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Estrus synchronization protocols for fixed-time artificial insemination (FTAI) during non-breeding season in ewes

Protocoale de sincronizare a ciclurilor sexuale în vederea însămânțării artificiale la moment fix (FTAI) în afara sezonului de reproducție la ovine

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ABSTRACT

This study aimed to compare three protocols used for induction and estrus synchronization followed by fixed-time artificial insemination (FTAI) during the non-breeding season in anestrous Cluj Merino ewes. Estrus was synchronized in 80 healthy multiparous ewes using intravaginal sponges containing 20mg of fluorogestone acetate (FGA) for 14 days. Ewes were previously divided into three groups. At the time of sponge removal, ewes in groups II and III received a dose of 500 IU eCG, while those in Group I received a half dose, respectively 250 IU eCG, followed by the administration of 50 µg of GnRH 30 hours later. Ewes from Group III were previously implanted with subcutaneous mini-implants of melatonin, 20 days before progesterone (P4) treatment. We could not find significant differences in terms of reproductive performances between Group I and Group II, with results showing that Group I had a conception rate of 47.05%, which is lower than that of Group II (56.52%). However, there were significant differences ($P < 0.05$) between melatonin-supplemented Group III and Group I (63.33% vs. 47.05%). Thus, the potential substitution of eCG with GnRH still needs to be optimized, while supplementation with melatonin can increase the conception rate during the non-breeding season in anestrous ewes.

Keywords: estrus synchronization, non-breeding season, melatonin, FGA, GnRH, eCG

REZUMAT

Scopul acestui studiu a fost acela de a compara trei protocoale de inducere și sincronizare a ciclurilor sexuale, urmată de însămânțare artificială la moment fix (FTAI) în afara sezonului de reproducție, la ovine din rasa Merinos de Cluj aflate în anestru sezonier. Estrul a fost sincronizat la 80 de oi multipare sănătoase, folosind bureți intravaginali care conțin 20 mg de acetat de fluorogeston (FGA), timp de 14 zile. Femelele au fost repartizate anterior în trei loturi experimentale. La momentul îndepărtării bureților, oilor din loturile II și III le-a fost administrată o doză de 500 UI eCG, în timp ce cele din lotul I au primit o jumătate de doză, respectiv 250 UI eCG, urmată de 50 µg de GnRH, 30 de ore mai târziu. Ovinelor din lotul III le-au fost aplicate anterior mini-implanturi subcutanate de melatonină, mai exact cu 20 de zile înainte de începutul tratamentului cu progesteron (P4). Nu am putut constata diferențe semnificative în ceea ce privește performanțele reproductive între lotul I (47,05%) și II (56,52%). Cu toate acestea, au existat diferențe semnificative ($P < 0,05$) între lotul III, suplimentat cu melatonină, și lotul I (63,33% față de 47,05%). Astfel, potențiala subtituire a eCG cu GnRH trebuie încă optimizată, în timp ce suplimentarea cu melatonină poate crește rata de concepție în afara sezonului de reproducție la ovine aflate în anestru sezonier.

Cuvinte cheie: sincronizarea ciclurilor sexuale, extrasezon, melatonină, FGA, GnRH, eCG

INTRODUCTION

Induction and estrus synchronization using hormonal treatments is of great interest to sheep farmers because it can increase a farm's income and boost genetic gain. Similar to other domestic species, sheep's reproductive management is frequently based on the induction and synchronization of estrus and ovulation, either during breeding or non-breeding seasons, for natural mating or artificial insemination (Abecia et al., 2012).

Wild animals use the seasonality of reproduction as an adaptive physiological strategy to acclimate to seasonal variations in temperature and food availability (Malpaux et al., 1996). The majority of sheep, goats and horses breeds that originated in temperate latitudes have retained this adaptation, whereas domestication has almost entirely eliminated it in cattle and pigs. Sheep (*Ovis Aries*) are seasonally polyestrous short-day breeders.

The diurnal cycle, or photoperiod, is a crucial environmental signal that determines when reproductive transitions occur in seasonal mammals (Lincoln and Short, 1980). In contrast to the long day lengths of spring and summer, which inhibit the reproductive axis in female sheep and result in an anovulatory state (Weems et al., 2015), the short day lengths of autumn and winter are stimulatory to the reproductive neuroendocrine axis, causing ovulatory cycles and fertility (Goodman et al., 2010). Plasma melatonin levels are highest at night and lowest during the day, and the fluctuating length of nocturnal melatonin secretion is a passive signal that informs the hypothalamus-pituitary-gonadal axis about the season (Abecia et al., 2011). The suprachiasmatic nuclei and superior cervical ganglia are involved in a multi-step neural pathway that transmits photoperiod information from the retina to the pineal gland, which then modulates the rhythm of melatonin secretion (Karsch et al., 1984).

Exogenous hormones that alter the physiological chain of events involved in the sexual cycle can be used to control sheep reproduction, but some "natural methods" such as light control (Yeates, 1949) or exposure to a male, also known as "ram effect" (Ungerfeld et al., 2004) are also used.

Ever since the first attempts to control the reproductive function in sheep via pharmaceutical methods (Dutt et al., 1948), there has been a special interest for a constant improvement of the protocols used, both during the breeding (Koyuncu and Altıcekic, 2010; Titi et al., 2010; Olivera-Muzante et al., 2011; Hasani et al., 2018; Martinez-Ros and Gonzalez-Bulnes, 2019; Yu et al., 2022;) and non-breeding season (Hashemi et al., 2006; Reyna et al., 2007; Payan et al., 2022). Also, some studies have compared the results obtained during the breeding season with those obtained outside the breeding season (Jackson et al., 2014; Santos-Jimenez et. al., 2020). With regard to the challenge of detecting estrus in these species, the use of hormones to induce heat has allowed for increased fixed-time artificial insemination (FTAI) in small ruminants, a very helpful management tool.

Hormones like progesterone or its analogs (progestagens) and prostaglandins can be administered to regulate the luteal phase of the cycle, whereas melatonin works by altering how the organism perceives the photoperiod and the annual pattern of reproduction (Abecia et al., 2012). Also, to stimulate estrus behaviour and folliculogenesis, equine chorionic gonadotropin (eCG) is administered after removing the progesterone sponge or device. Thus, eCG is used to regulate the follicular phase of the sexual cycle (García-Pintos and Menchaca, 2016).

Being described for the first time in the late 1950s (Lerner et al., 1958), melatonin began to be used in the 1980s in the UK, Australia, and New Zealand, in order to manipulate the seasonality of reproduction by using subcutaneous mini-implants (Palacin et al., 2011). In the last ten years, several studies have tried to test the effectiveness of melatonin administration in the non-breeding season, both on the reproductive performances of ewes (Cevik et al., 2017; El-Mokadem et al., 2019; Berean et al., 2021) and rams (Casao et al., 2013; Egerszegi et al., 2014; Cevik et al., 2017; Pool et al., 2020).

Also, studies from recent years have shown that melatonin can improve post-thaw sperm progressive motility and DNA integrity of rams (Pool et al., 2020), and in ewes, exogenous melatonin directly and indirectly influences oocytes quality (Chen et al., 2022).

Thus, this study aimed to compare three protocols used for induction and estrus synchronization followed by FTAI during the non-breeding season in anestrous Cluj Merino ewes. The effect of replacing half of the dose of eCG with GnRH, but also the influence of supplementing protocols with subcutaneous mini-implants of melatonin was tested.

MATERIAL AND METHODS

Animals and experimental design

This study was carried out in the non-breeding season (May-June 2023), at the USAMV Experimental Didactic Station, Hoia (latitude: 46°46'41.9"N; longitude 23°32'39.6"E; altitude: ~350 m). Healthy multiparous ewes (n = 80; Cluj Merino breed; 2–5 years old, mean body score of 2.5 ± 0.5), were divided into three groups. Each group was carefully structured to ensure an approximately equal distribution of age among the ewes within each respective group. The ewes lambed in February 2023, and the lambs were weaned approximately 60 days after lambing. The semen used for FTAI came from two healthy rams.

On day 0, all ewes received one intravaginal FGA-impregnated sponge (20 mg fluorogestone acetate, FGA, Chronogest®; MSD Animal Health, Madrid, Spain). For all three groups (Groups I, II, III), sponges were kept for 14 days. Additionally, ewes from group III were previously implanted with subcutaneous mini-implants of melatonin (Melovine® 18mg; CEVA santé animale) on day -20.

At the time of sponge removal, all animals in each group were administered a dose of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analog, for its luteolytic effect. Briefly, each ewe was injected intramuscularly with 250 µg of cloprostenol (Estrumate®, MSD Animal health). Additionally, to $PGF_{2\alpha}$, to stimulate estrus behavior and folliculogenesis, a dose of 500 IU eCG (Folligon®, MSD Animal Health) was administered to each sheep from groups II and III, with those in Group I receiving only half the dose (250 IU). After 30 hours from the time of sponge removal and hormonal treatments, ewes from Group I received a dose of 50 µg of gonadotropin-releasing hormone (GnRH; Ovarelin®, CEVA Santé Animale). Also, loss of sponge and vaginitis were recorded. Vaginitis was recorded when purulent discharge was presented at the time of sponge removal.

All the treatments performed in this study are presented in Figure 1.

Follicle diameter measurement

After 48 hours from the time of sponge removal, right before AI, the follicular diameter of each ovary was measured. The condition to be admitted to artificial insemination was that each ewe had at least one medium-sized follicle with a minimum diameter of 4 mm as described by Evans et al., 2000. The diameter of the follicles was determined by transvaginal ultrasonography with an endocavity transducer with a frequency of 5 – 10 MHz using a high-frequency standard ultrasound (US) machine (Vinno 6, Vinno Corporation, Suzhou, China).

Semen Collection, analysis and dilution

In order to boost fertility and DNA integrity, rams were also previously implanted with subcutaneous mini-implants of melatonin (Melovine® 18mg; CEVA santé animale) on day -20. Also, to facilitate semen collection, a dose of 250 µg of cloprostenol (Estrumate®, MSD Animal health) was administered 30 minutes before collection. Semen was collected from two Cluj Merino rams with both artificial vagina and electro ejaculator. The average ejaculation volume of each ram was 1.0–1.5 ml.

Semen concentration and dilution ratio were determined using a spectrophotometer (SDM5, Minitüb, Tiefenback, Germany). Sperm motility was determined using a phase contrast microscope (Olympus BX43; Olympus Co, Tokyo, Japan), and the semen with spermatozoa motility over 85% was permitted to be used. Semen was diluted with Tryladil® extender supplemented with 20% egg yolk in order to reach a final concentration of 800 x 106 spermatozoa/ml.

Artificial insemination (AI)

Cervical artificial insemination was performed with 0.2 ml of diluted semen (200×10^6 sperm/dose) with the insemination gun (Mintüb, Tiefenback, Germany), using a duckbill speculum and an external light source, while ewes were restrained with the hindquarter upwards on the AI cradle (Figure 2). AI was performed using fresh semen, immediately after dilution. Spermatozoa mobility was also evaluated one hour after the insemination of the last ewe, and the mobility remained >80%. We could not observe any notable difference regarding the quality of the semen between the two rams.

Transvaginal ultrasonography for pregnancy diagnosis

After 35 days from AI, the transvaginal ultrasonographic examination was performed using high-frequency US machine (Vinno 6, Vinno Corporation, Suzhou, China) equipped with a endocavity transducer with a frequency of 5 – 10 MHz. Ewes were restrained in a standing position by an assistant. The probe was lubricated with ultrasound gel and was gently introduced into the vagina at a 45° of upward angle, then forward and straight. The entire pelvic cavity was visualized with 90° rotations. If visualization was not proper, the abdominal wall of the animal was then lifted with the other hand. Pregnant ewes were determined by the detection of embryonic vesicles.

Pregnancy Outcomes

Pregnancy was identified in all ewes subjected to AI by detecting the presence of a conceptus 35 days post-AI by transvaginal ultrasonography. As a result, we were able to calculate conception rate = (no. of ewes identified as pregnant on day 35/no. of ewes mated) × 100.

Hormonal treatments cost

Drugs cost used for each group was calculated to determine the most cost-effective protocol. Hormonal prices have been established in accordance with the market price at the time this study was carried out.

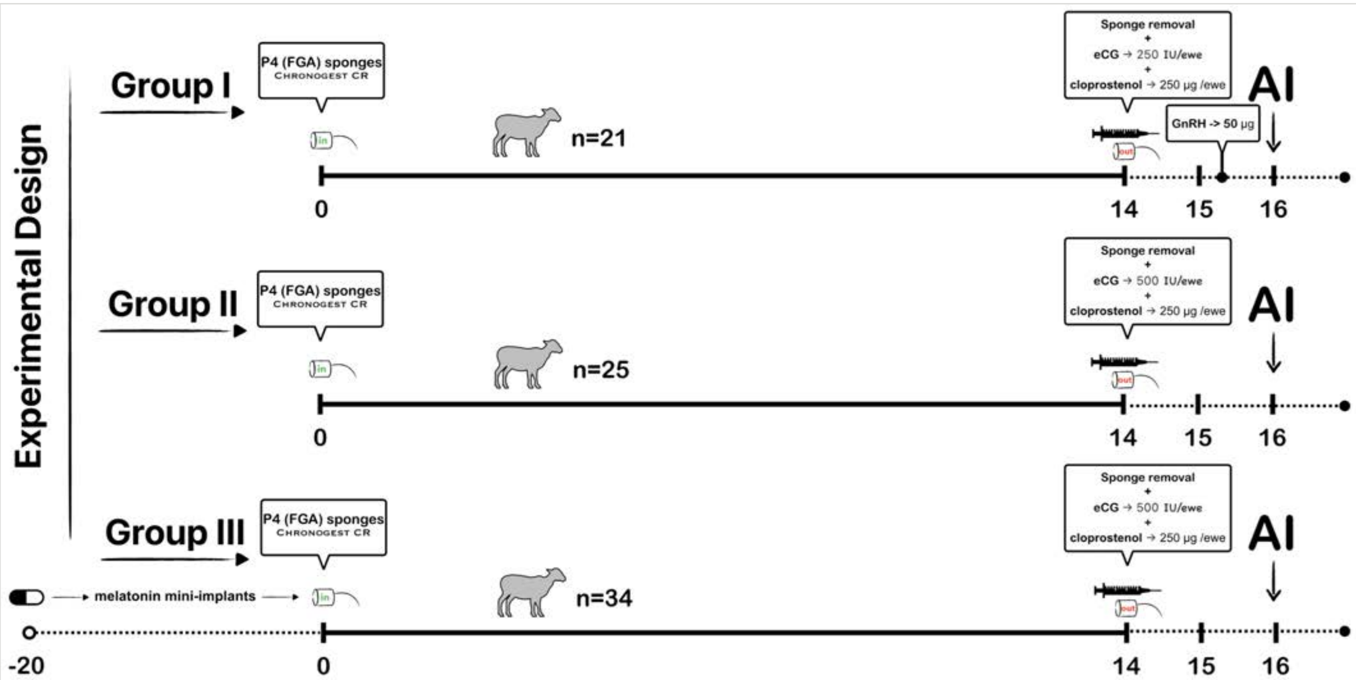

Figure 1. Experimental design in three groups of Cluj Merino ewes

Figure 2. Cervical artificial insemination

Statistical Analysis

Data was statistically analyzed using GraphPad Prism (Version 9.3.1). An exact Fisher's test was performed in order to compare the conception rate among the three protocols used.

RESULTS

At the time of sponge removal, the losses were recorded, and before AI, vaginal visual inspection was performed. There were no losses of sponges, and the rate of vaginitis was between 0.00% and 4.76% among groups. The reproductive performance of ewes is presented in Table 1.

Since the condition to be admitted to artificial insemination was that each ewe had at least one medium-sized follicle with a minimum diameter of 4 mm, all ewes that did not meet this requirement were not subjected to AI (Figure 3). Therefore, the highest rejection percentage for AI was identified within Group I (15%), while in Group II only 8% were not inseminated.

After 35 days from AI, the transvaginal ultrasonographic examination was performed in order to determine the conception rate. Consequently, the percentages determined for the conception rate were the highest in Group III (63.33%), while in Group I were the lowest (47.05%).

Table 1. Reproductive performance of ewes

Indexes	I	II	III
Vaginitis	4.76% (1/21)	0.00% (0/25)	2.94% (1/34)
Sponge loss	0.00% (0/21)	0.00% (0/25)	0.00% (0/34)
Ewes subjected to AI	85.00% (17/20)	92.00% (23/25)	90.90% (30/33)
Conception rate	47.05% (8/17)^{a}	56.52% (13/23)^{a,b}	63.33% (19/30)^{b}

^{a,b} – Values in the same row with different superscripts indicate that there is a significant difference (P < 0.05).

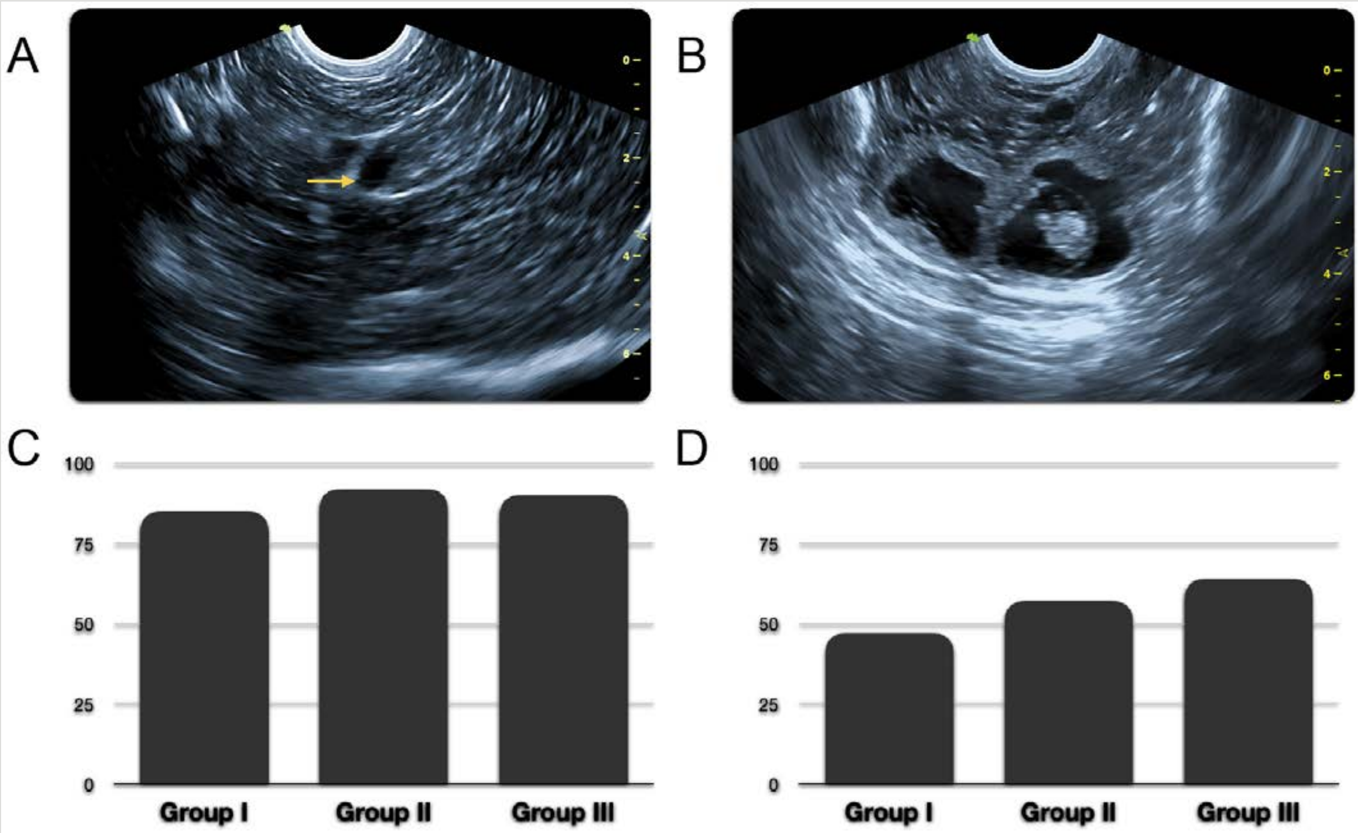

Figure 3. (A) Transvaginal ultrasonography for follicle diameter measurement (the yellow arrow indicates a medium-sized follicle); (B) Transvaginal ultrasonography for pregnancy diagnosis; (C) No. of ewes subjected to AI (%); (D) Conception rate (%)

Significant differences in terms of conception rate were found only between groups I and III ($P < 0.05$). Regarding the number of ewes admitted to AI after follicle diameter measurement, we could not identify significant differences between groups. Likewise, there were no significant differences in terms of sponge losses and vaginitis rate.

The costs of hormonal treatments reported per ewe are presented in Table 2. The cheapest protocol was the one determined for Group I (6.92 €/ewe), and the most expensive was the one used for Group III (13.60 €/ewe), as expected. Regarding the price for a successful conception per ewe, the lowest cost was recorded for Group II (14.41 €/conception), and the highest was for Group III (21.48 €/conception).

Table 2. Hormonal drugs (€/ewe)

Group	I	II	III
FGA	2.29	2.29	2.29
$PGF_{2α}$	1.16	1.16	1.16
eCG	2.35	4.70	4.70
GnRH	1.12	-	-
Melatonin	-	-	5.45
Total	6.92	8.15	13.60
Cost-effectiveness*	14.70	14.41	21.48

* – Cost-Effectiveness (€ per successful conception) = total cost/conception rate

DISCUSSION

In this study, we wanted to test two hypotheses, namely if GnRH can substitute half of the dose of eCG, as well as the effectiveness of supplementing the protocol with subcutaneous mini-implants of melatonin on the reproductive performance of ewes. In recent years, the possibility of replacing eCG with GnRH has been intensively studied (Luther et al., 2007; Martemucci and D'Alessandro, 2011; Sirjani et al., 2012; Lone et al., 2016; García-Pintos and Menchaca, 2018; Martinez-Ros and Gonzalez-Bulnes, 2019; Santos-Jimenez et al., 2020; Yu et al., 2022). Most of these studies concluded that GnRH can represent a viable alternative for replacing eCG used for induction and estrus synchronization in sheep.

The results of our study showed that by substituting half of the dose of eCG with GnRH the conception rate was not strongly affected during the non-breeding season, resulting in non-significant differences between Group I and Group II. Thus, Group I (GnRH and eCG) had a conception rate of 47.05%, which is lower than that of Group II (eCG; 56.52%).

Considering that in our study we used the FTAI technique, optimizing the GnRH dose administration time can improve the results. Since 15% of the ewes in Group I did not have at least one medium-sized follicle with a minimum diameter of 4 mm at the time of FTAI, compared to only 8% in Group II, a possible cause can be represented by the too-early administration of GnRH, which could lead to early ovulation. So, it is possible that an increase in the time interval between the dose of eCG and that of GnRH (>30 hours) can improve the results of the protocol. Thus, more studies are needed to determine the optimal moment of GnRH administration when used in combination with eCG.

Melatonin treatment has beneficial effects on reproductive function, such as reproductive recovery (Mura et al., 2017), proliferation and apoptosis of granulosa cells (Fu et al., 2014), *in vitro* oocyte maturation (Tian et al., 2017) and embryo development (Casao et al., 2010) and also maternal recognition of pregnancy (Abecia et al., 2019). All these beneficial effects lead to an improvement in reproductive performance when the induction and estrus synchronization protocol is supplemented with melatonin (Abecia et al., 2007; deNicolo et al., 2008).

Thus, regarding our second hypothesis, namely the effectiveness of supplementing the protocol with subcutaneous mini-implants of melatonin on the reproductive performance of ewes, Group III showed the best results in conception rate, 63.33%. From a statistical point of view, there were significant differences between Group III and Group I ($P < 0.05$). These differences once again confirm the beneficial effect of melatonin when used for induction and estrus synchronization protocols for FTAI during the non-breeding season in anestrous ewes. Nevertheless, subcutaneous mini-implants of melatonin had beneficial effects on the fertility of rams, as was shown in other studies (Casao et al., 2013; Egerszegi et al., 2014; Cevik et al., 2017; Pool et al. al., 2020).

The total costs of the pharmaceutical products used for Group I (6.92 €/ewe), Group II (8.15 €/ewe) and Group III (13.60 €/ewe) revealed that the prices were directly proportional to the performance in terms of conception rate. Considering the costs of each synchronization protocol per ewe related to the conception rate, Group II seems to be the most advantageous, the price for obtaining a pregnancy being the lowest (14.41 €/conception) and the one for Group I was very close (14.70 €/conception). The highest cost was recorded in Group III (21.48 €/conception). Choosing the most advantageous protocol is influenced by the profit margins for each lamb.

This is because even if the highest cost per conception was recorded for the protocol used in Group III, considering that by using this protocol it is possible to obtain a higher number of pregnant ewes, the profit margins per lamb can substitute the disadvantages of higher cost per conception, by increasing the number of lambs obtained and the total farm income and profit.

CONCLUSIONS

All three tested protocols are suitable for induction and estrus synchronization for FTAI during the non-breeding season in anestrous ewes. As expected, supplementation with subcutaneous mini-implants of melatonin determined the highest conception rate (63.33%), compared to Group I (47.05%) and Group II (56.52%). GnRH has the potential to substitute eCG in the non-breeding season, but the timing of administration must be optimized in future studies (>30 hours after sponge removal). Considering the prices of the three protocols per ewe (I: 6.92 €/ewe; II: 8.15 €/ewe; III: 13.60 €/ewe), but also the cost per conception (I: 14.41 €/conception; II: 14.70 €/conception; III: 21.48 €/conception), choosing the most cost-effective protocol depends on the specific needs and possibilities of sheep breeders.

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