groups (P>0.05; Table 3). Whereas, ewes in treatment group had higher (P<0.05) plasma concentrations of insulin (13.20 *vs*. 11.33 µIU/mL) at the day of CIDR removal than those in control group (Table 3). Injection ewes had greater plasma Ca (9.13 *vs*. 8.30 mg/dL), Mg (3.66 *vs*. 3.33 mg/dL) and Na (188.83 *vs*. 175.83 mEq/L) concentrations at the day of CIDR removal and Ca (8.87 *vs*. 8.03) at the day of estrous and mating compared to the control ewes (P<0.05). Whereas, plasma concentrations of K at the day of CIDR removal (4.02 *vs*. 4.14 mEq/L, respectively) and day of estrous and mating (4.17 *vs*. 4.22 mEq/L, respectively) were similar for Ca Mg and control groups (P>0.05). Plasma concentrations of total protein, total cholesterol and urea nitrogen (Table 5) at the day of CIDR removal (6.24 *vs*. 5.78; 41.48 *vs*. 38.44 and 14.50 *vs*. 15.00, respectively) and day of estrous and mating (6.57 *vs*. 6.55; 43.57 *vs*. 43.78 and 15.25 *vs*. 17.00, respectively) were similar between ewes of CaMg group and control group (P>0.05). Though, CaMg injection elevated plasma glucose (56.94 *vs*. 50.07 mg/dL) concentration at the day of CIDR removal compared to the control ewes (Table 5).

Estrous response (100 *vs*. 100% for control and CaMg groups, respectively), fertility rate (100 *vs*. 93.33% for control and CaMg groups, respectively), lambing rate (164.29 *vs*. 153.33% for control and CaMg groups, respectively) and twining rate (53.33 *vs*. 53.33% for control and CaMg groups, respectively) were similar between two groups (P>0.05). Injection of CaMg had no effect on lamb birth weight (6.69 *vs*. 7.00% for control and CaMg groups, respectively) and sex ratio (P>0.05). However, the proportion of female lambs (68.18 *vs*. 56.52% for control and CaMg groups, respectively) was numerically, but not significant, higher in ewes of CaMg group compared to the control group (Table 6). Reproductive well-being and performance of farm animals is largely dependent on their nutritional status (Smith and Akinbamijo, 2000).

Nutrition around mating is known to affect many aspects of the reproductive process of ewes (Martin *et al*. 2004; Scaramuzzi *et al*. 2006), largely through impacts on circulating concentrations of hormones and metabolites (Robinson *et al*. 2006). Minerals are involved in such a functions as intracellular detoxification of free radicals, synthesis of reproductive steroids and other hormones, carbohydrate, protein and nucleic acid metabolism. Thus, mineral deficiencies and/or excesses may impair fertility, embryonic development and survival and offspring development and survival (Smith and Akinbamijo, 2000). In the current study, CaMg injection resulted in a numerically higher (not significant) proportion of female lambs compared to control group. These results confirmed previous reports of the importance of dietary minerals affect around mating the sex ratio of offspring in different mammalian species (Chandraju *et al*. 2012; Arangasamy *et al*. 2015; Oun *et al*. 2016; Alhimaidi *et al*. 2021), who reported higher proportion of female offspring in mothers received more Ca and Mg compared to Na and K. This finding confirmed that the sex ratio of lambs may be affected primarily by events at or before conception rather than events post-conception.

The exact mechanisms linking nutrients with offspring sex ratio in mammals is not well determined. However, modulation of the ability of either the X or Y sperm to reach or penetrate the oocyte, chemical composition and pH of vaginal secretions, eicosanoids synthesis and plasma hormone and metabolite concentrations (Navara, 2018) may be involved.

In mammals, one of the most widely cited hypotheses in sex ratio biology is Trivers - Willard hypothesis (Trivers and Willard, 1973). They suggested a link between maternal condition and offspring sex ratios, such that mothers in good condition produce more male offspring. The BCS of ewe is 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 proportion of muscle and fat relative to skeletal size (Kenyon *et al*. 2014). As mentioned previously, BCS of ewes at the day of CIDR removal and its change from the beginning of experiment to day of CIDR removal tended to be lower in ewes of CaMg group than the control group, which may be attributed to numerically higher female lambs produce by these animals. This results were in line with Cameron and Linklater (2007) and Marei *et al*. (2018), who reported higher female offspring in feral horses (80 *vs*. 20%) and Holstein cows (58 *vs*. 15%) with better condition than those with poorer condition around mating.

Table 2 Effect of CaMg administration during mating on body condition score (BCS) and its changes in Kurdish × Romanov ewes

Parameters	Experimental treatment		SEM	P-value
	No CaMg injection	CaMg injection		
Body condition score				
Beginning of experiment	2.58	2.65	0.06	0.45
Day of CIDR removal	2.79	2.69	0.04	0.09
Day 30 after mating	2.91	2.79	0.05	0.12
Body condition score change				
Beginning of experiment to day of CIDR removal	0.21	0.04	0.04	0.06
Day of CIDR removal to day 30 after mating	0.12	0.10	0.03	0.72

CIDR: controlled internal drug release.
SEM: standard error of the means.

Table 3 Effect of CaMg administration during mating on plasma hormones concentration in Kurdish × Romanov ewes

Parameters	Experimental treatment		SEM	P-value
	No CaMg injection	CaMg injection		
Progesterone (ng/mL)				
Beginning of experiment	2.02	1.81	0.11	0.16
Day of CIDR removal	1.72	1.98	0.17	0.30
Day of estrous and mating	0.57	0.83	0.11	0.14
Day 30 after mating	3.90	3.52	0.25	0.27
Estradiol (pg/mL)				
Beginning of experiment	65.91	69.30	3.60	0.52
Day of CIDR removal	68.33	70.58	3.65	0.67
Day of estrous and mating	77.15	85.22	3.64	0.14
Insulin (µIU/mL)				
Beginning of experiment	12.07	13.01	0.31	0.06
Day of CIDR removal	11.33^{b}	13.20^{a}	0.37	0.02
Day of estrous and mating	12.24	13.44	0.56	0.18
Testosterone (ng/mL)				
Beginning of experiment	0.254	0.252	0.01	0.92
Day of CIDR removal	0.44	0.54	0.04	0.11
Day of estrous and mating	0.18	0.16	0.01	0.32

CIDR: controlled internal drug release.
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 CaMg administration during mating on plasma mineral concentration in Kurdish × Romanov ewes

Parameters	Experimental treatment		SEM	P-value
	No CaMg injection	CaMg injection		
Calcium (mg/dL)				
Beginning of experiment	8.15	8.35	0.15	0.04
Day of CIDR removal	8.30^{b}	9.13^{a}	0.26	0.05
Day of estrous and mating	8.03^{b}	8.87^{a}	0.27	0.05
Magnesium (mg/dL)				
Beginning of experiment	3.29	3.03	0.12	0.17
Day of CIDR removal	3.33^{b}	3.66^{a}	0.10	0.05
Day of estrous and mating	3.16	3.10	0.15	0.78
Sodium (mEq/L)				
Beginning of experiment	171.01^{b}	187.70^{a}	3.23	< 0.01
Day of CIDR removal	175.73^{b}	188.83^{a}	3.30	0.03
Day of estrous and mating	176.99	189.22	4.21	0.10
Potassium (mEq/L)				
Beginning of experiment	4.20	4.03	0.09	0.22
Day of CIDR removal	4.02	4.14	0.10	0.41
Day of estrous and mating	4.17	4.22	0.06	0.59

CIDR: controlled internal drug release.
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 Plasma metabolites concentration of Kurdish × Romanov ewes injected with CaMg solution during mating

Parameters	Experimental treatment		SEM	P-value
	No CaMg injection	CaMa injection		
Total protein (g/dL)				
Beginning of experiment	5.31	5.96	0.21	0.06
Day of CIDR removal	5.78	6.24	0.19	0.14
Day of estrous and mating	6.55	6.57	0.13	0.91
Blood urea nitrogen (mg/dL)				
Beginning of experiment	13.86	13.62	0.62	0.79
Day of CIDR removal	15.00	14.50	0.65	0.60
Day of estrous and mating	17.00	15.25	0.93	0.20
Glucose (mg/dL)				
Beginning of experiment	51.99[b]	62.01[a]	1.62	< 0.01
Day of CIDR removal	50.07[b]	56.94[a]	1.82	0.05
Day of estrous and mating	60.38	60.16	1.72	0.94
Total cholesterol (mg/dL)				
Beginning of experiment	39.71[b]	49.37[a]	1.62	< 0.01
Day of CIDR removal	38.44	41.48	1.40	0.22
Day of estrous and mating	43.78	43.57	1.55	0.94

CIDR: controlled internal drug release.
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 Reproductive parameters of Kurdish × Romanov ewes injected with CaMg during mating

Parameters	Experimental treatment		SEM	P-value
	No CaMg injection	CaMg injection		
Estrous response (%)	100.00	100.00	-	1.00
Fertility rate (%)	100.00	93.33	-	0.75
Lambing rate (%)	164.29	153.33	-	0.62
Twining rate (%)	53.33	53.33	-	1.00
Lamb birth weight (kg/lambed ewe)	6.69	7.00	0.48	0.65
Proportion of female lambs (%)	56.52	68.18	-	0.42
Proportion of male lambs (%)	43.48	31.82	-	0.42

SEM: standard error of the means.

In contrast to the results of the present study, however, Clayton *et al*. (2016) reported higher female lambs proportion (53.2 *vs*. 42.5% for high and low BCS, respectively) in ewes. Alhimaidi *et al*. (2021) did not observe any relationship between ewe body condition during mating and lamb sex ratio. The mechanism of Ca mediating adiposity might be due to its intracellular level and its impact on lipogenesis and lipolysis in adipocytes. Consequently, low Ca intake results in lower serum ionized Ca, stimulating PTH secretion, which promote Ca influx into adipocytes. This increased intracellular Ca in adipocytes stimulates lipogenesis and suppresses lipolysis resulting in conservation of fat. High Ca intake (resulting in higher serum ionized Ca) on the other hand, suppresses PTH response, stimulating lipolysis (Ilich *et al*. 2019). Furthermore, Mg enhance the body fat-reducing effects of calcium (Seki *et al*. 2013). This results strongly indicates that sex ratios respond to changing BCS surrounding the female, rather than an inherent trait carried by the female, herself. For this reason, studies testing for sex ratio adjustments in response to factors that are known to influence maternal BCS are important pieces of the sex ratio puzzle.

Reproductive hormones play a role in the process of sex ratio adjustment and these hormones act in both males and females to exert their effects. It was hypothesized that high concentrations of both testosterone and E_2 in both male and female parents result in the production of more male offspring, while high concentrations of P_4 have the opposite effect (James, 2008). In the current study, plasma E_2 + testosterone to P_4 ratio was lower at the day of estrous and mating in ewes received CaMg compared to the control group, which may be associated with numerically higher proportion of female lambs in these animals. A higher proportion of male offspring was observed when Holstein dairy cows were treated with estradiol just prior to insemination (Emadi *et al*. 2014) and when *in vitro* fertilization of mouse gametes occurred in an environment containing oestradiol (Zhang *et al*. 2006), which were in line with the results of the current study. Hormones produced by mother influence offspring during development. It is clear that there is a pathway by which steroid hormones can act to communicate information about the environment to the reproductive organs in a way that influences gamete and/or offspring growth and development.

Given such a pathway, it is logical to hypothesize that these steroid hormones may also mediate changes in sex ratios in response to the same types of environmental and social conditions (Navara, 2018). The exact mechanism linking estradiol with skewing offspring sex ratio is not well determined, however, improved binding and fertilization ability of Y-bearing sperm may be involved (Navara, 2018). Emadi *et al*. (2014) also suggest that estradiol might alter the timing between ovulation and fertilization, a factor that has been shown to influence sex ratios in cows (Martinez *et al*. 2004).

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 (Alfageeh and Alhimaidi, 2013; Navara, 2018). Although intra-uterine parameters were not measured in the current study, the numerical higher proportion of female lambs in ewes received CaMg compared with those in the C group may be attributed to higher plasma CaMg concentrations at the day of CIDR removal and estrous and mating in these animals. This result was in contrast to Alhimaidi *et al.* (2021), who reported no effect of feeding ewe a diet containing more Ca and Mg during mating on blood Ca, Mg, Na and K concentrations compared to those fed a diet with high Na and K. The balance between Na and K versus Ca and Mg could change the receptors of the oocyte wall to favor the attraction of either a male or female sperm. When there is a high Na and K intake, and a low Ca and Mg in the female's diet, the oocyte wall will change to attract the Y sperm. While, more Ca and Mg in the blood and a low Na and K will attract the X sperm (Vahidi and Sheikhha, 2007).

Another factor that could influence the sex ratio of lambs is the effect of minerals on circulating concentrations of glucose. In the current study, the numerical higher proportion of female lambs when ewes received a solution containing CaMg was concurrent with higher plasma glucose concentrations at the day of CIDR removal and estrous and mating. The results of the present study were in contrast to previous researches in field voles (Helle *et al.* 2008) and human (Cameron *et al.* 2008), who reported higher male offspring production by mothers with higher plasma glucose concentration. Male and female blastocysts have different glucose requirements (Navara, 2018). Given the potential of glucose to be a potent regulator of offspring sex ratios, it makes sense to consider the effects of the hormones that regulate glucose concentrations in circulation. Insulin is the first hormone that comes to mind when thinking about glucose regulation, and mammalian ovarian follicles are sensitive to insulin (Louhio *et al.* 2000). Insulin receptors are present and active in mammalian ovaries (Willis and Franks, 1995). Thus, insulin could act to coordinate information about food availability with modulation of offspring sex ratios at the level of the ovary. In the current study, increased plasma glucose and insulin concentration at the day of CIDR removal were concurrent with numerically higher female lambs in ewes received Ca and Mg. Therefore, future studies could monitor plasma glucose and insulin concentrations over a shorter time period in order to clarify potential effects on blastocyst survival.

CONCLUSION

In conclusion, injection of a solution containing Ca and Mg around mating had no effect on plasma progesterone, estradiol, testosterone, total protein, BUN and total cholesterol concentrations of Kurdish×Romanov ewes. Although, plasma insulin, Ca, Mg and glucose concentrations increased in ewes received Ca and Mg solution. The mechanism for sex ratio skewing in mammals remains unclear. Though, the results of the current study suggest that increasing the concentration of plasma Ca and Mg around mating could provide a means of controlling sex ratio of offspring born in ewe.

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