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Effects of different protocols for estrus synchronization in ewes on vaginal pH, estrus time and reproductive performance and change in vaginal electrical resistance values

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This study examined the effects of different intravaginal apparatus types used for estrus cycle management in ewes, as well as the timing of their insertion and withdrawal on vaginal pH and reproductive performance. Additionally, this study aimed to investigate changes in VER levels during estrus and anestrus in Kıvırcık ewes. A total of 90 Kıvırcık ewes in the estrus period were synchronized using fluorogestone acetate (FGA) sponges and controlled internal drug release (CIDR) protocols ranging from short (5 days) to medium (9 days) up to long-term (13 days). Additionally, 350 IU of equine chorionic gonadotropin (eCG) were injected intramuscularly when the FGA and CIDR were withdrawn from all the groups. Vaginal pH samples were taken on the days of vaginal apparatus removal (days 5, 9 and 13), as well as the first day of estrus and anestrus. Vaginal pH increased more markedly in ewes treated with FGA and CIDR when compared to those in anestrus, especially in the medium and long-term treated ewes, which had a pH value around 7.7. Both medium and long-term protocols had a high success rate for estrus occurrence, with all the tested ewes entering estrus. However, ewes in the FGA medium-term group presented the earliest estrus at 32.8 hours. Although statistically non-significant, the CIDR protocol resulted in numerically higher results for multiple birth rate, fecundity and litter size. The electrical resistance of vaginal mucus was measured during anestrus and immediately after estrus detection. VER values were recorded to be lower during estrus. VER values between 200 and 300 Ω might be indicative of estrus in ewes.

KEY WORDS: CIDR / estrus synchronization / fertility / sheep / vaginal electrical resistance

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Exogenous hormones are widely used in the Turkish sheep industry for estrus synchronization and superovulation, providing farmers with a high degree of control over flock mating timing and ensuring higher reproductive efficiency [Kusina *et al*. 2000]. In order to induce estrus in ewes during the non-breeding season, the administration of progesterone is the preferred method and results in prolonging of the luteal phase in these ewes. As a result, they are synchronized and after the cessation of hormonal treatment the ewes enter estrus simultaneously. Fluorogestone acetate (FGA) sponges or controlled internal drug release (CIDR) are commonly utilized for estrus synchronization in ewes [Rowe *et al*. 2009, Souza *et al.* 2011, Zohara *et al.* 2014]. In traditional estrus synchronization, FGA or CIDR remain in the vagina for 12-14 days, simulating the life of a corpus luteum regardless of the stage of the estrus cycle or the status of the ovaries at the time of apparatus insertion [Menchaca and Rubianes 2004]. It is observed that ewes enter estrus within 24-48 hours of receiving an equine chorionic gonadotropin (eCG) injection, generally following the removal of the intravaginal apparatus [Wildeus 2000, Swelum *et al*. 2015]. It is reported that during the withdrawal of intravaginal sponges from the vagina after 12-14 days of use generally purulent or haemorrhagic vaginal secretions are observed, resulting in low pregnancy rates in ewes [Martinez-Ros *et al*. 2018]. This is believed to occur mostly as a result of intravaginal sponges remaining in the vagina for an extended period of time and absorbing vaginal secretions, keeping them stationary [Al-Hamedawi *et al*. 2003]. This causes unnatural secretions or infections by altering the vaginal environment [Martins *et al.* 2009]. Moreover, in addition to its effect on the fertility of ewes the presence of vaginitis violates animal welfare principles. On the other hand, some studies have concluded that the application of progesterone-impregnated vaginal sponges for short periods such as 5-9 days is sufficient for successful estrus synchronization in sheep and goats [Viñoles *et al*. 2001, Fonseca *et al*. 2005, Ataman *et al*. 2006, Doğan *et al*. 2008, Özyurtlu *et al*. 2011, Machiya *et al*. 2012, Oliveira *et al*. 2016]. In recent years, independently from the type of intravaginal apparatus used, estrus synchronization methods have evolved to carry out shorter applications including durations of approximately 6-7 days [Cox *et al.* 2012]. It was reported during these procedures that CIDR provided a higher pregnancy rate than sponges, while also inducing higher progesterone production rates [dos Santos-Neto *et al.* 2011]. Moreover, it was reported during these short protocols that follicular response and ovulation could be better managed, acceptable fertility rates could be obtained and expenses could be decreased by reusing the intravaginal apparatus [Menchaca *et al*. 2017]. However, it is known that vaginal apparatuses frequently used for estrus synchronization alter the vaginal environment and contribute to the development of vaginitis [Suarez *et al*. 2006, Martins *et al.* 2009, Manes *et al*. 2010, Gatti *et al*. 2011, Oliveira *et al.* 2013, Manes *et al.* 2015]. According to Nakano *et al*. [2015], FGA significantly increased vaginal pH in all ewes whether it remained in the vagina for a short or a long period of time. Manes *et al*. [2010] observed that FGA decreased the vaginal pH, which normally ranges between 7.0 and 7.6. In another study Manes *et al*.

[2016] stated that the vaginal pH increased to 7.97 in ewes that showed signs of estrus following FGA treatment. Martinez-Ros *et al*. [2018] found that CIDR had no effect on vaginal pH, which was similar to that in the control group (6.8-6.9). While some studies reported that after the use of vaginal apparatus in ewes the pH level increased and the fertility rate decreased, because excessive alkalinity negatively affected spermatozoa viability and the fertility rate was higher at pH levels below 7.0, other studies reported that the optimal vaginal pH level should be between 7.0-8.5 to protect sperm viability and mobility in the ewe [Quinlan *et al.* 1941, Nakano *et al.* 2015, Manes *et al*. 2016]. Additionally, it was stated that when the vaginal pH was acidic, sperm mobility reduced, resulting in decreased fertility and significant economic losses [Al-Hilli and Ajeel 2015, Rasad and Setiawan 2017]. The primary factor limiting reproductive performance in many herds is connected with failure to detect estrus in a timely and precise manner. Estrus detection in ruminants is often challenging due to short periods of standing estrus, silent estruses, shifting dietary regimens as well as environmental temperatures. Efficient and accurate estrus detection is essential for successful mating and pregnancy. As stated above, estrus synchronization is an effective management tool that has been successfully employed to enhance reproductive performance in ruminants [Kusina *et al*. 2000]. A satisfactory conception rate is only possible if mating occurs at the correct time relative to ovulation. The onset of estrus is characterized by increasing levels of estrogen. Increased estrogen levels result in higher sodium chloride (NaCl) concentrations in the vaginal mucus, resulting in a significantly lowered electrical resistance [Fehring 1996]. In other words, during the phases of the estrus cycle the hormones estrogen and progesterone cause changes in mucus and in impedance values [Rezac 2008]. The variations in vaginal electrical resistance (VER) values associated with the estrus cycle correspond to its phases, providing critical information for efficient and effective breeding [Malakar *et al*. 2017]. The measurement of VER is a useful tool to detect estrus and ovulation, as well as determine the optimal time for mating [Gupta and Purohit 2001]. When applied correctly and effectively, vaginal resistance could be a very viable indicator of estrus [Yamauchi *et al.* 2009]. Nevertheless, considerable variation exists within and between female animals [Řezáč 2008].This study aimed to determine (i) changes in vaginal pH caused by the different types of intravaginal apparatuses (FGA and CIDR) used for estrus synchronization and the duration of their vaginal application (5-9-13 days), (ii) effects of the different types of apparatus and the duration of their vaginal application on the estrus response and reproductive performance in ewes, and (iii) changes in VER values during estrus and anestrus in Kıvırcık ewes.

Material and methods

Location and experimental conditions

This study was conducted during the non-breeding season (June), at the Agricultural Faculty Application and Research Farm at the Uludag University in

Bursa, a province in Turkey's north-western region. The farm was located in the humid lowland tropics at an altitude of 100 m above sea level, at 29°E longitude and 40°N latitude. Average minimum and maximum temperatures in the region are 3.2°C and 30.7°C, respectively.

Animal management

This study examined a total of 90 clinically healthy Kıvırcık ewes aged 2-3 years, weighing approximately 50-55 kg, and having given birth at least once. All ewes grazed on pastures from 07:00 to 18:00 h. During the mating period (4 weeks) and the final period of pregnancy (6 weeks) the ewes were administered concentrate feed. Depending on the condition of the grassland, it included hay (400-600 g/head) and a concentrate feed mixture (300 g/head) consisting of 57.5% wheat, 15% corn, 25% sunflower seed pulp, 1.3% marble powder, 1.0% salt and 0.2% vitamin-mineral given at the same times, each divided into two meals. A mineral salt stone and drinking water were available ad libitum.

Estrus synchronization

Ninety ewes were randomly assigned to one of two groups undergoing separate synchronization methods. Group 1 (n=45): Estrus synchronization was conducted by implanting CIDR (Controlled Internal Drug Release), (Intervet, New Zealand) constructed with silicone elastomer impregnated with 0.3 g natural progesterone. Thereafter, ewes were randomly assorted into 3 groups varying in progesterone administration durations: short (5 days-D5) (n=15), medium (9 days-D9) (n=15) and long-term (13 days-D13) (n=15). Group 2 (n=45): Estrus synchronization was performed by implanting intravaginal sponges impregnated with 20 mg fluorogestone acetate (FGA), (Chronogest/CR, Intervet, New Zealand). Additionally, 350 IU of eCG (Equine chorionic gonadotropin) were injected intramuscularly following the removal of the FGA and CIDR. Estrus was detected using a vasectomized ram starting 12 hours after apparatus removal and then every 12 hours thereafter (12, 24, 36, 48, 60 h). The acceptance of the male by the female was considered to be the commencement of estrus. Following estrus confirmation, ewes were exposed to proven fertile rams. The care and usage of animals were approved by the ethics committee of Bursa Uludag University and were in accordance with Turkish laws and regulations (License Number 2015-12/05).

Determination of vaginal pH

Vaginal pH was determined using pH-indicator strips (Merck KGaA, working range 6.5-10.0). The speculum was inserted and the indicator strips were held against the vaginal wall for a minimum of 3 seconds. The ewes' vaginal pH was determined during estrus and intravaginal apparatus removal (on the 5th, 9th and 13th days).

Determination of vaginal electrical resistance values

The VER values of ewes were measured during anestrus and after estrus detection by inserting the probe of an electrical heat detector (DRAMINSKI®, Poland) into the vagina as instructed by Theodosiadou and Tsiligianni [2015]. The average value of three consecutive measurements was recorded.

Reproductive parameters

The parameters calculated following the FGA and CIDR withdrawal were estrus response (percentage of ewes in estrus/total ewes treated), lambing rate (percentage of ewes lambing/ewes mated), rate of multiple births (percentage of multiple lambing/ total lambing), fecundity (number of lambs born/total ewes mated), litter size (number of lambs born/ewes lambing) and survival rate (number of living lambs/number of lambs born).

Statistical analysis

The Two-Sample T-Test was performed to compare VER values during the anestrus and estrus periods. While applying the Chi-square test to the data, ewes which gave birth to twins and those which gave birth to triplets were grouped together under the category of multiple birth. The model used in the analysis of variance is given below. The following linear model was applied for vaginal pH;

$$y_{ijk} = \mu + A_i + P_j + (AP)_{ij} + e_{ijk}$$

y_{ijk} – ijk-th observation;

μ – overall mean;

A_i – fixed effect of the i-th method (i= FGA, CIDR);

P_j – fixed effect of the j-th period (j= anestrus, the 5th, 9th, 13th days and the day of estrus);

$(AP)_{ij}$ – fixed effect of the method by period interaction;

e_{ijk} – effect of random error.

The Tukey test [SPSS 16] was used to conduct multiple comparisons.

Results and discussion

In this study the vaginal pH mean was determined to be 7.5±0.04. The effect of the method, the period (application duration) and the method x period interaction on vaginal pH was found to be significant (R^2=63.9%) (P<0.01). Table 1 contains the means, standard errors and results of the multiple comparison test for these factors and their levels. The vaginal pH was found to be higher in the CIDR (7.8) group

Table 1. Averages and standard deviations of vaginal pH changes in consecutive periods across two methods

Period	Method				Mean	
	FGA		CIDR			
	N	x (SD)	N	x (SD)	N	x (SD)
Anestrus	45	6.7 (0.46) ^{d}	45	6.7 (0.41) ^{d}	90	6.7 (0.43) ^{c}
D5	15	7.0 (0.60) ^{cd}	15	7.1 (0.64) ^{cd}	30	7.1 (0.61) ^{c}
D9	15	7.5 (0.63) ^{bc}	15	8.4 (0.51) ^{a}	30	8.0 (0.72) ^{ab}
D13	15	7.3 (0.41) ^{c}	15	8.2 (0.59) ^{ab}	30	7.7 (0.67) ^{b}
Estrus	45	8.0 (0.47) ^{ab}	45	8.4 (0.54) ^{a}	90	8.2 (0.54) ^{a}
Mean	135	7.3 (0.73) ^{a}	135	7.8 (0.91) ^{b}	270	7.5 (0.04)

^{abcd}Values in rows (or columns) with different letters differ significantly at $P<0.01$.

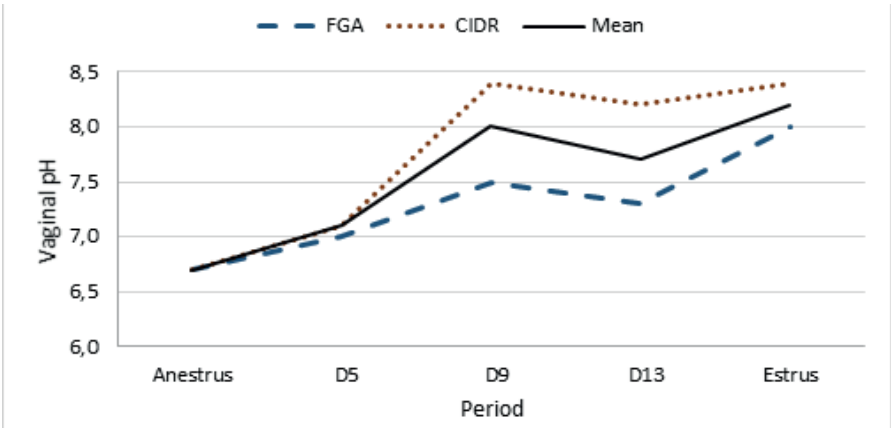

Fig. 1. Changes of vaginal pH in the FGA and CIDR groups depending on the periods. FGA – fluorogestone acetate, CIDR – controlled internal drug release.

than the FGA group (7.3). As indicated in the column 'Mean', the difference between the periods covering both methods is significant ($P<0.01$). The change in vaginal pH depending on time for the FGA and CIDR protocols is given in Figure 1.

As can be seen, pH increased until the 9th day with the insertion of the vaginal apparatus. The difference between the ewes in the CIDR group and those in the FGA reached a maximum on the 9th day. Later, on the 13th day, a slight decrease was recorded. However, during estrus the vaginal pH reached 8.0 in the FGA group and 8.4 in the CIDR group, with an approximate pH increase of 1.3 and 1.7, respectively. These results indicate that CIDR-treated ewes had a greater increase in vaginal pH. In this study the vaginal pH, which was acidic during anestrus, became neutral within 5 days and turned alkaline within 9 days with both the FGA and CIDR treatments. This is consistent with the findings reported by Nakano *et al*. [2015]. In contrast, Swartz *et al*. [2014] determined that vaginal pH was close to neutral in ewes treated with short and long-term CIDRs. In this study it was observed that CIDR increased

vaginal pH significantly faster than FGA did. In an earlier study FGA was found to increase vaginal pH to a greater extent than CIDR [Martinez-Ros *et al*. 2018]. A high vaginal pH was associated with the observation of purulent and bloody vaginal secretions in 80% of the ewes when FGAs were withdrawn. Moreover, in the current study vaginal pH was alkaline during estrus as well. Similarly, Suarez *et al.* [2006], Manes *et al.* [2016] and Rasad and Setiawan [2017] found that the ewes' vaginal pH values showed an increase during estrus. Rasad and Setiawan [2017] reported that this increase was a result of the physiological changes associated with the rise in estrogen levels in the blood during estrus. Moreover, in this study the vaginal pH values in the ewes in the CIDR (D9) and the CIDR (D13) groups on the day the apparatus was removed were as high as those in estrus. Even while both CIDR and FGA increased vaginal pH in the current investigation, this was not observed during the withdrawal of the apparatus. Hence, it can be concluded that this discrepancy resulted from the breed's susceptibility or the farm's conditions. Quinlan *et al*. [1941], Błaszczyk *et al.* [2004], Khalifa *et al*. [2010] and Martinez-Ros *et al*. [2018] stated that vaginal pH decreased during estrus. In some previous studies it was reported that the decrease observed in vaginal pH during estrus coincided with the ovulation period, induced by LH release [Błaszczyk *et al*. 2004, Khalifa *et al.* 2010, Martinez-Ros *et al*. 2018]. On the other hand, Mahmoud [2013] stated that vaginal pH did not differ greatly between ewes treated with FGA (6.74) and those in the control group (6.80) during estrus.

Although the estrus synchronization protocols resulted in a 2-hour reduction in the estrus duration in the FGA group, the effect was found to be non-significant (Tab. 2). The rate of the ewes experiencing estrus for different durations as a result of the FGA and CIDR applications is given in Figure 2. According to this, estrus rates in the first 36 hours were calculated as 77.8 and 62.2% for the FGA and CIDR groups, respectively. On the other hand, all ewes participating in the D9 and D13 groups entered estrus within 48 hours in both protocols. However, when the apparatus remained in the vagina for 5 days, estrus in all ewes was delayed until the 60th hour. As a result, regardless of the fact whether the ewes'

Table 2. Effects of the FGA and CIDR protocols on reproductive characteristics

Trait	FGA				CIDR				P-value
	D5	D9	D13	mean	D5	D9	D13	mean	
Estrus response (hour)	36.8	32.8	36.0	35.2	40.8	36.8	35.2	37.6	0.057
Multiple birth rate (%)	33.3	40.0	33.3	35.6	53.3	46.7	60.0	53.3	0.608
Fecundity (head)	22	22	21	21.6	23	23	25	23.6	
Litter size (head)	1.47	1.47	1.40	1.44	1.53	1.53	1.67	1.58	
Survival rate (%)	95.5	95.5	85.7	92.3	87.0	91.3	72.0	83.1	0.135
Pregnancy prolificacy (kg)	493	553	558	534.6	565	534	609	569.3	
Total prolificacy (kg)	4129	4504	4182	4271.6	3942	4320	3869	4043.6	

FGA – fluorogestone acetate, CIDR – controlled internal drug release.

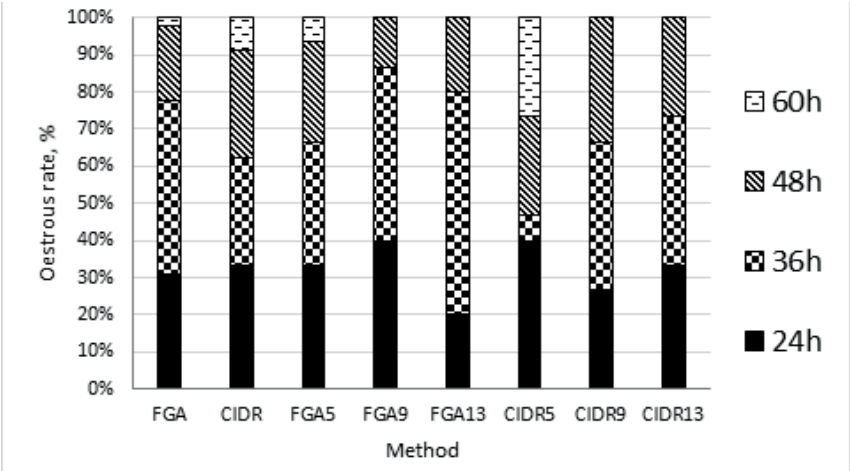

Fig. 2. Estrus values indicated by the FGA (fluorogestone acetate) and the CIDR (controlled internal drug release) protocols at different periods of time.

estrus was synchronized using CIDR or FGA, it was concluded that the apparatus needed to remain in the vagina for at least 9 days in order for all ewes to enter estrus within 48 hours. Thus, the risk of vaginal secretion and infection, which are typically encountered during long-term synchronization protocols, is likely to be minimized. These results corroborate those of Hashemi *et al*. [2006] and Moeini *et al*. [2007]. In contrast to this study, Moradi *et al*. [2012] discovered that the rate of ewes entering estrus was significantly higher in the FGA group than in the CIDR group ($P<0.05$). However, in numerous prior studies it was determined that ewes in the CIDR group entered estrus earlier and remained in estrus longer than those in the FGA group [McNatty *et al*. 1988, Fukui *et al*. 1999, Zonturlu *et al*. 2008, Swelum *et al*. 2015]. Hence, these discrepancies can be attributed to animal care and feeding conditions playing a role in the success of synchronization, ewes' body condition scores or a range of environmental stress factors.

In this study both treatments had a positive effect on estrus synchronization of Kıvırcık ewes during anestrus. In addition, all ewes gave birth to healthy lambs. The influence of the type of application on multiple birth and survival rates were found to be non-significant ($P>0.05$). However, it was observed that the multiple birth rate in the ewes in the CIDR group was 18% higher compared to the FGA group. Although it was reported in some previous studies that alkaline vaginal pH decreased fertility [Nakano *et al*. 2015, Manes *et al*. 2016], it was observed in this study that the high vaginal pH during estrus did not adversely affect fertility or prolificacy in ewes both in the FGA and CIDR groups. Since the slight secretion observed during the removal of the apparatus reverted to normal until estrus in all the ewes, it did not cause any fertility problems. Similarly to these findings, also Peek and Matthews [1986] and Eggert-Kruse *et al*. [1993] discovered that the survival rate and sperm motility

decreased when vaginal pH was acidic and that the optimal vaginal pH value for sperm motility in ewes is between 7.0 and 8.0. On the other hand, it was determined in this study that the effects of the vaginal apparatus types (FGA and CIDR) on reproductive performance did not differ statistically. However, CIDR provided a higher multiple birth rate, fecundity, litter size and pregnancy prolificacy than FGA. It appears to be possible to increase fecundity, perhaps through improved sperm survival and/or fertilization rate as a result of a more favourable vaginal environment. This result shows similarity to those obtained by Zonturlu *et al*. [2008], Moeini *et al*. [2007] and Knight *et al*. [1988]. On the contrary, while some studies reported that reproductive performance of ewes treated with CIDR was higher than that of ewes treated with FGA [Swelum *et al*. 2015], others report that reproductive performance of ewes treated with FGA was higher than that of ewes treated with CIDR [Moradi *et al.* 2012]. However, the current investigation discovered that the fatality rate in the CIDR group was approximately 10% higher. The fact that birth weights of multiple-birth lambs are generally rather low and these lambs cannot grow sufficiently due to lower milk and colostrum consumption may be contributing to the high fatality rate.

Moreover, duration of the apparatus remaining in the vagina did not create a statistically significant difference in terms of reproductive performance in this investigation. In addition to some studies reporting successful short-term (e.g., 5-7 days) sponge applications in ewes [Fitzgerald *et al.* 1985, Beck *et al*. 1993, Viñoles *et al*. 2001, Ataman *et al*. 2006], there are others reporting that the apparatus remaining in the vagina for 6 or 12 days had no effect on fertility [Khalilavi *et al.* 2016] or that the apparatus was required to remain in the vagina for 12 days for a high reproductive performance [Hosseinipanah *et al*. 2014].The VER values (Ω) measured during estrus and anestrus were 246.9±30.0 and 449.1±82.5, respectively. Vaginal electrical resistance was found to be significantly (P<0.001) lower during estrus than during anestrus. All ewes had VER values ≤300 ohm during the estrus period. The present study used the average value of three VER measurements performed immediately after teaser rams detected estrus and before free mating to determine the optimal time for mating in Merino ewes. Because mating typically occurs during this period, VER was determined immediately after estrus detection by teaser rams. The VER in heat ewes was 246.9±30.0 Ω (ranging from 200.0 to 300.0 Ω) and the VER in non-heat ewes was 449.1±82.5 Ω (from 320 to 690.0 Ω). The VER values were significantly (P<0.001) lower in ewes in estrus compared to those in anestrus, regardless of estrus synchronization. Similarly, Rahman *et al*. [2020] identified the VER values of <300 ohm during the estrus period. In turn, Tsiligianni [2014] reported that <400 ohms of VER values in three ewe breeds that conceived during the estrus period. Theodosiadou and Tsiligianni [2015] reported that VER values in Chios ewes during the estrus period were <300 ohms (ranging from 267 to 297 Ω) and <400 ohms (ranging from 276 to 362 Ω) in Kymi ewes during anestrus. The VER values reaches the lowest value in sheep during proestrus and estrus [Bartlewski *et al*. 1999]. In the current study the findings support the reports of Bartlewski *et al.* [1999]. Tsiligianni [2014]

reported that cervical mucus volume and crystallization increase following estrus synchronization by MGA in ewes, and that these differences can be attributed to the electrical resistance of mucus. It is stated that there is a strong correlation between reduced electrical resistance of mucus in ewes and improved sperm transformation and spermatozoa survival in the cervix. Hence low VER values of vaginal mucus during estrus could be a useful indicator of successful mating time in ewes.

In conclusion, it was determined that the vaginal apparatus used to successfully synchronize estrus in ewes in the anestrus period was fit to remain in the vagina for 9 days regardless of the type (FGA vs. CIDR) and that it is likely to be used as a valid alternative to long-term procedures. When the types of apparatus were compared in terms of reproductive performance, despite the difference being statistically non-significant, CIDR provided a higher multiple birth rate, fecundity, litter size and pregnancy prolificacy than FGA. Additionally, the duration of the vaginal apparatus application did not create a statistically significant difference in terms of reproductive performance in this study. The vaginal pH varied and increased in both groups (FGA and CIDR) when compared to anestrus ewes, especially following the medium and long-term protocols. Moreover, it was determined in this study that the vaginal pH was alkaline while the ewes were in estrus. Breeders may choose to consider using vaginal pH to detect estrus and as part of routine gynaecological examinations. Lower VER values were recorded during estrus than during the anestrus period. All ewes showed VER values ≤300 ohm during the estrus period. The determination of vaginal pH and measurement of the electrical resistance of vaginal mucus could be useful in the selection of ewes for mating, even if rams are used to detect estrus.

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