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Original article

Real-time assessment of the superovulatory effect of FSH and eCG with laparoscopy at different seasons in Akkaraman ewes

U. Şen

Ondokuz Mayis University, Faculty of Agriculture,
Department of Agricultural Biotechnology, 55139, Samsun, Turkey.

Abstract

Conventional methods for determining the reproductive performance of sheep bred either after estrus synchronization during the breeding season or after induction of estrus/ovulation during the non-breeding season take a long time and may give misleading results due to the effect of environmental factors. Laparoscopic observations allow real-time monitoring of ovarian activity around estrus or ovulation. This study was aimed at assessing the superovulatory effects of follicle-stimulating hormone (FSH) and equine chorionic gonadotropin (eCG) treatments by laparoscopy during breeding (September-November, n=12) and non-breeding (April-June, n=12) seasons in Akkaraman sheep. In both seasons, after CIDR withdrawal, the ewes were injected either with 600 IU eCG or 300 µl (20 mg/ml) FSH twice at 12 hour intervals. Plasma P_4, E_2 and LH concentrations were determined at the time of intra-vaginal CIDR insertion (day 0) and then at its withdrawal (day 12), followed by 3 and 6 days of eCG or FSH injections. After 3 (first observation) and 6 (second observation) days of hormone injections, laparoscopy was performed to record ovarian activity in both seasons. The eCG increased ($p<0.05$) the numbers of large follicles (first observation) and CL (first and second observations) in the breeding season compared to FSH treatment. CL, small-moderate and large follicle numbers of eCG treated ewes were higher ($p<0.05$) than those of FSH at both observations in the non-breeding season. In the breeding season, eCG treated ewes had higher ($p<0.05$) plasma P_4 (3 and 6 days after hormones injections) and E_2 (3 days after hormones injections) concentrations than those of FSH. In conclusion, the results of the present study indicate that treatment with eCG during the non-breeding season can support ovarian activity, and thus increase ovulation rate and plasma hormone concentrations around induced estrus/ovulation in Akkaraman ewes.

Key words: superovulation, season, laparoscopy, plasma hormones, eCG, FSH

Correspondence to: U. Şen, e-mail: ugur.sen@omu.edu.tr, tel.: +90 362 3121919, fax: +90 362 4576034

Introduction

Estrous cycle or ovarian activity of ewes can be controlled using exogenous hormone treatments (Quintero-Elisea et al. 2011, Santos et al. 2011) to improve reproductive efficiency. In particular, such treatments have been commonly used to induce an out-of-breeding season for lambing programs aimed at meeting consumer demand for lamb meat (Kohno et al. 2005, Ozyurtlu et al. 2008, Santos et al. 2011). In the commercial protocols eCG and FSH are the most frequently used hormones due to their effects on ovarian activity (Boscos et al. 2002, Titi et al. 2010, Neto et al. 2012). The main mechanisms of eCG and FSH hormones are explained on the basis of FSH receptors present in the small and medium sized follicles and their involvement in the stimulation of follicle development (Cahoreau et al. 2015). Previous studies have shown that the administration of eCG or FSH only, without progesterone treatment, has little influence on estrus stimulation (Ware et al. 1986). Therefore, eCG or FSH hormone treatments in ewes are generally given after intra-vaginal progesterone treatment for approximately 12 days (Boscos et al. 2002).

Ovarian activity in terms of follicular dynamics and plasma concentration of reproductive hormones is the greatest indicator of reproductive performance in sheep (Hafez et al. 2016). Therefore, the success of estrus synchronization programs is related to their impact on plasma concentrations of reproductive hormones, the follicular development waves, the number of ovulatory follicles and the number of formed corpora lutea (CL). However, traditionally, the reproductive performance of the animals in such programs is determined by conventional parameters (Sen and Onder 2016) such as percentage of animals in estrus and mated, pregnancy rate and infertility rate, birth related yields (such as lambing rate), fecundity and/or litter size (Sen and Sirin 2017).

Conventional methods for determining the reproductive performance following estrus synchronization take a long time and may not give true results due to the effect of environmental factors such as temperature, maintenance, feeding etc. Also, the superovulatory effect of eCG and FSH, which are most common used hormones in estrus synchronization, can be affected by many factors such as breed, body weight, body condition score, age, parity, nutritional level and season (Quinlivan and Robinson 1969). All of these factors lead to the need for real-time identification of the effects of estrus synchronization treatments on ovarian activity and reproductive performance. The aim of the present study was, therefore, to determine the effect of eCG and FSH administration on ovarian activity and plasma progesterone (P_4), estrogen (E_2) and luteinizing hormone (LH) concentrations during the breeding and non-breeding seasons in Akkaraman ewes.

Materials and Methods

The experimental procedures were approved by the Local Animal Care and Ethics Committee of Ahi Evran University, Kirsehir, Turkey, ensuring compliance with directive 86/609/EEC for animal experiments. The study was carried out on adult Akkaraman sheep both during the breeding (September, n=12) and non-breeding (April, n=12) seasons at a private farm in Kirsehir, Turkey (38°55′56.8″N, 34°10′45.6″E and 985 m above sea level). The ewes were of similar age (ranging from 3 to 4 years) and body weight (51.3±1.5 Kg). One week prior to the estrus synchronization in both seasons, one ml of $PGF_{2\alpha}$ (Dinolytic; 5 mg $PGF_{2\alpha}$/ml, Pharmacia, Belgium) was injected intramuscularly to lutalyse the CL on the ovaries of all the ewes. The animals were then treated with an intravaginal CIDR (Controlled Internal Drug Releasing, Inter Ag, Hamilton, New Zealand) device containing 30 g natural P_4 for 12 days in both seasons. Following withdrawal of the CIDR the ewes were allocated randomly to two treatment groups balanced for body weight in both seasons. Ewes in the first group were injected with 600 IU eCG (Folligon, Intervet, Boxmeer, Holland) and ewes in the second group were injected twice with 300 µl (20 mg/ml) FSH (Folltropin-V; Bioniche Animal Health, Belleville, Ontario, Canada) at 12 hour intervals. In both seasons, blood samples were taken from the jugular vena of the ewes on CIDR application (day 0), at the time of CIDR withdrawal (day 12), and then 3 (day 15) and 6 days (day 18) after eCG and FSH injections. Blood samples were collected in sodium ethylenediaminetetraacetic acid (sodium heparin) containing vacutainer tubes and the plasma was separated following centrifugation at 2500 × g for 10 min at 4°C and then stored at −20°C until analyzed for the hormones. In all the samples plasma concentrations of P_4 (EU0398), E_2 (ESH0035) and LH (ESH0034) were determined in duplicate by ELISA (enzyme linked immunosorbent assay) using commercial kits (Wuhan Fine Biological Technology Co., Ltd., Hubei, China).

Ovaries of all the animals in both the groups were examined by laparascopy 3 (day 15; first observation) and 6 (day 18; second observation) days after eCG and FSH injections as described by Alfaris et al. (2012) and Souza-Fabjan et al. (2014) to record the number of follicles and corpora lutea (CL) . The ewes were deprived of feed and water for 24 h prior to the laparoscopic procedure. Anesthesia was carried out using an anesthetic cocktail containing 2 ml of Ketasol

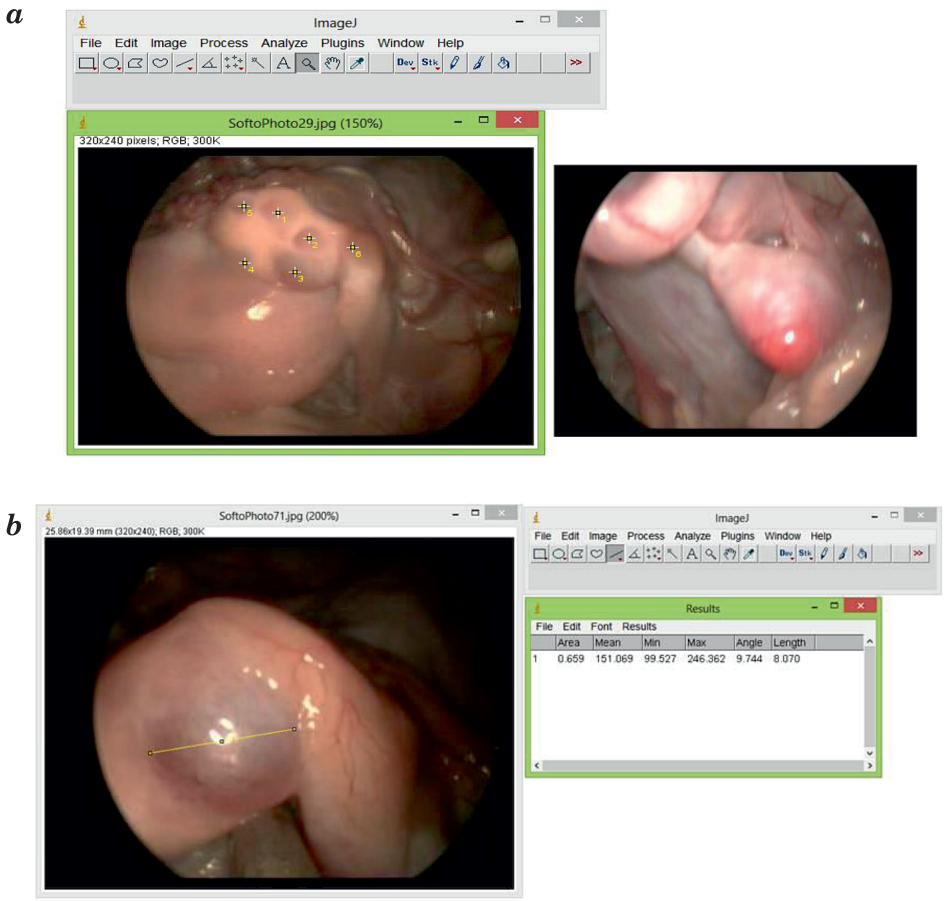

Fig 1. Picture showing CL, follicle numbers (a) and follicle diameter (b) on the ovaries as recorded during the laparascopic examination.

(Richter Pharma, Wels, Austria) and 0.04 ml of Romphun (Bayer Turk, Istanbul, Turkey). The ewes were placed in laparoscopic cradles and a 5-mm laparoscope (Karl Storz Endoscopes GmbH & Co., Tuttlingen, Germany) attached to a video system was inserted into the abdominal cavity through a canula (cranial to the udder and to the left side of the midline). An atraumatic grasper was inserted to hold the ovary, making it possible to find and count follicles and CL into the right side of the abdomen through a second canula. Following laparoscopic observations, the trocars/canulas wounds were treated with a topical antibiotic spray (Tiamphenicol + Cetrimide + Gention Viole, Piyedif Aerosol, Cavadif). Images of the ovaries of the all ewes in both the treatment groups were recorded with laparoscopy linked to an image capture system. The number and diameter of the follicles and number of CL were determined using the Image J 1.19Z free software image analysis program (Fig. 1). Follicles were classified according to their diameters into two groups; small--moderate (SM; 2-8 mm) and large (L; >8 mm).

To analyze the data, the Mann-Whitney U-test and one-way ANOVA for ovarian activity traits (number and diameter of the follicles and number of CL), and repeated measurement analysis for blood traits (P_4, E_2 and LH) were carried out according to the structure of the data using the SPSS 17.0 package program (SPSS, Chicago, IL, USA).

Results

Follicle and corpus luteum numbers determined after three (first observation) and six (second observation) days of FSH (n=6) or eCG (n=6) administration to Akkaraman ewes during the breeding season are presented in Table 1. There were no significant differences between FSH or eCG administration after three days in terms of small-moderate and total follicle numbers on both ovaries, but eCG administration increased the number of large follicles compared to FSH administration ($p<0.05$). Additionally, total CL number on both the ovaries in eCG treated ewes was higher ($p<0.05$) than those in FSH treated ewes. FSH and eCG treated ewes had a similar number of small-moderate, large and total follicles on both ovaries following six days of hormone administration. However, eCG administration increased ($p<0.05$) the number of CL on both the ovaries compared to FSH administration.

Follicles and corpus luteum numbers determined after three (first observation) and six (second observa-

Table 1. Follicles and corpus luteum numbers determined after three (first observation) and six (second observation) days of FSH (n=6) or eCG (n=6) administration to Akkaraman ewes during breeding season.

Treatments		Follicles diameter						TFN	CLN		
		2 - 8 mm			8 mm <						
		Ovarium			Ovarium				Ovarium		
		Right	Left	Total	Right	Left	Total		Right	Left	Total
FO	FSH	4.3 ±0.9	4.0±0.8	8.3±1.7	0.5±0.3^{b}	0.3±0.3^{b}	0.8±0.5^{b}	9.0±1.1	0.0±0.0	0.0±0.0	0.0±0.0^{b}
	eCG	3.8±0.5	3.8±0.6	7.3±0.7	1.3±0.3^{a}	1.5±0.3^{a}	2.8±0.7^{a}	10.3±2.1	0.5±0.1	0.3±0.1	0.8±0.3^{a}
SO	FSH	4.5±0.7	3.5±0.3	8.0±0.9	1.8±0.3	1.0±0.4	2.8±0.6	8.2±2.3	0.5±0.2	0.5±0.2	0.7±0.2^{b}
	eCG	4.0±0.4	4.3±0.5	8.3±0.8	1.3±0.6	1.3±0.3	2.5±0.9	9.3±2.4	1.0±0.3	0.8±0.2	1.5±0.3^{a}

$^{a-b}$ Different letters in the same column indicate significant difference ($p<0.05$).
FO = first observation, SO = second observation, FSH = follicle-stimulating hormone, eCG = equine chorionic gonadotropin, TFN = total follicle number, CLN = total numbers of corpora lutea.

Table 2. Follicles and corpus luteum numbers determined after three (first observation) and six (second observation) days of FSH (n=6) or eCG (n=6) administration to Akkaraman ewes during non-breeding season.

Treatments		Follicles diameter						TFN	CLN		
		2 - 8 mm			8 mm <						
		Ovarium			Ovarium				Ovarium		
	Right	Left	Total	Right	Left	Total			Right	Left	Total
FO	FSH	3.0±0.3	2.8±0.4	5.8±0.5^{b}	0.2±0.1^{b}	0.2±0.1	0.4±0.2^{b}	6.2±0.6^{b}	0.0±0.0	0.0±0.0	0.0±0.0
	eCG	4.4±0.8	4.0±0.6	8.4±0.5^{a}	0.8±0.2^{a}	0.6±0.3	1.4±0.4^{a}	9.8±0.7^{a}	0.2±0.1	0.0±0.0	0.2±0.1
SO	FSH	3.5±0.3	3.2±0.2	6.7±0.2^{b}	0.6±0.3^{b}	0.2±0.1^{b}	0.8±0.4^{b}	7.5±0.5^{b}	0.2±0.1	0.4±0.2	0.6±0.2^{b}
	eCG	4.5±0.5	4.6±0.5	9.1±0.5^{b}	1.6±0.4^{a}	1.4±0.3^{a}	3.0±0.9^{b}	12.1±1.4^{b}	0.6±0.3	0.6±0.3	1.2±0.4^{a}

$^{a-b}$ Different letters in the same column indicate significant difference ($p<0.05$).
FO = first observation, SO = second observation, FSH = follicle-stimulating hormone, eCG = equine chorionic gonadotropin, TFN = total follicle number, CLN = total numbers of corpora lutea.

tion) days of FSH (n=6) or eCG (n=6) administration to Akkaraman ewes during the non-breeding season are presented in Table 2. After 3 days of hormone administration a significant increase in small-moderate, large and total number of follicles was recorded in eCG treated ewes compared to FSH treated ewes ($p<0.05$). However, the total number of CL did not differ between FSH or eCG treated ewes after 3 days of hormone administration. FSH treated ewes had a lower ($p<0.05$) number of small-moderate, large and total follicles compared to eCG treated ewes after six days of hormone administration. Similarly, the total number of CL number in FSH treated ewes was lower ($p<0.05$) than those in eCG treated ewes.

The effect of eCG and FSH administration on the numbers of small-moderate (2-8 mm), large (>8 mm), total follicles and CL on both ovaries of Akkaraman ewes in first (a) and second (b) laparoscopic observations in the breeding and non-breeding seasons is shown in Figure 2. Following three days of hormone administration, eCG treated ewes had similar small-moderate follicle numbers during breeding and non-breeding seasons, but FSH treated ewes had a higher ($p<0.05$) number of small-moderate follicles during the non-breeding season compared to the breeding season. Both eCG and FSH administration increased ($p<0.05$) the number of large follicles at the first laparoscopic observation during the breeding season compared to the non-breeding season. There were no significant differences between eCG treated ewes in terms of total number of follicles at the first laparoscopic observation during the breeding and non-breeding seasons. However, the total number of follicles in FSH treated ewes was higher ($p<0.05$) during the breeding season than the non-breeding season. FSH treated ewes had a similar CL number at the first laparoscopic observation during breeding and non-breeding seasons, but eCG treated ewes had a higher ($p<0.05$) CL number during the breeding season compared to the non-breeding season. FSH or eCG administration did not change small-moderate and large follicle numbers and CL numbers at the time of the second laparoscopic observation during the breeding and non-breeding seasons. Similarly, eCG administration did not change the num-

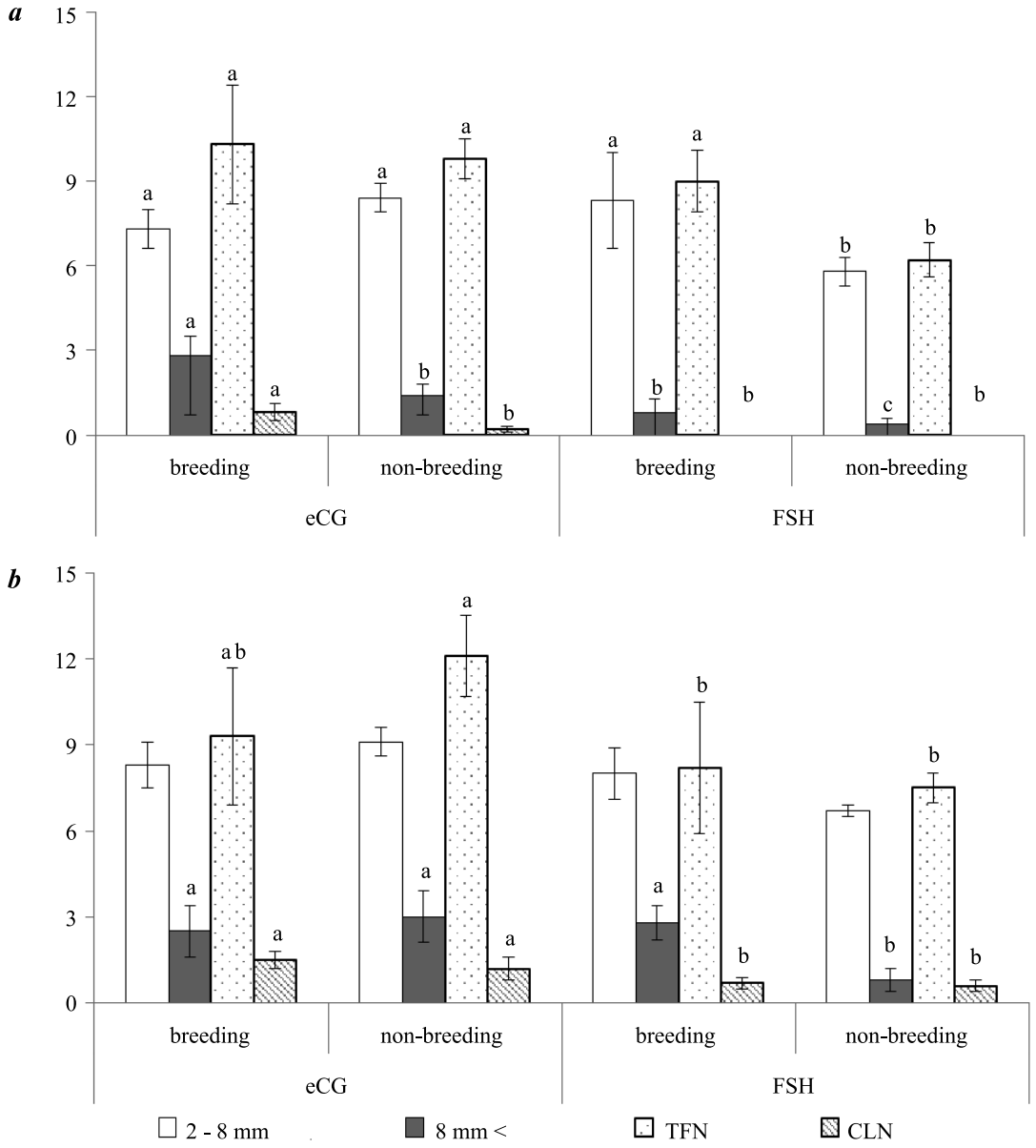

Fig 2. Effect of follicle-stimulating hormone (FSH) and equine chorionic gonadotropin (eCG) administration on the numbers of small-moderate (2-8 mm), large (>8 mm), total follicles and CL on both ovaries of Akkaraman ewes in first (a) and second (b) laparoscopic observations in the breeding and non-breeding seasons. [a-b] Different letters in the same color bars indicate significant difference ($p<0.05$).

ber of large follicles at the second laparoscopic observation during the breeding and non-breeding seasons, but FSH administration increased ($p<0.05$) the number of large follicles on both ovaries during the breeding season compared to the non-breeding season.

The effect of eCG and FSH administration on plasma P_4, E_2 and LH levels in Akkaraman sheep at various days relative to CIDR insertion during the breeding (a) and non-breeding (b) seasons are shown in Figure 3. In the present study, eCG administered sheep had higher ($p<0.05$) plasma P_4 concentrations than those of FSH on day 3 (15 days) and day 6 (18 days) after hormone administration in the breeding season. In contrast to these results, there were no significant differences in terms of plasma P_4 concentration between treatment groups during the non-breeding season. The eCG administration increased ($p<0.05$) the plasma E_2 concentrations compared to FSH on day 3 after hormone administration in the breeding season, but there were no significant differences in terms of plasma E_2 concentrations between treatment groups in the non- breeding season. In the present study, eCG administration tended to increase ($p=0.095$) plasma LH concentrations compared to FSH on day 3 after hormone administration in the breeding season. However, plasma LH concentrations were similar between eCG and FSH administered sheep in the non-breeding season.

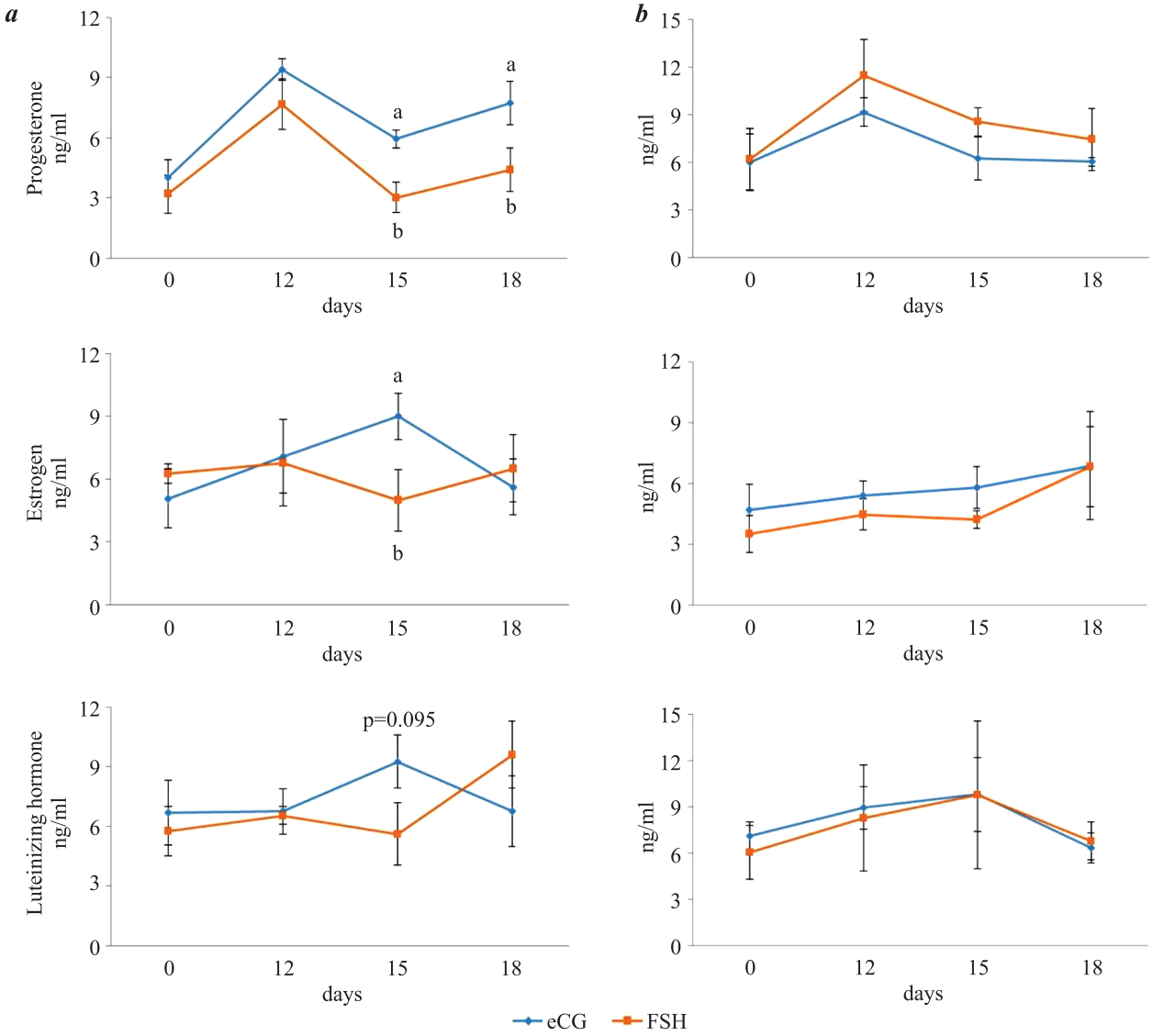

Fig 3. Effect of follicle-stimulating hormone (FSH) and equine chorionic gonadotropin (eCG) administration on plasma Progesterone (P_4), Estrogen (E_2) and Luteinizing hormone (LH) levels in Akkaraman sheep at various days relative to CIDR insertion during the breeding (a) and non-breeding (b) seasons. [a, b] $p<0.05$.

Discussion

In the present study, to our knowledge, the effect of FSH and eCG treatments on ovarian follicular dynamics of Akkaraman ewes at different stages of the estrus cycle (beginning of follicular activity; first laparoscopic observation and the end of follicular activity; second laparoscopic observation) during the breeding and non-breeding seasons were observed for the first time by laparoscopy. Our results indicate that FSH and eCG treatments had a similar effect on small-moderate follicle development, but eCG treatment increased the development of large follicles from both ovarian follicle pools when compared to FSH treatment at the first laparoscopic observation in the breeding season. Similarly to the results of our study, Driancourt and Fry (1992) reported that eCG application shows an earlier effect on follicular development in the ovary than that of FSH application in sheep. Additionally, Moakhar et al. (2010) found that eCG treatment after CIDR application increased the number of large follicles on the ovaries on the basis of ultrasound scanning. However, ultrasound follicular dynamics assessments of Neto et al. (2012) showed that eCG and FSH treatments had similar effect on the largest and second follicle size during the early follicular development phase in Santa Inês breed sheep. These differences may be explained by the dose of hormones used or genetic differences between sheep breeds.

The differences between the FSH and eCG treatments in terms of large follicle development were gone, but a higher number of CL was observed in ewes with eCG treatment at the second laparoscopic observation. The number of CL showed that the ovulation rates

of ewes treated with eCG were higher than those of FSH treated ewes. Similarly, O'Hara et al. (2016) have observed that administration of eCG resulted in increased luteal tissue area compared with controls in the ovaries of cows in which follicular growth was monitored by daily ultrasound examinations. The main reason for the absence of differences between hormonal treatments in terms of follicular features during the end of follicular activity (the second laparoscopic observation) may have been due to the later effect of FSH on ovarian activity compared to eCG (Driancourt and Fry 1992). Previous studies reported that eCG has the capacity to induce both FSH and LH hormone activity, which are synthesized and secreted by the gonadotropic cells of the anterior pituitary gland (Stenbak et al. 2003, Murphy 2012). Moreover, eCG causes early activation of oocyte into follicle (Moor et al. 1985) and follicles exposed to eCG synthesize larger amounts of P_4 (Driancourt and Fry 1992). The stimulated secretion of FSH in a dose-dependent manner does not influence secretion of LH (Kile and Nett 1994). Therefore, the eCG hormone treatment may have acted similarly to both the FSH and luteinizing hormone (LH) resulting in the increased number of follicle ovulation and CL formation in the present study. Rawling et al. (1977) reported that the plasma LH concentration and release frequency were lower in sheep towards the end of the breeding season and in the early anestrus period than in the breeding season. Previous studies reported that early development of follicles with eCG stimulation increases secretion of follicular E_2, which may have an earlier positive feedback effect on the hypothalamus (Hafez et al. 2016) in terms of preovulatory LH surge. The results of the present study support the conclusion of Driancourt and Fry (1992), who reported that the eCG hormone has an earlier and greater effect on the follicular development and activity of the ovary relative to the FSH hormone. Additionally, FSH administration may not have been sufficiently effective on ovulation of the graafian follicles on the ovary and only may have resulted in fewer CL due to an insufficient or delayed LH surge. These observations are consistent with the findings of previous studies (Driancourt and Fry 1992, Moakhar et al. 2010, Neto et al. 2012), who reported that eCG treatment increased the number of ovulations or CL on ovaries in sheep.

Gonadotropins are routinely used in progesterone-based induction/synchronization of estrus protocols to stimulate ovarian activity and ovulation in small ruminants during the non-breeding season (Wildeus 2000). eCG has a much greater effect on ovarian activity than FSH due to its considerably longer biological half-life, approximately 20 h for eCG, compared with 2 h or less for FSH (Akbar et al. 1974, Mclntosh et al. 1975). In the present study, laparoscopic observations showed that FSH and eCG exhibited different effects on ovarian activity between breeding and non-breeding seasons. Unlike the breeding season, during the non-breeding season eCG treatment increased the total number of small-moderate and large follicles on the ovaries when compared to FSH treatment at the first laparoscopic observation. Similar results were obtained on the second laparoscopic observation during the non-breeding season. The eCG treated ewes had higher small-moderate and large follicle numbers than in the FSH treated ewes on the second laparoscopic observation.

The eCG shows high LH- and FSH-like activities and has a high affinity for both FSH and LH receptors in the ovaries (De Rensis and López-Gatius 2014). On the granulosa and thecal cells of the follicle, eCG has long-lasting LH- and FSH-like effects that stimulate E_2 and P_4 secretion (De Rensis and López-Gatius 2014). Driancourt and Fry (1992) reported that eCG administration influences ovarian activity by recruiting small follicles, causing up to a threefold increase in follicular growth rate, and altering the size distribution of the largest follicles at estrus but not by reversing atresia. In addition, eCG increases ovulation rate and stimulates formation of CL because of its protein structure which is identical to that of LH (Bousfield et al. 2001). In the present study, a higher rate of development of CL was observed in eCG treated ewes than FSH treated ewes during the non-breeding season at the second laparoscopic observation. Our results clearly demonstrate a better effect of eCG administration following CIDR removal in terms of ovulation and CL formation compared to administration of FSH during the non-breeding season.

Determination of plasma P_4 hormone levels in blood is an important indication for monitoring and controlling ovarian activity (Kawu et al. 2007). In the present study, eCG administration increased plasma P_4 levels compared to FSH administration following 3 and 6 days after hormone administration in the breeding season. However, plasma P_4 concentrations between treatment groups were similar at various days during the non-breeding season. The results of the present study are consistent with the results of Horoz et al. (1997) on the Kıvırcık sheep breed. It is thought that eCG administration in the breeding season may result in an increase in plasma P_4 concentration, by affecting the hypothalamus region for promoting gonadotropin releasing hormone (GnRH) production, resulting in early CL formation and increasing the amount of P_4 naturally produced from the ovary.

The high levels of plasma E_2 hormone in the follicular phase of the estrus cycle is the most obvious indi-

cation of effectiveness of estrus (Çam et al. 2004). Although eCG administration increased plasma E_2 levels compared to FSH administration 3 days after hormone administration in the breeding season, plasma E_2 levels between the treatment groups were similar on various days during the non-breeding season in the present study. Previous studies have shown that an increase in the number of follicles during the follicular phase increases the amount of plasma E_2 and leads to earlier behavioral estrus (Hafez et al. 2016). Results of the present study indicate that eCG treatment increases the plasma E_2 concentrations in the period when potential aggressive behaviors may be exhibited because of FSH treatment in breeding season in Akkaraman sheep.

In conclusion, laparoscopic observations have shown that eCG treatment following CIDR removal in the non-breeding season may increase ovarian activity, resulting in improved ovulation rate and development of CL in Akkaraman ewes. Moreover, eCG administration had a better effect on plasma P_4, E_2 and LH concentrations in the breeding season compared to the non-breeding season. The results of this study also suggest that the use of eCG after CIDR insertion and its removal after 12 days in the out-of-breeding season protocols may improve reproductive performance and increase more multiple lamb births.

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