Acta Scientiae Veterinariae, 2023. 51: 1920.

RESEARCH ARTICLE
Pub. 1920

ISSN 1679-9216

Effect of GnRH Administration on Pregnancy-Associated Glycoproteins in Dairy Sheep with Different Reproductive Status

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ABSTRACT

***Background*:** Measurement of pregnancy-associated glycoproteins (PAGs) by radioimmunoassay or enzyme-linked immunosorbent assay has been commonly used for early pregnancy diagnosis in ruminants. However, an accurately pregnancy detection depends on test antibody, breed and number of embryos. Only few studies have conducted to detect or predict animals at risk of late embrionic mortality (LEM) and to use hormonal interventions for embryo losses reducing, but this area is still open in sheep. The current study aimed to evaluate the effect of gonadotropin releasing hormone (GnRH) administration on Day 4 after artificial insemination on the pregnancy-associated glycoproteins in dairy sheep with different reproductive status in relation to pregnancy and late embrionic mortality detection.

***Materials, Methods & Results*:** Sixty-five East Friesian sheep were divided in 2 groups - I (Control group, n = 35) and II (GnRH group, n = 30) and sublected to estrus synchronization and artificial insemination (AI). Group I was not treated and Group II received 50 µg GnRH on Day 4 after AI. PAGs in blood serum were measured by Alertys Ruminant Pregnancy test on Days 4, 12, 20, 25 and 35 after AI and ultrasound pregnancy test was conducted on Days 20, 25, 35 and 60. Reproductive status (non-pregnant, pregnant and animals with LEM) was determined by ultrasound and the results between different groups were compared. The PAGs mean values according to reproductive status and Day after AI were analysed. Accuracy, sensitivity and specificity of the PAGs test for pregnancy diagnosis on Day 35 were calculated. On Day 20 after AI the pregnat sheep (83.3%) in group I tended to be higher than those (77.1%) in group II with decrease of 25.7% and 20% for the same groups on Day 25. On day 25 LEM was recorded in 33.3% and 24% in group I and II, respectively. The ultrasound exams on Day 60 confirmed the results from Day 25 after AI. A total value of non-pegnant, pregnant animals and LEM was 20%, 56.9% and 28.8%, respectively. The mean values of PAGs in animals with the same reproductive status in group I and II no differed statistically between Days 4 and 35 after AI. On Day 25 the PAGs values in LEM groups (0.126 ± 0.072 and 0.179 ± 0.029) were higher than those (0.062 ± 0.038 and 0.083 ± 0.023) in the non-pregnant groups, but no significan difference was deternined. On Day 35 after AI the mean values of PAGs (0.414 ± 0.125 and 0.421 ± 0.121) for the pregnant groups were significantly ($P < 0.05$) higher than those (0.078 ± 0.053 and 0.093 ± 0.034) for the non-pregnant groups. The values of PAGs in LEM groups on Day 25 (0.099 ± 0.062 and 0.113 ± 0.058) were decreased and close to the mean values in non-pregnant sheep. A significant effect of the day after AI on the PAGs values was evidenced in pregnant animals only (control r = 0.97 and GnRH r = 0.98; $P < 0.05$). The PAGs started to rise rapidly between Days 25 and 35 after AI. On Day 35 the accuracy (98%), the sensitivity (97.3%) and the specificity (100%) of the PAG test for pregnancy diagnosis were similar to the values (100%) for ultrasound method.

***Discussion*:** The gonadotropin treatment on Day 4 after AI tended to improve the reproductive performance in the sheep, but no significant effect of GnRH on the PAGs values in the animals with different reproductive status was found. The PAGs profiles in non-pregnant and LEM sheep were close and distinguishing between non-pregnant and LEM ewes on Day 35 was impossible. The PAGs values in pregnant sheep showed significantly ($P < 0.05$) increase between Days 25 and 35 after AI with higher mean values in pregnant than non-pregnant groups on Day 35 ($P < 0.05$). Alertys Ruminant Pregnancy test was a reliable for pregnancy detection in sheep on Day 35 after artificial insemination.

Keywords: sheep, GnRH, PAGs, reproductive status, pregnancy.

DOI: 10.22456/1679-9216.129955

Received: 25 March 2023 | Accepted: 10 July 2023 | Published: 8 August 2023

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INTRODUCTION

The measurement of PAGs by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) is commonly recommended as an alternative method of progesterone and ultrasonography for pregnancy diagnosis in sheep [8,13,18]. Recently, the pregnancy-associated glycoproteins (PAGs) have been investigated extensively in respect to pregnancy and embryonic mortality detection in sheep [1,3,7,8]. Different authors [11,14,19] used commercial ELISA tests conclude that accurately pregnancy detection and the ability to predict embryo survival during early gestation are antibody dependent, and influence by age, breed and number of embryos/fetuses. Several studies [11,13,21] have conducted to detect or predict ruminants at risk of late embryo mortality on the base of PAGs assay. Hormonal control and genetic factors contribute to ovulation and preimplantation events responsible for embryonic health [6]. A few researches determine a positive effect of GnRH treatment beween Days 4 and 12 after AI on embryo loss in sheep [5,9,12]. A single injection of GnRH on Day 35 after AI resulted in increase of PAG concentration and recovery of embryo wellbeing in buffaloes, allowing a reduction in pregnancy loss during the early pregnancy [15]. It seemed that the luteotropic effect of exogenous GnRH may add to the luteotropic effect of PAGs in supporting the pregnancy in animals considered at risk of low embryo survival. The information about combined use of PAGs assay and GnRH administration as a tool to improve reproductive status and to reduce embryonic mortality in sheep is confined.

The current study aimed to evaluate the effect of GnRH administration on Day 4 after artificial insemination on the pregnancy-associated glycoproteins in dairy sheep with different reproductive status in relation to pregnancy and late embrionic mortality detection.

MATERIALS AND METHODS

Animals

The experiment was carried out with 65 sheep from East Friesian breed at the end of lactation, housed in intensive sheep farms, located in south Bulgaria. Animals were aged from 2 to 3 years, body weight 65-70 kg, and housing technology in group boxes. The feeding was in accordance with the recommendations for breed, age and lactating status of the animals with water intake *ad libitum*. The study was conducted during the breeding season (September - October). All procedures were in an agreement with the requirements for welfare and animal's protection included in Bulgarian legislation.

Estrus synchronization and artificial insemination, ultrasound examination, PAGs assay and reproductive status registration

Both groups were subjected to estrus synchronization by intravaginal sponges (Syncro-part® - 30 - 30 mg flurogeston acetate)[1] for 12 days and intramuscular injection of equine chorionic gonadotropin (Folligon® - 500 UI, 2.5 mL, i.m)[2] on Day of a sponge removal. Artificial insemination with fresh-diluted semen was conducted from 52 to 58 h after the sponge withdrawal. The fresh-diluted semen of 0.2 mL with 300 x 10^6 motile sperms was deposited deep cervically by the experienced operator. Group I was not treated while group II received (Ovarelin® - 50 µg gonadorelin as diacetate, 1 mL, i.m.)[1] on Day 4 after AI.

Ultrasound examination on Days 20, 25, 35 and 60 was conducted by ultrasound scanner SonoScape S2 Vet[3] and multifrequency (7-12 MHz) linear transducer. A transrectal and transabdominal approach were used on Days 20 and 60, respectively. On Days 25 and 35 all sheep were subjected firstly to a transabdominal ultrasography. In case of negative pregnancy result, a transrectal ultrasonography was performed. On the base of ultrasound data sheep were recoded as non-pregnant, pregnant, and sheep with late embrionic mortality. A positive pregnancy diagnose on Days 20 and 25 was set in visualization of echogenic embryo, located in echogenic uterine lumen, while on Day 60 it was recorded in observation of fetus with visible cardiac activity and placentomas. The cases with a lack of embryo after previous detected pregnancy were considered late embryonic mortality. It was calculated on the base of ultrasound results on day 20, 25, 35 and 60 after AI and presented as a percentage of not confirmed positive pregnancy diagnoses, compared to all positive diagnoses detected in the previous ultrasound exams.

Blood samples for PAGs measurement were collected on Days 0, 12, 20, 25 and 35 after artificial insemination. The serum from the coagulated blood samples was separated by centrifugation (3000 *g* for 15 min) and stored in a sterile tube at -200°C until analysis. PAGs concentrations were measured by ELISA using a commercial Alertys Ruminant Pregnancy Test[4], following the manufacturer instruction. The results of the assays were measured as optic density (OD) of the sample, corrected by subtracting OD of the sample (S) and the OD of the negative control (N) measured with 450 nm wavelength in interpretation of the final result as S-N. When S-N value for each sample were equal or greater than 0.300 the animal was recorded pregnant and less than 0.300 non-pregnant [19]. Two non-pregnant animals in group I with minus S-N values were counted to zero in the statistical calculation. An accuracy, sensitivity and specificity of PAGs test for pregnancy determination on Day 35 were recorded on the base of obtained ultrasound results on Day 60 after AI.

Statistical analysis

The results were processed by a computer program Statistica version 7.0 (Stat-Soft. - 1984-2000)[5]. Values of non-pregnant animals, pregnancy rate and late embryonic mortality in different groups were presented in a percentage and compared by non-parametric method for comparison of proportions, using Student's *t*-criterion. The PAGs values (mean ± standard deviation) of control and GnRH group and according to reproductive status (pregnant, non-pregnant, late embryonic mortality) and Day after AI were compared by ANOVA and post-hoc Tukey-test. The effect of the Day after AI on the PAGs values was determined by correlation analysis. Differences were considered significant at $P < 0.05$ level.

RESULTS

During the examination on Day 20 after AI, a percentage of pregnant sheep in the GnRH treated group (83.3%) tended to be higher than those (77.1%) in the control group, but no differed significantlly ($P > 0.05$) [Table 1]. The percentages of non-pregnant sheep in both groups (22.9% and 16.7%) were the same until the last ultrasound exam on Day 60 after AI. On Day 25 after AI the pregnant aninals in both groups decreased with 25.7% and 20% and reached 51.4% and 63.3% for in 1st and 2nd group, respectively. Ultrasonography on Day 25 was indicative for 33.3% and 24% late embryonic death in control and GnRH injected sheep, respectively. The ultrasound exams in both group on Day 60 confirmed the results obtained on Day 25 after artificial insemination. A total value of non-pegnant, pregnant aninals and LEM was 20%, 56.9% and 28.8%, respectively.

The mean values of PAGs in animals with the same reproductive status in the control and the GnRH group no differed statistically between Days 4 and 35 after artificial insemination (Figure 1). On Day 25 the mean values of the PAGs for both groups with late embryonic death (0.126 ± 0.072 and 0.179 ± 0.029) were higher than those (0.062 ± 0.038 and 0.083 ± 0.023) in the non-pregnant groups, but significance of the differences was not registered. On Day 35 after AI the mean values of PAGs for pregnant animals in groups I and II (0.414 ± 0.125 and 0.421 ± 0.121) were significantly ($P < 0.05$) higher, compared to the means obtained (0.078 ± 0.053 and 0.093 ± 0.034) in the non-pregnant sheep. One pregnant animal in the control group had value (0.296) below the cut-off value (S-N ≥ 0.300) for pregnancy. The values of PAGs in the groups with LEM (0.099 ± 0.062 and 0.113 ± 0.058) were lower than the values detected on Day 25 without statistical significance, and were close to the mean values in non-pregnant sheep.

A correlation analysis showed significant effect of the day after AI on PAGs values in pregnant animals only (control r = 0.97 and GnRH r = 0.98; $P < 0.05$) [Figure 2B]. The PAGs started to increase on day 20 with rapid rise between Days 25 and 35. A similar effect of the Day after AI in the other groups was not recorded ($r \leq 62$; $P > 0.05$), but the PAGs in sheep with LEM decreased between Days 25 to 35 (Figure 2C). Additional analysis about an ability of Alertys Ruminant Pregnancy Test to distinguish non-pregnant and pregnant sheep on Day 35 after AI showed high accuracy (98%), sensitivity (97.3%) and specificity (100%) close to the obtained 100% for ultrasound method (Table 2).

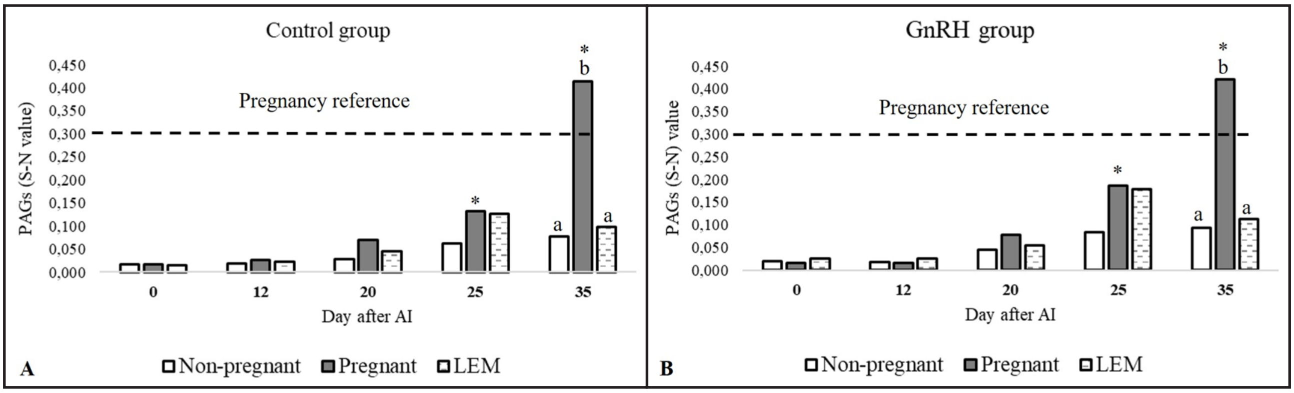

Figure 1. Pregnancy-associated proteins (mean ± sd) in control (A) and GnRH treated (B) sheep according to reproductive status and test pregnancy reference. [GnRH: gonadotropin releasing hormone; LEM: late embryonic mortality. Mean values with different superscripts at the same day differ at $P < 0.05$. Mean values at the same group marked with asterisks in different days indicate differences at $P < 0.05$].

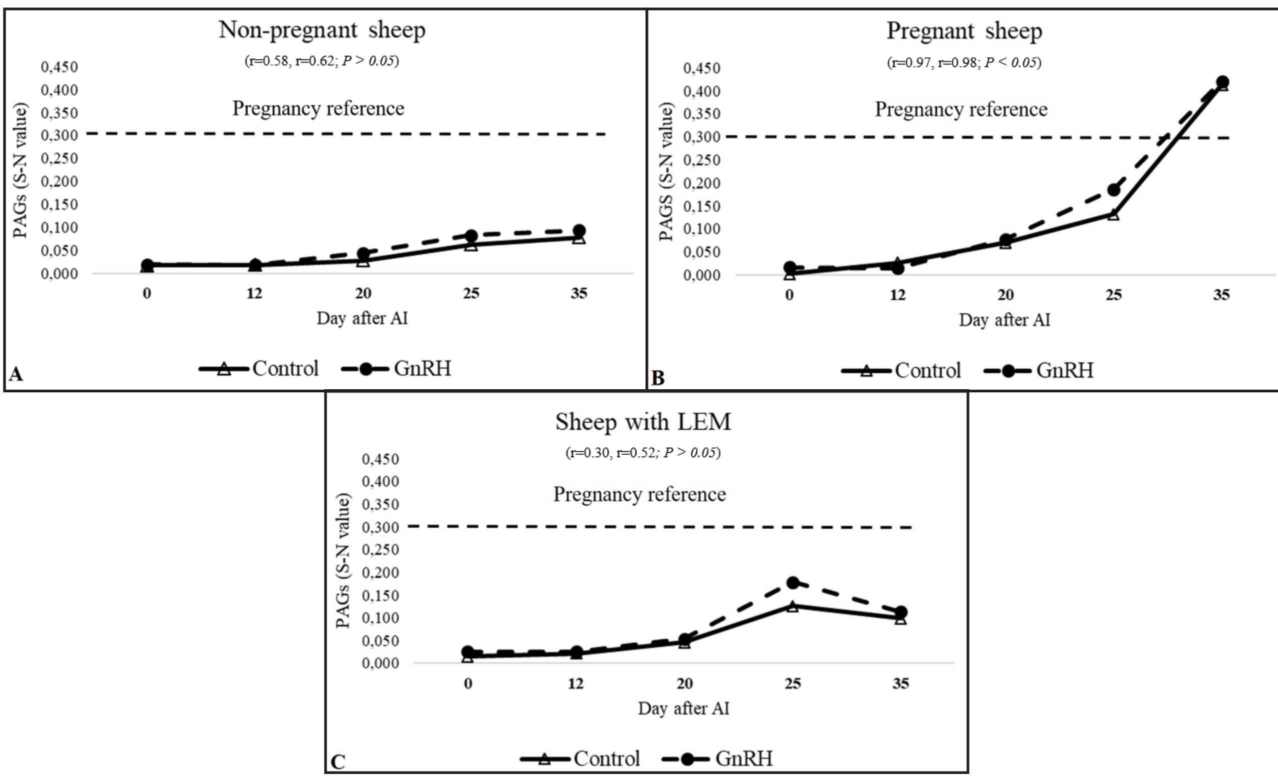

Figure 2. Pregnancy-associated proteins in non-pregnant (A) pregnant (B) and sheep with late embryonic mortality (C) in different groups according to Day after AI and test pregnancy reference. [GnRH: gonadotropin releasing hormone; LEM: late embryonic mortality; AI: artificial insemination]

Table 1. Reproductive status of sheep in control and GnRH group through different Days after AI based of ultrasound diagnoses.

Day after AI	Groups					
	Non-pregnant		Pregnant		Late embryonic mortality	
	Control n=35	GnRH n=30	Control n=35	GnRH n=30	Control n=27	GnRH n=25
	% (n)	% (n)	% (n)	% (n)	% (n)	% (n)
20	22.9 (8)	16.7 (5)	77.1 (27)	83.3 (25)	0 (0)	0 (0)
25	22.9 (8)	16.7 (5)	51.4 (18)	63.3 (19)	33.3 (9)	24 (6)
35	22.9 (8)	16.7 (5)	51.4 (18)	63.3 (19)	0 (0)	0 (0)
60	22.9 (8)	16.7 (5)	51.4 (18)	63.3 (19)	0 (0)	0 (0)
Total	20 (13/65)		56.9 (37/65)		23.1 (15/65)	

AI: artificial insemination; GnRH: gonadotropin releasing hormone.

Table 2. Sensitivity, specificity and accuracy of ultrasound method and Alertys Ruminant Pregnancy test for pregnancy detection on Day 35 after artificial insemination*.

	Sensitivity[1] % (n/n)	Specificity[2] % (n/n)	Accuracy[3] % (n/n)
Ultrasound	100 (37/37)	100 (13/13)	100 (50/50)
PAGs ELISA	97.3 (36/37)	100 (13/13)	98 (49/50)

*Adapted by Ricci *et al.* [23]. [1]Proportion of pregnant sheep with a positive ultrasound or PAGs outcome. [2]Proportion of not-pregnant sheep with a negative PAGs outcome. [3]Proportion of pregnancy status, pregnant and not pregnant, that was correctly classified by ultrasound and PAGs.

DISCUSSION

The efficacious of reproduction in sheep is extremely focused on pregnancy status of females after artificial insemination and reduction of embryofetal losses [2,10]. Different strategies for improvement of the reproductive performance include estrus synchronization and AI, ealrly pregnancy diagnosis by ultrasound or PAGs assy, and/or hormonal treatment for reduction of embryonic and fetal losses [9,18,19]. The PAGs are main proteins secreted by trophoblast cells of the pregnant ruminants and have identical structure and immunological properties in sheep and cattle [20]. They cross into maternal circulation by passive diffusion and are presented in detectable concentrations by RIA or ELISA as early as 18 - 20 days after conception [22,23]. During the pregnancy, the PAGs concentrations in small ruminants differ according to age, breed, fetal number and sex [14,16, 21]. It is important for veterinarians to recognize the limitations of reading results of the different PAGs test and potential for misclassification of open or pregnant animals associated with changes in PAGs secretion throughout pregnancy or persistence of PAGs in maternal circulation after pregnancy loss [18].

This study presents data regarding an effect of GnRH treatment on day 4 after artificial insemination on the PAGs in high prolific East Friesian sheep breed with diferent reproductive status. The insignificant differences in the PAGs values until Day 20 between not treated and GnRH treated sheep indicated no effect of the early GnRH administration on PAGs production during the first 20 days after AI, irrespective of their reproductive status. In this period the PAGs profiles in the sheeep with different reproductive status were close and there was no ability for distinguishing non-pregnant and pregnant ewes or to predict LEM occurred between Days 20 and 25 after AI. Aforementioned data was in agreement with the results of previous authors [8,19] that used a similar ELISA tests and determined pregnancy detectable concentrations of PAGs in ovine serum on days 28 or 33 post mating. However, De Carolis [7] and Meshref *et al*. [14] present information for pregnancy diagnosis on Day 18 and 16 after insemination, respetively. This discrepancy can be explaned by different methods and tests for PAGs measurement, differences in breed, age and number of embryos of the sampled sheep or individual characteristics of the animals. In this aspect, an investigation of the combinations of antibodies with a laboratory-based and a commercial ELISA test reports an antibody dependency of the test accuracy for pregnancy detection and prediction of embryo survival during early gestation in sheep [11]. A large individual variation of PAGs among ewes with single or multiple pregnancy have determined. Moreover, the breed and the gender of the fetus also could influence ovine PAGs production [7].

In the current research the Day after AI influenced PAGs profile in pregnant animals only, while in the non-pregnant and sheep with LEM occurred between Days 20 and 25, significant effect was not determined. A similar progressively rise of the PAGs from the day of conception until day 60 was observed in pregnant Sarda and Lacaune ewes [7]. The GnRH administration had not effects on the pregnancy-associated profile in non-pregnant animals and sheep with LEM between Days 4 and 35 after AI. It was confirmed by the low values of the correlation coefficients and a lack of significance ($P > 0.05$). Determination of discriminative value of PAGs for distinction of non-pregnant and animal with LEM on Days 25 and 35 was impossible, because of similarity between the values. However, it should be noted that in sheep recorded with LEM, the mean values of PAGs started to decline in a period of 25 - 35 days. It can be hypotized that decreasing in PAGs concentrations in 2 consecutive measurements could be indicator for an occurred or pending embryonic death. Hussein *et al*. [13] concluded that a decrease in the serum concentrations of PAGs between days 28 and 45 may be predictive for embryonic/fetal mortalities in cross-bred ewes with an average age of 21-28 months. Verberckmoes *et al*. [21] used RIA test based on ovine PAGs and determined that some Suffolk and Texel ewes showed an abnormal PAG profile between Days 25 and 35 of conception. The average ovPAG concentration of the these ewes at Day 25 was in pregnancy reference. In contrast to the normal pregnant ewes, they had significantly lower ovPAG concentrations at Day 35. On this base the authors accepted a homologous RIA as useful tool for the detection of late embrionic mortality in sheep. However, Roberts *et al*. [18] used commercial Bovine Pregnancy Test to detect PAG1 and BioPRYN assay to detect PSPB in pregnant sheep and determined unique gestational profiles based on assay specificities. They suggested that PAGs would not be useful predictors of fetal growth and development in sheep, but PAG1 can be effectively used as a predictor of fetal number in Polypay x Dorset cross-breeds. In our study the detection of LEM was based on ultrasound disgnoses. Animals with occured LEM were not recorded as pregnant on Days 25 and 35, because they had PAGs values below the pregnancy reference of used PAGs test. On the other hand, the PAGs concentration in getational period differed according to the breed and between singleton and multiple pregnancies [1,7]. Future studies with a large number of sampled animals can explore an effect of different factors (age, breed, embryo/fetus number etc.) on the accuracy of the comersial ELISA PAGs tests for prediction of embrionic mortality in sheep [3,18]. Adaptation of the used method and PAGs tests according the specific influence factors may inprove their accuracy to predict embryo mortality in sheep. Determination of correct predictive values to an acceptable point for use in applied reproductive management can provde the PAGs to be utilized as a diagnostic tool in order to improve farm reproductive management through intervention strategies for ruminants identified to be at risk for embryo loss [4].

The information about an effect of early gonadotropin adninistration on the survival of embryo or fetus is contradictory. Many questions related to controlling of embrionic mortality in sheep remain an area with scope for improvement [6]. Hashem *et al*. [12] used a single dose of GnRH 7 Days post-mating and achieved successfully decrease of pregnancy loss from day 40 after mating to parturition. No effect on pregnancy rate and embryo after administration of hCG or GnRH on Day 4 post AI was determined by Fernandez *et al*. [9], but the treatment with hCG reduced pregnancy losses at Day 33 post AI. The ultrasound data indicated a tendency to increasing the pregnant sheep and reducing the late embryonic mortality occurred between Days 20 to 25 in the GnRH treated group. Other studies also report a positive effect of GnRH administration between Days 4 and 12 post mating on pregnancy rate and emryo survival [5,9,13]. The beneficial effect of GnRH was explained with enhancement in luteal activity of current CL in the ovaries and formation of accessory corpora lutea, resulting in an increase in circulating progesterone. The altetered progesterone leves lead to changes in the uterus necessary for embryo or fetal growth and development, thus prevent the embryo mortality [9]. The obtained total values for pregnancy and LEM in East Friesian sheep were close to reported total pregnancy rate (66.7%) and embryo losses (23.1%) in Rahmani ewes subjected to a similar treatment [12]. Regardless of abovementioned data, additional experiments are needed to better understanding of time and dose dependent effects of gonadotropins on embryo mortality in sheep.

CONCLUSIONS

Administration of gonadotropin on Day 4 after artificial insemination tended to impove the reproductive performance in sheep, but no significant effect of GnRH on the mean PAGs values in the animals with different reproductive status was found. The PAGs values in pregnant East Friesian sheep showed significantly ($P < 0.05$) increase between Days 25 and 35 after AI with higher mean values in pregnant than non-pregnant groups on Day 35 ($P < 0.05$). Alertys Ruminant Pregnancy test was a reliable for pregnancy detection in sheep on Day 35 after artificial insemination.

MANUFACTURERS

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***Ethical approval*.** The experiment was approved by the National Ethics Committee for animals in accordance with the Bulgarian legislation (Ordinance No 20/1.11.2012) on the minimum requirements for protection and welfare of experimental animals and requirements for use, rearing and/or their delivery (Approval №3610/18.02.2021).

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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