

5 **Comparison of Reproductive Performance Using Progesterone Vaginal
Implant and eCG in Out of Breeding Season in Cyclic and Anestrus Dairy
Ewes**

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ABSTRACT

BACKGROUND: Estrus synchronization is widely used in induction of ovulation in dairy ewes
in out of breeding season.

OBJECTIVE: The aims of this study were to investigate the differences in reproductive performance between cyclic and anestrus Lacaune dairy ewes in synchronization protocol using vaginal implant of progesterone (CIDR) and eCG in out of breeding season programming.

METHODS: Eighty-four Lacaune ewes were chosen that ranging in 15 to 20 month old and they had one delivery, which at least 90 days had passed. Two blood samples were taken for progesterone (P4) evaluation apart from 10 days. Plasma P4 concentrations were determined using an ELISA. Ewes with <1 ng/ml P4 in two blood sample were considered acyclic and compared with cyclic ewes in estrus expression, Pregnancy rate, Lambing rate, Litter size, Fecundity, Birth weight and sex of lambs. The ewes were kept in separated place about 5 km far away from the rams for more than 2 months.

RESULTE: Out of 84 ewes, 31 ewes were cyclic (36.90 %) and 53 ewes were anestrus (63.10 %). In anestrus ewes, P4 concentration was 0.43 ± 0.4 ng/ml and 0.45 ± 0.04 ng/ml in the first and second blood sampling, respectively. In cyclic ewes, P4 concentration was 2.46 ± 0.39 ng/ml and 2.77 ± 0.34 ng/ml in in the first and second blood sampling, respectively. Expression of behavioral estrus did not differ between anestrus and cyclic ewes ($P > 0.10$). Pregnancy rate and lambing rate tended to be greater in cyclic ewes as compared with anestrus ewes (Odds ratio = 3.35, 95 % confidence interval = 0.88-12.77, $P = 0.08$). Litter size, fecundity, birth weight of lambs and sex ratio of offspring were not different between anestrus and cyclic ewes ($P > 0.10$).

CONCLUSION: The cyclic ewe when using synchronization protocols outside the breeding season could cause high pregnancy and lambing rates in comparison with anestrus ewe.

Key words: Dairy ewe, eCG, Out of breeding season, P4, Reproductive indices.

Introduction

Sheep and goats are seasonal polyestrous animals. The ovarian activity decreases in the seasons when the day length is longer, and they are so-called out of the breeding season. Outside the breeding season, ewes enter the anestrus phase. Anestrus in ewes is characterized by the absence of estrus and ovulation due to a decrease in LH pulse frequency in response to increased sensitivity of the hypothalamus to the negative feedback effect of estradiol (Bedenbaugh *et al.*, 2018). Various studies have shown that seasonal estrus in sheep is a result of changes in the response of the hypothalamus-pituitary axis to the ovarian estradiol hormone (Legan *et al.*, 1977). In order to resume ovarian activity outside of the breeding season, brain control centers must be re-sensitized (Grattan, 2015). For this purpose, progesterone P4 priming is used. The source of P4 that is provided to the animal actually captures this artificial induction that has a P4 producing corpus luteum. Before creating an active corpus luteum on the ovary, the dominant follicle or follicles must grow from the follicular wave and reach the dominance stage, and then ovulation occurs and then become a P4-producing corpus luteum. Using an external P4 source, artificial induction occurs to re-sensitize brain centers to restart ovarian activity (Kuru *et al.*, 2020). There are various methods to induce ovarian activity outside the breeding season of ewe, such as: changing the amount of light exposure (Gómez *et al.*, 2008), using melatonin implants (Mura *et al.*, 2017), using hormones such as natural and artificial P4. Each of these techniques has a percentage of success and failure. The presence of male animals among female animals can play a significant role in the release of LH and FSH and lead to increased ovulation and subsequent fertility and pregnancy (Ferdowsi *et al.*, 2020). The use of natural or artificial P4 in ewes to synchronize estrus has different forms (sponge and CIDR and oral and injectable forms). Each of these P4 sources release a different amount of this hormone into the blood (Gonzalez *et al.*, 2020). When there is an intention to use P4 outside of the reproductive season, eCG hormone

65 can be used as a source of gonadotropins along with it to increase the percentage of success in
inducing ovarian activity and also the number of lambs with the presence of a ram in each birth
(Martinez *et al.*, 2018). The method of using CIDR or sponge along with eCG hormone outside
the breeding season is a common and well-known method all over the world and has a high
pregnancy rate (Pugh and Baird, 2012). The success rate of synchronization in sheep without
70 eCG administration outside the breeding season is low (Boscos *et al.*, 2002). Increasing the dose
of eCG improves the ovulation rate (Khan *et al.*, 2022). The administration of eCG in sheep
increases the ovulation rate by mobilizing small ovarian follicles, increasing the growth rate of
dominant follicles and changing the proportions of follicle size classes in estrus (Martemucci and
Alessandro, 2011). eCG does not seem to rescue follicles from atresia in sheep (Barrett *et al.*,
75 2004). eCG causes early oocyte activation (Hameed *et al.*, 2021). Follicles that are exposed to
eCG synthesize more P4 (Şen, 2020). There are various methods to detect some degree of
acyclicity of sheep. Laparoscopy method, ultrasound method, estrus monitoring and plasma
progesterone measurement, which are considered the easiest and most accurate methods for
detecting anestrous ewe. According to the amount of P4, it is possible to find out if the animal is
80 non-cyclical. According to previous studies, the value of this number has been reported
differently. From 0.2 ng/ml (Husein and Ababneh, 2008), 0.5 ng/ml (Talebkhani *et al.*, 2012) and
1 ng/ml (Mirzaei *et al.*, 2017) in two occasions and 9 to 10 days apart, they are considered as the
basic amount of progesterone, if the amount of progesterone is lower than this number it is
considered as acyclic. We considered the highest value mentioned in the articles as the basic P4
85 level so that we can argue that if the P4 level of an ewe is higher than 1 ng/ml, it is definitely a
cyclic ewe.

The aims of this study were to investigate the difference between cyclic and non-cyclic dairy
Lacuane ewes in the synchronization program outside the breeding season and to compare the

differences using P4 among the ewes regarding the reproductive performance such as: estrus
90 expression, pregnancy rate, lambing rate, litters size, fecundity, birth weight and gender.

Materials & Methods:

Study location

This study was carried out in an industrial dairy sheep farm in Qazvin province-Iran (longitude
50°18', and altitude 121 m). This study started in April, 2022. The average temperature recorded
95 at this time was 14°C.

Animals

In total, 84 Lacuane dairy ewes, based on plasma P4 levels were divided into cyclic and non-
cyclic groups. These two groups were compared based on reproductive indices. The animals
were kept in dense conditions. In short, these dairy ewes received 1 kg of corn silage, 420 gr of
100 corn flour, 420 gr of barley flour and 750 gr of alfalfa.

For prevention of ram effects (sight, smell and sound), the ewes were kept in separated pens
about 5 km far away from the rams for more than 2 months. All rams had at least one mating
season experience. The animals were fed with a mix of alfalfa hay, barley, concentrate with trace
elements, and wheat straw in the zone. They were treated by antihelmantic for external and
105 internal parasites. The management of the ewes did not change throughout the entire period. All
rams and ewes were fed properly for flushing 20 days before the start of this study. In the
flushing, they got higher amount of corn silage and flour with higher amount of barley flour.

Progesterone assay

Two blood samples were taken from the jugular vein before the start of the synchronizing program and subsequent mating, at an interval of 9 days, to evaluate the amount of progesterone in the ewes. To obtain plasma in order to determine progesterone concentration, centrifugation was performed at 1000 rpm for 10 minutes. ELISA method and Monobind kit belonging to USA were used to determine plasma concentration of progesterone. If the amount of P₄ in the plasma was less than 1 ng/ml, it was considered as anestrus.

115 **Protocol for synchronization of estrus**

CIDR[®] (Eazi-Breed[™] Zoetis, Pfizer, New York, USA) contains 0.3 gram was used for 14 days. In addition, 400 IU of eCG (Gonaser, Hipra, Spain) was injected intramuscularly on the day of CIDR removal.

Estrus detection

120 Estrus detection was performed by ram. The rams were introduced to the flock after implant removal. They were within the flock for 40 days. Sheep marking crayon or Ewe Harness Crayon (Carmel[®]) was used. The back of the ewes was checked for coloring and the effect(s) of ram jumping that were recorded.

Management of mating and pregnancy diagnosis

125 Rams were introduced to ewes for natural mating in a ratio of one to ten. The rams were in the groups until 25 days after the start of the program. Transabdominal B-mode ultrasonography by WED 3100 (Well, D Schenzhen, China) with a 5 MHz convex array transabdominal transducer was performed for pregnancy diagnosis almost between days 30 and 45 of gestation. The second ultrasonic test was performed to confirm pregnancy in 80-90 days.

130 **Reproductive performance**

The reproductive performance indices were calculated such as Estrus expression (EE), lambing rate (LR), pregnancy rate (PR), fecundity rate (FR), prolificacy rate (PR), or litter size rate (in the Table). Lamb genus and the average birth weight are shown in the Table.

Statistical analysis

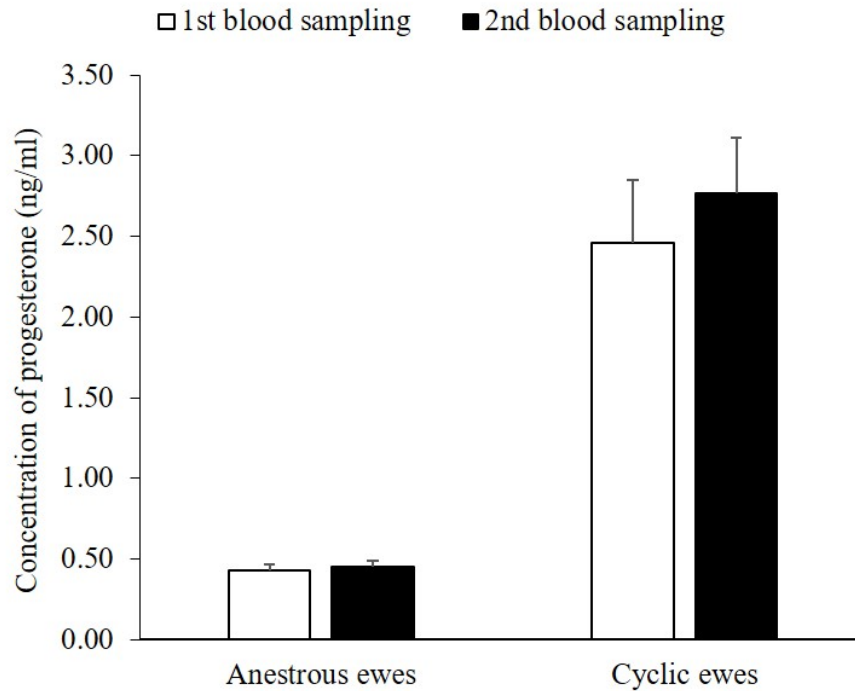
135 Continuous data (i.e., litter size, fecundity and birth weight of lambs) were analyzed by GLM procedure. Binary data (i.e., estrus expression, pregnancy rate, lambing rate and sex ratio of offspring) were analyzed using GENMOD procedure including function link logit in the model. Multiple comparisons were performed using LSMEANS statement. All analyses were conducted in SAS version 9.4 (Statistical Analysis Systems, Cary, NC, USA). Differences at $P < 0.05$ and
140 $0.05 \leq P < 0.10$ were considered significant and tended to be significant, respectively.

Results

Progesterone concentration

Out of 84 ewes that were evaluated for concentration of progesterone by two consecutive blood sampling 10 days apart, 31 ewes were cyclic (36.90 %) and 53 ewes were anestrus (63.10 %).

145 In anestrus ewes, progesterone concentration was 0.43 ± 0.4 ng/ml and 0.45 ± 0.04 ng/ml in the first and second blood sampling, respectively (in the Figure). In cyclic ewes, progesterone concentration was 2.46 ± 0.39 ng/ml and 2.77 ± 0.34 ng/ml in in the first and second blood sampling, respectively (in the Figure).



150 Figure. Concentration of P4 in anestrus and cyclic ewes in the first and the second blood sampling in out of breeding season programming.

Expression of behavioral estrus did not differ between anestrus and cyclic ewes ($P > 0.10$; in the Table). Pregnancy rate and lambing rate tended to be greater in cyclic ewes as compared with
 155 anestrus ewes (Odds ratio = 3.35, 95 % confidence interval = 0.88-12.77, $P = 0.08$; in the Table). Litter size, fecundity, birth weight of lambs and sex ratio of offspring were not different between anestrus and cyclic ewes ($P > 0.10$; in the Table).

Variable	Anestrous ewes (n = 53)	Cyclic ewes (n = 31)
Estrus expression (%)	75.47 (40/53)	90.32 (28/31)
Pregnancy rate (%)	73.58 (39/53) ^a	90.32 (28/31) ^b
Lambing rate (%)	73.58 (39/53) ^a	90.32 (28/31) ^b
Litter size	1.44 ± 0.09	1.39 ± 0.11
Fecundity (%)	105.66 ± 10.37	125.81 ± 13.56
Birth weight of lambs (kg)	3.86 ± 0.12	3.96 ± 0.14
Sex ratio of offspring (%)	57.14 (32/56)	64.10 (25/39)

^{ab}Various small letters indicate tendency to statistical difference between two categories of ewes ($0.05 < P < 0.10$).

160 Table. Reproductive performance of anestrous and cyclic ewes in estrous synchronization in out of breeding season. Data are presented as mean ± SEM and percentages. Values in parenthesis are actual numbers.

Discussion

165 The findings of this study showed that the rate of pregnancy and lambing in cyclic ewes was higher compared to anestrous ewes in the synchronization program out of the breeding season using a synchronization protocol consisting of CIDR and eCG (in the Table). The lack of comparison between cyclic and non-cyclic ewes in synchronization protocols out of the breeding season and the only citation being the off-season at this period, it is clear that the percentage of ewes in the off-season are also cyclic, it has been tried to study the response difference between

these two groups of ewes in the common synchronization protocol of using CIDR and eCG hormone. Naturally, if the amount of P4 before the synchronization program extracted, the proper program for each group of ewes can be presented separately. Reyna *et al.*, (2007) showed that the synchronization and response to eCG hormone is better in outside of the breeding season than inside. Within the breeding season, ewes are cyclic and this study showed that the pregnancy rate was reduced in cyclic ewes in out of breeding season in dairy ewes. The main reason for this topic is the insemination method. In the study of Reyna and his colleagues, the insemination was done at a fixed time and laparoscopically, which indicates that it is more suitable to create synchronization with CIDR and then inject eCG hormone in off-season (Reyna *et al.*, 2007). In cyclic ewes, the response to CIDR may be completely different. From luteinizing follicles to preventing ovulation and etc. In the case of anestrous ewes, the only purpose of using CIDR is progesterone priming. It is important to mention that the results of our study were obtained by natural mating and artificial insemination was not performed in these groups. This suggests that cyclic ewes may only affect the timing of estrus symptoms. The results of this study also showed that the estrus expression is not lower in cyclic ewes compare in non-cyclic ones, whereas in terms of percentage, 15% more than in estrus expression shown in cyclic ewes. Another important issue is the difference in ovulation time. In cyclic ewes and in the breeding season, if the synchronization program is used, the time of ovulation in these groups is very different from each other, and this also causes the low pregnancy rate in insemination at a fixed time (Santos *et al.*, 2020). This does not mean that the response to the synchronization program is low in these groups, however, in cyclic animals, the response to the hormone should be stronger than in non-cyclic animals (Barrett *et al.*, 2004). Cyclic cows can be easily induced with two injections of prostaglandin and there is no need to use CIDR and inject hormones. But, sheep are very different from cows. If P4 is measured outside the breeding season, usually 50% of the herd is non-cyclic based on P4 levels, and it is expected that in case of natural mating without

any intervention, approximately 50% of the herd should become pregnant. Whereas only 10 to
195 15 percent of the cyclic animals became pregnant and remnant need to performed the
synchronization program (Arjmandi *et al.*, 2021). This is the reason why it is not recommended
to inject prostaglandin in any herd of sheep and goat. In another study, a transient peak of FSH
was observed in both reproductive and non-reproductive seasons in ewes before the appearance
of follicular waves. Thus, eCG administration is expected to affect follicular wave dynamics
200 (Barrett *et al.*, 2004; Viñoles *et al.*, 2001). Synchronization success rate in sheep without eCG
administration outside the breeding season is low (Martinez *et al.*, 2015). The administration of
eCG in sheep increases the ovulation rate by mobilizing small ovarian follicles, increasing the
growth rate of dominant follicles and changing the proportions of follicle classes in estrus (Uslu
et al., 2012). eCG does not appear to rescue follicles from atresia in ewes (Martemucci *et al.*,
205 2011). eCG induces early oocyte activation (Dogan *et al.*, 2018). Follicles exposed to eCG can
produce more P4 (Garoussi *et al.*, 2019. Ghasemzadeh-nava *et al.*, 2017). It was expected that in
cyclic ewes, Litter size & fecundity increased in the flock and higher versus anestrus ewes. In
our study, no significant difference was observed between the two groups (in the Table). This
possibility was given because the response to the hormone in cyclic ewes should have shown
210 itself better. In the studies, it was shown that outside the breeding season, the ratio of multiple
ovulation in the first ovulation is higher than the second ovulation (MK, 2013). Perhaps the most
likely reason is that the ovulation rate in non-cyclic animals is high during the first ovulation.
Another possible reason is due to the effect of the ram effect in increasing the amount of secreted
LH, and this has caused an increase in ovulation and subsequent multiple births in non-cyclic
215 ewes. In the studies carried out, it has been shown that the mating of a male animal and the direct
contact of the ewe with a ram lead to an increase in the secretion of LH, and an increase in the
growth of follicles and the production of estradiol, and subsequently, estrus, LH surge and
ovulation (Scaramuzzi and Downing, 1997; Berean *et al.*, 2019; Mirzaei *et al.*, 2017; Ayaseh *et*

220 *al.*, 2021). Ewes that are constantly accompanied by rams become accustomed to their presence
and have the same level of estrus as ewes without rams. 65% of Romney ewes ovulate within 65
hours after the ram enters (Nakafeero *et al.*, 2020) and 50% of Merino ewes ovulate within 41
hours after the ram enters. When the ram enters at the same time as the sponge is removed,
ovulation occurs along with estrus symptoms (Ungerfeld *et al.*, 2020). If the ram enters with a
225 delay of 48 to 52 hours, ovulation occurs, but the ewes do not show signs of estrus (Mahmoud *et*
al., 2019). Lambing rate in our study was higher as pregnancy rate in cyclic ewes than non-cyclic
ewes and that was due to the absence of abortion in the studied ewes. Many studies have been
done to replace GnRH with eCG (Reyna *et al.*, 2007; Santos *et al.*, 2020). The main reason for
these studies is to lower the price and comply with the ethical principles of animal treatment,
because the eCG hormone is extracted from pregnant horses. But in most studies, GnRH in
230 outside the breeding season and in non-cyclic ewes showed a much lower pregnancy percentage
than eCG, and it is not recommended at all. But GnRH has shown a very appropriate and similar
response to eCG within the reproductive season or in cyclic ewes (Reyna *et al.*, 2007). In
addition, use of eCG with GnRH on the day of insemination increased reproductive performance
(Hosseinzadeh *et al.*, 2016). In another study showed that injection of 300 IU of hCG hormone
235 after insemination improves pregnancy rate in ewes (Didarkhah and vatandoost, 2022). So, if you
know that the ewe is cyclical outside the breeding season, it can be suggested that instead of eCG
hormone, GnRH can be used instead. Perhaps the importance of this study lies in the fact that
until now, outside of the reproductive season, a comparison between cyclic and non-cyclic ewes
included in the synchronization protocol had not been done in terms of reproductive indices.

240

Conclusion

The cyclic ewe when using synchronization protocols outside the breeding season can cause high pregnancy and lambing rates compared to anestrus ewe.

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Authors' declaration of interests

No competing interests have been declared.

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مقایسه عملکرد تولیدمثلی با استفاده از کاشت واژینال پروژسترون و eCG در خارج از فصل تولید
مثل در میش های شیرسیکلک و آنستروس

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چکیده

زمینه مطالعه: همزمان سازی فحلی برای القای تخمک گذاری در خارج از فصل تولید مثلی در گوسفند شیری به صورت گسترده استفاده می شود.

420 هدف: هدف از این مطالعه بررسی تفاوت عملکرد تولیدمثلی بین میش های شیری سیکلیک و آنستروس نژاد لاکون در برنامه همزمان سازی با استفاده از کاشت واژینال پروژسترون (CIDR و eCG) در خارج از فصل تولید مثل بود.

مواد و روش کار: هشتاد و چهار راس میش لاکون در محدوده سنی 15 تا 20 ماهه انتخاب شدند که یک بار زایمان داشتند و حداقل 90 روز از زمان زایش آنها گذشته بود. دو نمونه خون برای ارزیابی پروژسترون (P4 به فاصله 10 روز) گرفته شد. غلظت P4 پلاسما با استفاده از ELISA تعیین شد. میش هایی با کمتر از 4 ng/ml P4 در دو نمونه خون غیر سیکلیک در نظر گرفته شدند و از نظر فحلی، میزان آبستنی، میزان بره زایی، تعداد بره، باروری، وزن تولد و جنسیت بره ها با میش های سیکلیک مقایسه شدند. 425 میش ها به مدت بیش از 2 ماه در محل جداگانه‌ای با فاصله حدود 5 کیلومتری قوچ ها نگهداری شدند.

نتایج: از 84 راس میش، 31 میش سیکلیک (36/90 درصد) و 53 میش غیر سیکلیک (63/10 درصد) بودند. در میش های غیر سیکلیک، غلظت پروژسترون به ترتیب 0.4 ± 0.43 و 0.45 ± 0.04 در خون گیری اول و دوم بود. در میش های سیکلیک، غلظت پروژسترون در خونگیری اول و دوم به ترتیب 2.46 ± 0.39 و $2/77 \pm 0/34$ نانوگرم بر میلی لیتر بود. بیان رفتار فحلی بین میش های سیکلیک و غیر سیکلیک تفاوتی نداشت ($P > 0/10$). نرخ آبستنی و نرخ بره زایی در میش های سیکلیک در مقایسه با میش های غیر سیکلیک بیشتر بود (نسبت شانس = 3.35، 95٪ فاصله اطمینان = 0.88-12.77، $P=0.08$). چندقلو زایی، تزاید گله، وزن تولد بره ها و جنسیت بره ها بین میش های غیر سیکلیک و سیکلیک تفاوتی نداشت ($P > 0/10$).

نتیجه گیری: سیکلیک بودن میش در هنگام استفاده از پروتکل های همزمانی در خارج از فصل تولید مثلی، می تواند سبب بالا بودن نرخ آبستنی و بره زایی نسبت به میش های غیر سیکلیک گردد.

435 کلمات کلیدی: میش شیری، eCG، خارج فصل تولید مثلی، پروژسترون، شاخص های تولید مثلی.

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