TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2020) 44: 1-8

doi:10.3906/vet-1909-74

Structural and functional changes in corpus luteum of single- and/or double-ovulated pregnant and nonpregnant ewes during the spring and autumn seasons

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Received: 24.09.2019 • **Accepted/Published Online:** 14.12.2019 • **Final Version:** 10.02.2020

Abstract: This study was conducted to investigate the effect of ovulation numbers on the progesterone and luteal volume in nonpregnant and pregnant Lori-Bakhtiari ewes. In the spring season 48 reproductive tracts of nonpregnant ewes (treatments included single ovulation, unilateral double ovulations, and bilateral double ovulations) and in the autumn season 144 reproductive tracts of nonpregnant and pregnant ewes (single ovulation, unilateral double ovulations, bilateral double ovulations, single ovulation with 1 foetus, single ovulation with 2 foetuses, unilateral double ovulations with 1 foetus, unilateral double ovulations with 2 foetuses, bilateral double ovulations with 1 foetus, bilateral double ovulations with 2 foetuses) were collected. The results indicated that the mean volume of the individual corpus luteum (CL) and the total volume of CL in the autumn season were significantly higher than the spring season. However, there were no significant differences in the progesterone concentration between unilateral and bilateral double ovulations. The results also indicated that in ewes with double ovulations and 1 foetus, the total volume of CL and the progesterone concentration were higher than those of the ewes with single ovulation and 1 or 2 foetuses. It was concluded that increasing the incidence of twinning rate does not appear to be a prerequisite for the presence of 2 CLs in the ewes.

Key words: Double ovulation, progesterone, spring and autumn seasons, Lori-Bakhtiari ewes

1. Introduction

Changes in the volume and function of the corpus luteum (CL) secreting progesterone, which is responsible for the continuation of pregnancy in many species, are related to pregnancy with multiple foetuses [1,2]. The adequate secretion of progesterone has a significant effect on the achievement of a successful pregnancy and oestrous cycle regulation in females [3]. The vast majority of domesticated breeds of sheep show 1 or 2 ovulations in a single oestrus cycle [4]. Evidence shows that the highest ovulation rate occurs in the late summer and early autumn, the beginning of the natural breeding season, and decreases gradually in the late autumn and winter, which are out of breeding season for ewes [5]. Double ovulations are more common in sheep breeds and can lead to the desirable occurrence of twinning. It was found that the numbers of large follicles in ewes were 2–3 times more than the ovulation rate [6]. Although twinning rate is positively correlated with the rate of double ovulations, the incidence of double ovulations is more common than the twinning rate. Ovulation ratios are affected by many environmental and physiological factors. For sheep, the influence of these factors may differ within and between breeds of sheep, so breed can be a major factor that determines the ovulation rate [7,8].

Several studies have reported that there is a relationship between the incidence of single and double ovulations and the blood hormones in cows [9,10], ewes [11,12], and mares [13]. It is expected that a larger CL is able to secrete more progesterone. However, results can sometimes be inconsistent. For example, Lopez et al. [14] found that dairy cows with double ovulations had increased luteal volume but decreased concentration of progesterone compared with cows with a single ovulation. In contrast, Voelz et al. [10] reported that cows with 1 or 2 CLs had similar plasma concentrations of progesterone. On the other hand, cows with 2 CLs had similar luteal volumes than cows with 1 CL. Mann et al. [9] demonstrated that double ovulation had no impact on the blood progesterone concentration in nonlactating cows. Luteal volume can be used as an indicator of luteal function and the concentration of plasma progesterone [15]. Karami-Shabankareh et al.

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[12] showed a significant positive correlation between the serum progesterone concentration and the total luteal volume in Sanjabi ewes ($r = 0.715$, $P < 0.01$). Another study reported a positive correlation between the luteal volume and the concentration of progesterone ($r = 0.39$) [10].

Based on the current knowledge, there are limited reports about the comparison of the effect of the ovulation numbers on the progesterone levels, luteal volume, and especially the twinning rate in pregnant ewes during the autumn season. Therefore, the initial objective of this study was to investigate the influence of single and double ovulations on the CL volume, the blood progesterone concentration, and the number of foetuses in pregnant ewes, which was not previously defined. The second objective was to determine whether the serum progesterone concentration actually affects the ovulation rate and the number of foetuses in ewes or not.

2. Materials and methods

2.1. Site of the study and breed description

The experiment was performed in the Kohgiluyeh and Boyerahmad province of Iran (30°41′N, 51°33′E). The Lori-Bakhtiari ewe breed is one of the most numerous and widespread sheep breeds mainly in the southwestern parts of Iran (the Zagros Mountains), in the Kohgiluyeh and Boyerahmad, Isfahan, and Khuzestan provinces, and particularly in the Chaharmahal and Bakhtiari province [16]. The physical characteristics and the productive performances of Lori-Bakhtiari ewes include an adult live weight of 68–77 kg, height of 70–75 cm, fat-tail weight of 8–10 kg, milk yield of 85–100 L per 6 months, 1.17 lambs per year, and fleece weight of 2.5–3 kg per year [17]. Average body weight of rams is 95–125 kg. Since the breeding season for ewes is between the late summer and the late autumn, births normally begin in early spring. The ewes usually conceive a single offspring.

2.2. Experiment 1

This experiment was conducted in Kohgiluyeh and Boyerahmad province from May to June (spring season). On different days, the reproductive tracts of 48 Lori-Bakhtiari ewes (3–4 years old) were collected from the Yasouj abattoir and were transported to the laboratory (Department of Animal Science of the Faculty of Agriculture at Yasouj University of Iran) within 2 h, and were washed with 0.9% NaCl containing 2 million IU of procaine penicillin G and 2 g of dihydrostreptomycin. The CL numbers of each animal were then categorized as single and double ovulations. Treatments in this experiment were ($n = 16$ ewes per group): (1) single ovulation (none pregnant), (2) unilateral double ovulations (none pregnant), and (3) bilateral double ovulations (none pregnant). In this experiment we tried to choose similar reproductive tracts as indicated in Figure 1.

2.3. Experiment 2

A total of 144 reproductive tracts from Lori-Bakhtiari ewes, which were 3–4 years old, were used in the trial.

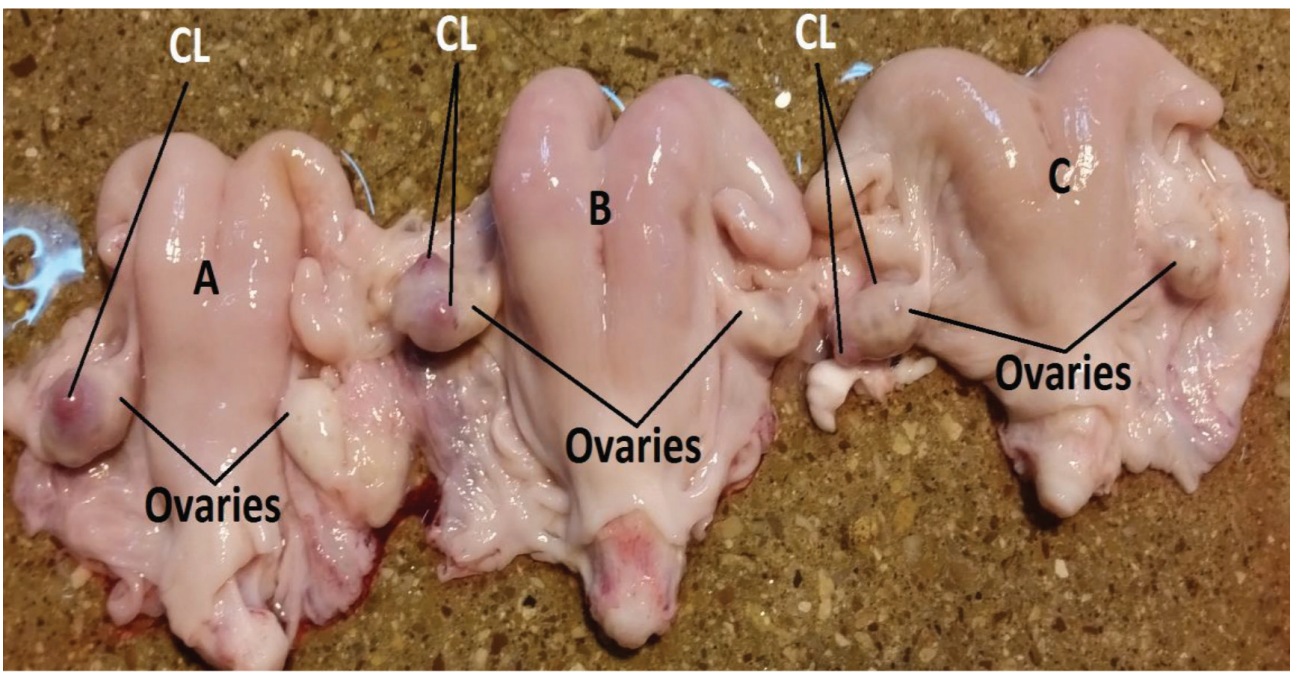

Figure 1. Reproductive tracts of Lori-Bakhtiari ewes used in experiment 1 (spring season). A = Single ovulation; B and C = Unilateral double ovulations, CL = Corpus luteum.

The experiment was carried out from September to December (the autumn season). Ovaries were removed immediately after slaughtering and were transported to the laboratory within 2 h. In this experiment it was tried to select reproductive tracts with CLs similar to those in experiment 1 from nonpregnant ewes (as shown in Figure 2). In pregnant ewes it was also tried to choose reproductive tracts with similar ages of embryos (as shown in Figure 3). Treatments in this experiment were (n = 16 ewes per group): (1) single ovulation (none pregnant), (2) unilateral double ovulations (none pregnant), (3) bilateral double ovulations (none pregnant), (4) single ovulation with 1 foetus (pregnant), (5) single ovulation with 2 foetuses (pregnant), (6) unilateral double ovulations with 1 foetus (pregnant), (7) unilateral double ovulations with 2 foetuses (pregnant), (8) bilateral double ovulations with 1 foetus (pregnant), and (9) bilateral double ovulations with 2 foetuses (pregnant). Then, in nonpregnant and pregnant ewes, the number of CLs on the surface of the ovaries of each animal was categorized as single and double ovulations, and the respective numbers were recorded. Visible foetuses inside the uterus horns were classified into single and twin-bearing categories, and the respective numbers were also recorded. The volumes of all luteal structures (both experiments) were determined by using a formula described previously [18].

2.4. Blood sampling and progesterone analysis

In both experiments, before slaughtering, approximately 10 mL of blood samples were collected by means of jugular venepuncture from all ewes. The tube samples were placed into ice immediately after collection and then were transported to the laboratory. The samples were centrifuged at 2500 rpm for 15 min, within 2 h after the collection, and the serum was harvested and stored at –20 °C until the progesterone concentration analysis. A commercial ELISA kit (ELA-4825-300, Monobind, Inc., Lake Forest, CA, USA) was used to measure the progesterone levels in the blood sera. The suitability of the assay was determined by a new standard curve for serum progesterone. The sensitivity and intra- and interassay coefficient variations of the kit were 0.105 ng/mL and 4.46% and 9.39%, respectively. Total progesterone concentration of each animal was calculated on each slaughter day and the correlation between volume of CLs (P4/CL vol.) and progesterone per experiment was tested.

2.5. Statistical analysis

The ovaries of each animal were categorized as having 1 or 2 CLs and the progesterone concentrations of single- and double-ovulated ewes were evaluated on each day of the slaughtering. Luteal characteristics and the mean value of the serum progesterone levels in single and double ovulation cases were analysed by analysis of variance in SAS software (SAS Institute, Cary, NC, USA). The correlation between the progesterone concentration and the total volume of CL in nonpregnant ewes during the out of breeding season and in nonpregnant and pregnant ewes during breeding season was separately analysed by the Pearson correlation test. The differences between 2 means (for volume of CL of 1 and 2) were analysed by Student's t-test and the differences among more than 2 means were compared by using Duncan's multiple range test. Data were presented as mean ± SD.

3. Results

Although the individual CL volume in single-ovulated ewes was significantly larger ($P < 0.05$) than in double-ovulated

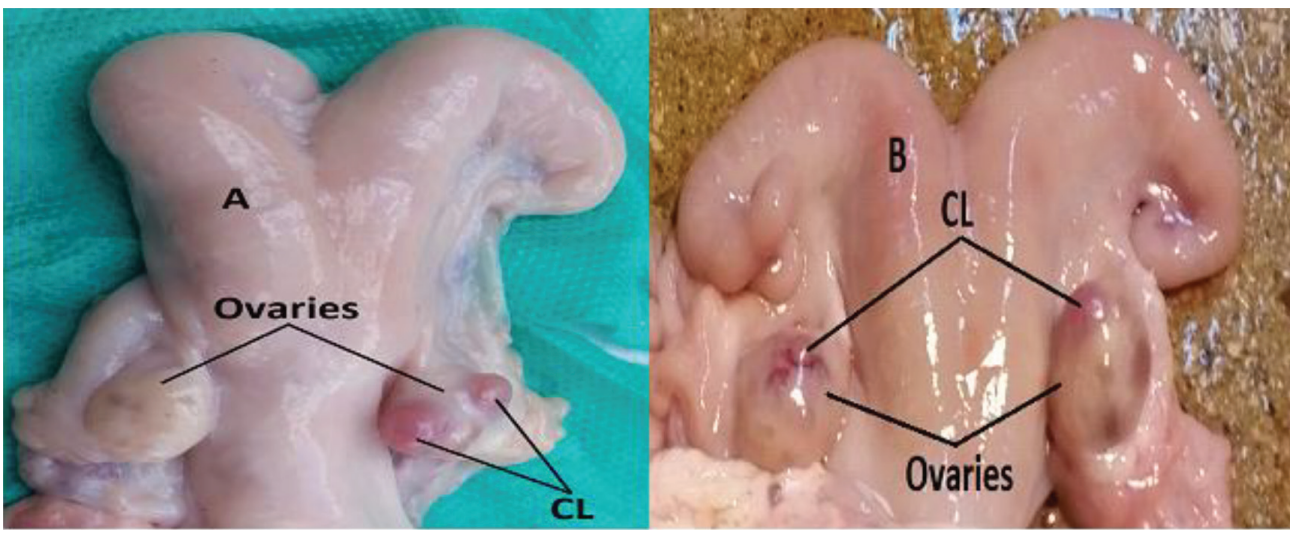

Figure 2. Reproductive tracts of Lori-Bakhtiari ewes used in experiment 2 (autumn season). A = Unilateral double ovulations, B = Bilateral double ovulations, CL = Corpus luteum.

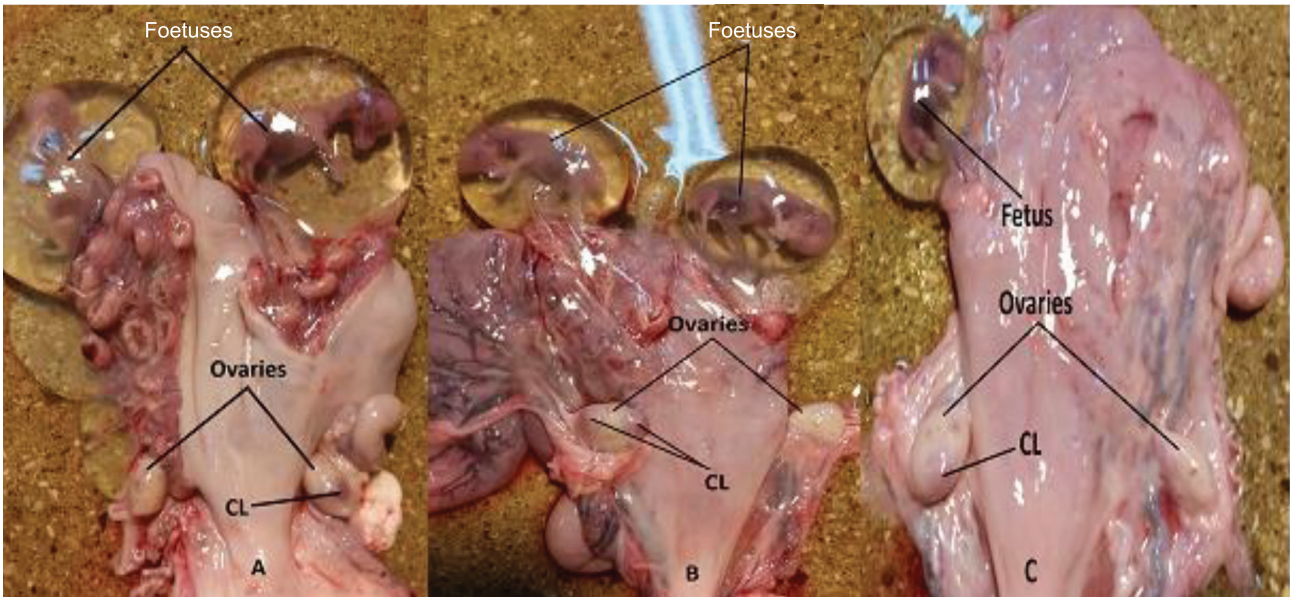

Figure 3. Reproductive tracts of Lori-Bakhtiari ewes used in experiment 2 (autumn season). A = Single ovulation with 2 foetuses, B = Unilateral double ovulations with two foetuses, C = Single ovulation with 1 foetus, CL = Corpus luteum.

ewes for nonpregnant ewes during the spring and autumn seasons, the total volumes of CLs in single-ovulated ewes were significantly smaller ($P < 0.05$) than in double-ovulated ewes. The results showed that the mean volumes of individual CL and the total volumes of CLs during the autumn season were significantly higher ($P < 0.05$) than in spring season. While no significant differences were found ($P > 0.05$) between unilateral and bilateral double ovulations in the number of CLs, the number of CLs in bilateral double ovulations was numerically more than in unilateral double ovulations during the spring season, but it was not similar with the autumn season (Table 1).

The results showed that in nonpregnant ewes during the spring and autumn seasons, the serum progesterone concentration in single-ovulated ewes was significantly lower ($P < 0.05$) than the double-ovulated ewes. There were not significant differences ($P > 0.05$) between the spring and autumn seasons for the single ovulation cases. However, in double ovulations cases, the serum progesterone concentration during the autumn season was significantly higher ($P < 0.05$) than in the spring season. The results indicated that there were no significant differences in the serum progesterone concentration between unilateral and bilateral double ovulations during the autumn and spring seasons ($P > 0.05$). However, in unilateral double ovulation cases the serum progesterone concentration was numerically higher than the bilateral double ovulations (Table 1).

During the autumn season in pregnant ewes, the individual CL volume in single-ovulated ewes (with 1 or 2 foetuses) was significantly larger ($P < 0.05$) than that of double-ovulated ewes (with 1 and 2 foetuses). However, the results showed that the total volumes of CL and the serum progesterone levels in ewes with double ovulations (with 1 and 2 foetuses) were significantly higher ($P < 0.05$) than those in ewes with single ovulation (with 1 and 2 foetuses). The results also indicated that there were significant differences ($P < 0.05$) in ewes with single ovulation (with 1 and 2 foetuses). In ewes with single ovulation and 2 foetuses, the total volumes of CL and also the serum progesterone levels were higher ($P < 0.05$) than in ewes with single ovulation and 1 foetus. The results notably indicated that during the autumn season in ewes with single ovulation (with 1 and 2 foetuses) and double ovulations (with 1 and 2 foetuses) there were significant differences ($P < 0.05$) in both the total volume of CL and the serum progesterone level. In ewes with double ovulations and 1 foetus the total volumes of CL and also the serum progesterone levels were higher ($P < 0.05$) than in ewes with single ovulation (with 1 and 2 foetuses). On the other hand, although there were significant differences ($P < 0.05$) in the total volumes of CLs between unilateral and bilateral double ovulations, there were not significant differences ($P > 0.05$) in the serum progesterone concentration. However, the progesterone concentrations in the unilateral double ovulation cases with 2 foetuses were numerically higher (Table 2).

When the individual CL volumes in unilateral double ovulations of nonpregnant ewes were compared during the autumn season, significant differences ($P < 0.01$) were determined between the 2 CLs. Additionally, significant differences were found between the 2 CLs in unilateral

Table 1. Luteal characteristics (mean ± SD) and the progesterone level (mean ± SD) in single- and double-ovulated nonpregnant ewes during the spring and autumn seasons.

	Spring			Autumn		
CL (n)	Single	Double		Single	Double	
Position	Single	Unilateral *	Bilateral	Single	Unilateral	Bilateral
CL volume (mm^3)	226.81 ± 6.69 ^{b}	142.43 ± 5.47 ^{d}	143.75 ± 1.79 ^{d}	235.43 ± 3.90 ^{a}	148.36 ± 1.83 ^{c}	147.60 ± 1.71 ^{c}
Total CL volume (mm^3)	226.81 ± 6.69 ^{d}	284.86 ± 10.93 ^{b}	287.50 ± 3.58 ^{b}	235.43 ± 3.90 ^{c}	296.73 ± 3.67 ^{a}	295.21 ± 3.41 ^{a}
Serum P4 (ng/mL)	3.91 ± 0.60 ^{c}	5.44 ± 0.64 ^{ab}	5.16 ± 0.47 ^{b}	4.17 ± 0.39 ^{c}	5.74 ± 0.41 ^{a}	5.54 ± 0.39 ^{a}

The small superscript letters (a, b, c, d, e, and f) in the same line show significant differences ($P < 0.05$).

Table 2. Luteal volumes (mean ± SD) and the progesterone level (mean ± SD) in single- and double-ovulated pregnant ewes during the autumn season.

	Single ovulation		Double ovulations*			
CL (n)	Single	Single	Double (I)	Double (II)	Double (III)	Double (IV)
Foetus (n)	1	2	1	1	2	2
CL volume (mm^3)	236.88 ± 2.50 ^{b}	240.66 ± 2.58 ^{a}	149.26 ± 1.04 ^{e}	151.4 ± 1.9 ^{d}	152.59 ± 1.13 ^{cd}	153.91 ± 1.74 ^{c}
Total CL volume (mm^3)	236.88 ± 2.50 ^{f}	240.66 ± 2.59 ^{e}	298.53 ± 2.07 ^{d}	302.80 ± 3.8 ^{c}	305.19 ± 2.27 ^{b}	307.83 ± 3.47 ^{a}
Serum P4 (ng/mL)	4.52 ± 0.40 ^{d}	4.84 ± 0.35 ^{c}	5.96 ± 0.36 ^{b}	6.04 ± 0.36 ^{b}	6.34 ± 0.25 ^{a}	6.17 ± 0.27 ^{ab}

* I and III = Unilateral (ewes exhibited both ovulations on the right or left ovaries), II and IV = Bilateral (ewes exhibited ovulations on the right and left ovaries). The small superscript letters (a, b, c, d, e, and f) in the same line show significant differences ($P < 0.05$).

double ovulations in pregnant ewes with 2 foetuses and in nonpregnant ewes during the spring season ($P < 0.001$). However, bilateral double ovulations in nonpregnant ewes during both the autumn and the spring seasons, bilateral double ovulations in pregnant ewes with 1 and 2 foetuses, and unilateral double ovulations in pregnant ewes with 1 foetus had significant ($P < 0.0001$) differences in volume (Table 3).

Among the CL 1 (Group 1), the CL in bilateral double ovulations in pregnant ewes with 1 foetus had the maximum volume and the CL in unilateral double ovulations in nonpregnant ewes during the spring season had the minimum volume ($P < 0.05$). However, the volume of CL 2 (Group 2) in unilateral double ovulations in nonpregnant ewes and unilateral double ovulation in pregnant ewes with 2 foetuses during the autumn season were significantly higher than those of the other ovulation groups ($P < 0.05$) and also the volume of CL 2 in bilateral double ovulations in pregnant ewes with 1 foetus was significantly smaller than the others (Table 3). The positive correlation between the progesterone level and the total volume of CL during this experiment in nonpregnant ($r = 0.865$, $P < 0.0001$) and pregnant ewes ($r = 0.886$, $P < 0.0001$) was statistically significant.

4. Discussion

The relation between the ovulation status with luteal characteristics and the serum progesterone concentrations has been the aim of several research groups in different species. Echternkamp et al. [19] found that in beef cattle genetically selected for twinning, cows with double ovulations had greater total volumes of CL and progesterone concentrations than the cows with a single ovulation. The total volume of CL and the progesterone level differed in both single- and double-ovulated ewes in this study. The results showed that during both the spring and autumn seasons in nonpregnant and pregnant ewes, the total volume of CL and the serum progesterone concentration in double-ovulated ewes was significantly higher than in the single-ovulated ewes, supporting the previous observation from the spring season in nonpregnant Sanjabi ewes [12]. Therefore, the presence of 2 CLs was correlated with the increased serum progesterone concentrations that support the strategies of inducing the formation of an additional CL leading to the increased progesterone concentration in ewes. However, the results of the present study are in contrast with the findings of Lopez et al., who reported that in cows with double ovulation, the luteal volume

Table 3. The individual volume of CL (mean ± SD) in double-ovulated ewes.

	CL volume 1 (mm^3) (Group 1)	CL volume 2 (mm^3) (Group 2)
Double ovulations (1) Ψ	145.42 ± 5.02 ^{h**}	139.44 ± 2.48 ^{b}
Double ovulations (2)	195.38 ± 5.76 ^{c***}	92.12 ± 2.85 ^{e}
Double ovulations (3)	151.18 ± 6.33 ^{g*}	145.55 ± 3.74 ^{a}
Double ovulations (4)	208.14 ± 4.53 ^{b***}	87.07 ± 4.67 f
Double ovulations (5)	164.42 ± 2.53 ^{e***}	134.11 ± 3.22 ^{c}
Double ovulations (6)	219.32 ± 3.64 ^{a***}	83.48 ± 1.69 ^{g}
Double ovulations (7)	158.64 ± 2.74 ^{f**}	146.55 ± 2.22 ^{a}
Double ovulations (8)	185.72 ± 3.19 ^{d***}	122.11 ± 2.54 ^{d}

Ψ 1 and 2 = Unilateral and bilateral double ovulations in nonpregnant ewes during the spring season, respectively; 3 and 4 = Unilateral and bilateral double ovulations in nonpregnant ewes during the autumn season, respectively; 5 and 7 = Unilateral double ovulations in pregnant ewes with 1 and 2 foetuses, respectively; and 6 and 8 = Bilateral double ovulations in pregnant ewes with 1 and 2 foetuses, respectively. Asterisks indicate significant differences in the same row for volume of CL 1 and 2 (* = $P < 0.01$, ** = $P < 0.001$, *** = $P < 0.0001$). The small superscript letters (a, b, c, d, e, f, g, and h) in the same column show significant differences ($P < 0.05$) for each ovulation position.

increased but the concentration of progesterone decreased [14]. Additionally, Voelz et al. [10] found that cows with 2 CLs had similar luteal volumes and serum concentrations of progesterone to those of cows having 1 CL. On the other hand, in a study conducted by Mann et al. [9], nonlactating cows were reported to have a high incidence of double ovulations, which did not affect the progesterone level.

The results in nonpregnant ewes with a single ovulation notably showed that the total volume of CL during the autumn season was significantly higher than that during the spring season. However, there were no significant differences in the serum progesterone concentrations between the spring and autumn seasons. A significant positive correlation was detected between the total volume of CL and the blood progesterone level in nonpregnant and pregnant ewes. Lucy et al. suggested that there is no correlation between the CL size and the progesterone level [20]. Nevertheless, Sartori et al. [21] found positive relationships between the CL weight and the progesterone concentration in heifers and lactating cows. Spell et al. [22] found a significant relationship between the CL size and the plasma progesterone concentration. On the other hand, in different studies a positive correlation between the total CL volumes and the serum progesterone concentrations was found in cattle [10,15]. Rizos et al. [23] found that the concentration of progesterone depends on the volume of the CL tissue. However, a previous study reported that the rapid physical development of the CL is an important phase for progesterone production [24]. Indeed, during the early stage of CL development, CL volume is associated with the blood progesterone levels. In the mature stage of CL, CL volume has no impact on the circulating progesterone concentrations [24].

The most important result in the present study was the differential effect of the ovulation rate on the serum progesterone concentration and the number of foetuses in sheep. The results notably indicated that ewes with double ovulations and 1 foetus had higher total volumes of CL and serum progesterone concentrations compared with ewes with a single ovulation and 1 or 2 foetuses. Our data support a previous observation in dairy cows [10], which showed that an accessory CL induced the concentration of progesterone which was necessary to maintain the pregnancy but could not determine the number of foetuses. Therefore, the incidence of double ovulations and higher serum progesterone concentration cannot predict the incidence of the twinning in ewes. Evans et al. [25] found that lower dosages of progesterone had no deleterious effect on embryo quality or fertility in sheep. Interestingly, in the present study, a single CL with 2 foetuses was observed extensively. Thus, increasing the incidence of the twinning rate does not appear to be a prerequisite of the presence of 2 CLs in Lori-Bakhtiari ewes. However, Silva-del-Rlo et al. [26] reported that after double ovulations, similar proportions of single and twin pregnancies were observed in lactating Holstein cows. When the volumes of CL 1 (Group 1) in double ovulating animals were compared, the results showed that, for bilateral double ovulations in pregnant ewes with 1 foetus, the volumes of CLs of Group 1 were maximum, and for unilateral double ovulations in nonpregnant ewes during the spring season, the volumes of CLs of Group 1 were minimum among the total ovulation groups. Mann et al. [9] reported that on day 5 of the oestrous cycle there were no significant differences in the weight of individual CLs of animals having double ovulations, while on day 8 of the oestrous cycle, one of the CLs was significantly bigger than the other. The results of the present study also showed that the volumes of CLs of Group 1 in pregnant ewes with bilateral double ovulations and 1 foetus were higher than those of the other ovulation groups. However, the total volume of both CLs and the serum progesterone concentrations in bilateral double ovulations with 1 foetus were significantly lower than in double ovulations with 2 foetuses. Consistent with our findings, it has been reported that double ovulations are

related to individual CLs having smaller volumes, but not to the total luteal weight or the blood progesterone levels [9].

In conclusion, higher serum progesterone concentration may determine ovulation rate but cannot actually determine the number of foetuses in ewes; in nonpregnant and pregnant ewes, the total volume of the CL is larger and the serum progesterone concentration is higher in double-ovulated ewes than in single-ovulated ewes during both the spring and autumn seasons; and increasing the twinning rate does not appear to be a prerequisite of the presence of 2 CLs in ewes.

Acknowledgments

The authors would like to thank the director of the Yasouj abattoirs for providing the research material, such as reproductive tracts and blood samples used in this study, and also the staff who volunteered to assist in ewe age detection for their proficiency, reliability, and also for providing a friendly environment during the sample collection. The authors thank Dr Ahmad Azizi for his technical assistance in the progesterone assay.

Conflict of interest

The authors have no conflict of interest.

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