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Estrus Synchronization and Artificial Insemination with Fresh and Chilled Semen in Assaf Ewes

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Abstract

This paper aims to study the efficiency of two short-term progestagen (FGA vs. MAP) + eCG treatments in estrus synchronization and artificial insemination (AI) with fresh or chilled semen in Assaf ewes fertility rate. All ewes received a subcutaneous implant of exogenous melatonin 45 days before been treated with short-term progestagens + eCG. By June 1st, ewes were divided in two groups: half was treated with an intravaginal sponge impregnated with 20 mg of FGA and the other half with an intravaginal sponge impregnated with 60 mg of MAP. Progestagen treatments lasted for 6 days. At sponge withdraw, all ewes were injected with 750 IU of eCG. Ovarian activity was assessed by plasmatic progesterone levels before and after progestagens + eCG treatment. Semen was collected by electro ejaculation and extended with Andromed® or OviXcell®. AI was performed 55 hours after eCG administration with fresh or chilled semen. During AI several factors were assessed: vagina mucosa color and lubrication, external cervical *Os* type, cervical mucous viscosity, semen deposition place and seminal cervix outflow. Semen was deposited as deep as possible without distress or trauma cervix mucosa. All Assaf ewes presented cyclic activity before progestagen + eCG treatments (2nd fortnight of May). Short-term progestagen + eCG treatments were equally efficient (100.0%). About 76.5% of Assaf ewes were pregnant 41 days after AI. Fertility rate was influenced by external *Os* type, semen deposition place and seminal cervix outflow. However, this rate was not conditioned by vaginal color or lubrication, cervical mucus viscosity, semen preservation technic and semen extender.

Keywords

Sheep, Assaf, Estrus Synchronization, Artificial Insemination

1. Introduction

Reproduction control and AI hold several advantages to commercial flocks: they improve farm management [1] [2] [3] and production [4]-[9], reproduction data recording accuracy [4] [7], genetic progress and animals production value [4] [5] [6] [7] [10]-[19] and allow the use of temporary physic, physiology or behavior defective animals as breeders [7] [14], precocious detection of infertility or sub-fertility [5] [20], control sexual transmitted diseases [4] [6] [9] [10] [14] [15] [21] and improve profitability [4] [6] [7] [22]. Nevertheless this technic is scarcely used in the Northeast of Portugal due to breeders' age, poor education, size and scarce property ownership, tradition, among other factors.

2. Material and Methods

This study took place in Carviçais, Torre de Moncorvo County (Portugal), at the Mateus Lda. commercial farm (Latitude: 41˚10'N, Longitude: 6˚55'W and Altitude: 701 meters) between April 1st and July 20th, 2017.

Ewes were permanently raised indoors, fed in group with natural meadow hay (*ad libitum*) and supplemented with 1.0 kg/ewe/day of Lucerne hay and 1.0 kg/ewe/day of a commercial food product. During the milking process, twice a day, ewes were individually supplemented with 0.5 kg of a commercial food product.

Ewes were weighted in a cage scale and the body score was classified according to the Australian Body Score Condition (BSC) table [23].

2.1. Animals

Thirty-four ewes (primiparous: 33 and multiparous: 1) and two adult (2 - 3 years old) Assaf rams were used in this study. Last lambing happened 3 - 4 months earlier. All ewes were milked twice a day.

2.2. Ovarian Activity

Ovarian activity was assessed by plasmatic progesterone levels. Blood samples were collected to vacuum tubes with heparin by jugular vein puncture. After blood centrifugation at 3,000 rpm, for 15 minutes, at room temperature, blood plasma was collected to previously identified Eppendorf tubes and briefly preserved in an ultra-freezer at −70˚C. RIA assessed Progesterone plasmatic levels using a DPC® Gamma C12 scintillation counter and DiaSource® kits (*DiaSource ImmunoAssays* S.A., Louvain-la-Neuve, Belgium). Intra and inter assays mean coefficients were 7.7% and 15.8%, respectively.

2.2.1. Initial Assessment

At the second fortnight of May, blood samples were collected in the morning, every 3 - 4 days, to evaluate the initial physiology state of all ewes by progesterone blood plasmatic levels.

Ewes were considered in anestrous when, in all collected samples, progesterone plasmatic levels were below 0.5 ng/ml [24].

2.2.2. Post-Progestagens + eCG Treatments Assessment

The formation of the first *corpus luteum* (CL) post hormonal treatments was assessed by blood samples collection for 5 days. First collection took place 24 hours post eCG administration.

Ewes were considered to have formed the first CL when progesterone plasmatic levels rose over 0.5 ng/ml for the first time [24].

2.3. Hormonal Treatments

All ewes received a melatonin (18 mg) subcutaneous implant (Melovine, CEVA, Portugal) on April 1st. On July 15th ewes were divided in two groups: FGA (n = 17) and MAP (n = 17) (Table 1). FGA ewes were treated with an intravaginal sponge impregnated with 20 mg of fluorgestone acetate (Chrono-Gest®, Intervet, Portugal) and MAP ewes with an intravaginal sponge impregnated with 60 mg of medroxyprogesterone acetate (Sincro-Gest®, Laboratorios Ovejero, Spain). At the same time all ewes were injected i.m. with 100 μg of cloprostenol (Estrumate®, MSD Animal Health, Portugal), a Prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) synthetic analogous.

Progestagens treatments lasted for 6 days. At sponge withdraw (June 7th) ewes were injected i.m. with 750 IU of eCG/ewe (Intergonan®, Intervet, Portugal).

2.4. Semen Collection and Seminal Analysis

Semen was collected by electro ejaculation (Electrojac™ Ideal, Minesota, USA). Eight ejaculates were collected: 2/ram in the morning and 2/ram in the afternoon. Rams did not ejaculate for 3 days before collection. Ejaculates were transported to lab and placed in a water-bath at 37˚C (Neslab® RTE 221, Newington, USA). Semen extenders were also kept in the same equipment.

Each ejaculate was evaluated for volume, concentration and wave motility.

Table 1. Assaf ewes divided by the ovarian control activity treatment.

Assaf Ewes (n = 34)	
Melatonin Implant (18 mg)	
20 mg FGA + 100 μg of Cloprostenol (n = 17)	60 mg MAP + 100 μg of Cloprostenol (n = 17)
Intravaginal Sponge Removal + 750 IU eCG	

Only ejaculates with volume higher than 1.0 ml, good wave motility and a minimum concentration of 3.0×10^9 spermatozoa/ml were used. Each seminal dose was prepared to contain at least 200×10^6 spermatozoa.

2.5. Semen Doses

Morning collected ejaculates from the same male were well mixed before been divided into two tubes and extended (1:1) with Andromed® (Minitüb, Tiefenbach, Deutschland) or OviXcell® (IMV Technologies, L'Aigle, France). Ten minutes later extended semen was cooled from 37˚C to 15˚C, for 120 minutes, in a refrigerator water-bath (Neslab® RTE 221, Newington, USA). After been stabilized for 10 minutes, extended semen was packed in 0.25 ml French mini straws and sealed with polyvinyl chloride powder.

Afternoon collected ejaculates from the same male were well mixed before been divided into two tubes and extended (1:1) with Andromed® (Minitüb, Tiefenbach, Deutschland) or OviXcell® (IMV Technologies, L'Aigle, France). After been stabilized for 10 minutes at 37˚C, extended semen was packed into 0.25 ml French mini straws and sealed with polyvinyl chloride powder. AI started less than 30 minutes later.

2.6. Time Fixed Artificial Insemination

AI started 55 hours after eCG administration. Ewes began to be inseminated with fresh extended semen. One skilled technician performed AI.

Near half ewes treated with FGA + eCG were inseminated with fresh diluted semen and the other half with chilled semen. The same was done in ewes treated with MAP + eCG. Within both groups some ewes were inseminated with semen extended with Andromed® and the others with semen extended with OviXcell® (Table 2).

During AI the following factors were assessed: vagina mucosa color (Pale pink vs. Pink vs. Dark pink), vagina lubrication (Good vs. Poor), external cervical *Os* type (Figure 1), cervical mucus viscosity (Liquid vs. Viscose), semen deposition place (Vaginal vs. 1st fold vs. 2nd fold) and seminal cervical outflow (No outflow vs. Light outflow vs. Plentiful outflow).

Semen was preferentially placed as deep as possible in the cervical canal, but

Table 2. Fresh or chilled semen doses extended with Andromed® or OviXcell® used to AI treated ewes.

Assaf Ewes (n = 34)							
FGA + eCG (n = 17)				**MAP + eCG** (n = 17)			
Fresh (n = 8)		**Chilled** (n = 9)		**Fresh** (n = 8)		**Chilled** (n = 9)	
Andromed (n = 3)	**OviXcell** (n = 5)	**Andromed** (n = 6)	**OviXcell** (n = 3)	**Andromed** (n = 6)	**OviXcell** (n = 2)	**Andromed** (n = 3)	**OviXcell** (n = 6)

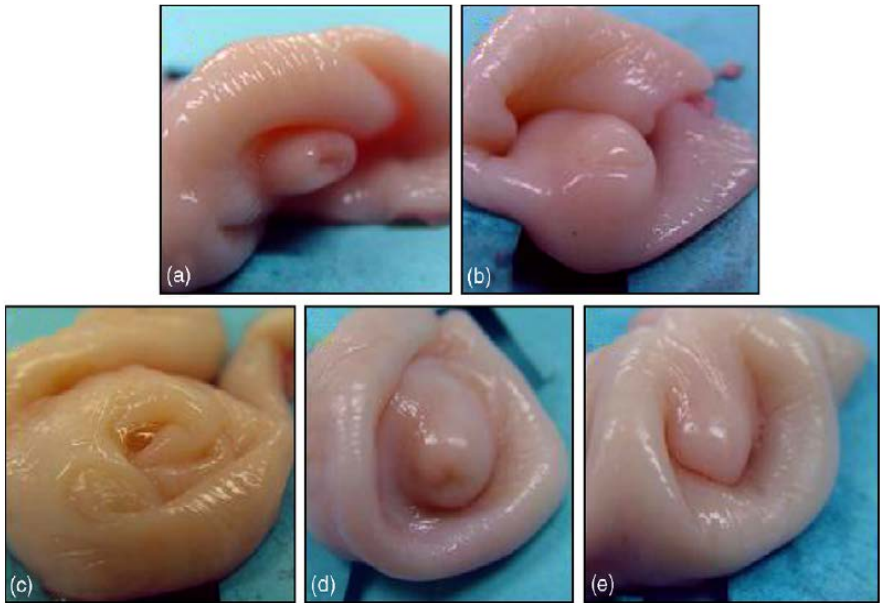

Figure 1. External cervical *Os* types: (a) Duckbill; (b) Slit; (c) Rose; (d) Papilla; and (e) Flap [25].

avoiding distress or trauma the mucosa. AI was performed using a vaginal speculum fitted with a white LED light and Quicklock® guns covered with a Minitub sheath (Minitüb, Tiefenbach, Deutschland).

2.7. Pregnancy Diagnosis

Forty-one days (20/07/17) after AI (09/06/17) pregnancy diagnosis were conducted by real-time ultrasonography, using a Mindray Z5Vet ultra-sounder and a multi-frequency rectal probe (5.0 - 10.0 MHz).

2.8. Statistical Analysis

Descriptive statistics are presented as mean ± standard deviation (coefficient of variation—c.v.). Data were statistically analyzed according to the ANOVA [26] and Bonferroni\Dunn test [27] to detect difference between means. Distributions of external *Os* types were analyzed using the Chi-square (χ^2) test [28]. All statistical analyses were performed using SAS Statistical Software, version 9.2 [29].

3. Results and Discussion

Assaf ewes were quite young - 2.0 ± 0.3 years old (c.v. = 15.2%). Age difference between FGA and MAP treated groups, inseminated with diverse semen extenders (Andromed® vs. OviXcell®), after semen preservation with unlike technics (Fresh vs. Chilled) was not significant (P > 0.05) (**Table 3**). These results are probably related to age homogeneity of all ewes.

Ewes weighted 69.4 ± 11.2 kg (c.v. = 16.1%) and presented a BSC of 3.5 ± 0.5 points (c.v. = 13.3%). Weight and BSC differences between FGA and MAP

Table 3. Mean age, body weight and body score condition (BSC) of Assaf ewes by hormonal treatment, semen extender and preservation technique.

	Age (years)	Weight (kg)	BSC (points)
FGA	1.9[a] ± 0.4	68.9[a] ± 11.0	3.5[a] ± 0.5
MAP	2.0[a] ± 0.0	69.9[a] ± 11.7	3.5[a] ± 0.4
Andromed®	1.9[a] ± 0.4	66.9[a] ± 12.3	3.4[a] ± 0.5
OviXcell®	2.0[a] ± 0.0	72.1[a] ± 9.5	3.5[a] ± 0.4
Fresh	2.0[a] ± 0.4	72.4[a] ± 13.6	3.7[a] ± 0.5
Chilled	1.9[a] ± 0.2	66,9[a] ± 8.2	3.3[a] ± 0.4

a = a, for P > 0.05 (between lines).

treated groups, semen extenders groups and preservation technics groups were not significant (P > 0.05). Assaf ewes presented a body weight and BSC suitable to breeding activity, regardless been at the 3 - 4th month of lactation. O'Brian [30], Scaramuzzi and Martin [31] and Karikariand and Blasu [32] says ewes should be bred with a BSC ranging between 2.5 - 3.0 points.

3.1. Physiological State Previous to Short-Term Progestagen Treatments

Before starting a reproduction control and AI program researchers should always check for ewes' ovarian activity [33]. At the second fortnight of May all ewes (n = 34) presented plasmatic levels of progesterone higher than 0.5 ng/ml, meaning they were cycling (100.0%). This outcome may reflect the pre-treatment of all ewes with melatonin implants or simply be the result of warmer environmental temperatures (late spring), more suitable to thermoregulation and consequently to reproduction activity [34] [35] [36] [37].

Age, body weight and BSC had no significant effect in the physiological state prior to short-term progestagen treatments + eCG (P > 0.05).

3.2. Ovarian Response to Short-Term Progestagen Treatments

Both short-term progestagen treatments + eCG were 100.0% efficient. Highly favorable body weight and BSC, warm environmental temperatures and prior ovarian cyclicity may have determined this result.

Progesterone plasmatic levels rose above 0.5 ng/ml for the first time around 28.9 ± 11.5 hours (c.v. = 39.7%) after eCG administration. Ovulatory response was rather fast, maybe due to the administration of 750 IU/ewe. eCG has been shown to reduce the interval between sponge withdrawal and estrus and improve the efficiency of synchronization of estrus and ovulation during the breeding season [38]. However, fertility rate tends to be maximum when AI is performed at the second half of the estrous, a few hours before ovulation [39].

Progestagens treatment (FGA—28.3 ± 12.7 hours vs. MAP—29.6 ± 10.5 hours) had no significant influence in this parameter (P > 0.05). In small rumi-

nants, FGA has a shorter half-life than MAP [40]. FGA induces an earlier return to ovarian cyclic activity (higher precision) than MAP, although, with no significant effect in estrus length [41]. Treatment with MAP sponges does not adequately synchronize estrus and ovulation among cyclic ewes [41]. However, Abecia *et al.* [2], Ungerfeld e Rubianes [42], Zeleke *et al.* [43], Mateus [44], Afonso [45] and Dendena [46] were not able to establish any synchronization efficiency difference between FGA and MAP treatments. Present results meet these last investigators' observations. Exogenous gonadotropin administration does advance ovulation and higher estrous synchronization precision [1] [9] [47]. They support ovarian mechanisms affecting follicular growth and maturation and promoting the proper luteinization of the CL [4] [9] [40].

The interval between synchronization treatment and AI is very important [48]. Different investigators propose unlike intervals: 46 hours (Fernandez-Abella *et al.*, 2003; cited by [48]), 48 - 72 hours (Karagiannidis *et al.*, 2001; cited by [48]), 55 hours [46] [49] and 58 - 63 hours [50] [51]. The decision to inseminate 55 hours post eCG administration originated a reasonable fertility rate.

3.3. Artificial Insemination Response

About 76.5% of all Assaf ewes were pregnant 41 days after AI. This result was higher than many indicated in the bibliography for sheep: 50% - 65% [4] [49] [52], 65% - 75% (Colas and Guérin 1979; cited by [53]), although Assaf ewes were mainly primiparous and the short interval between progestagens sponge removal and first CL formation. Ewes tend to present estrus 36 hours after progestagens sponge removal [5] and to ovulate in 58 - 60 hours [33]. Other investigators report higher fertility rates 70% - 82% [50] [51], 82.2% (Hill *et al.*, 1998 and Ehling *et al.*, 2003; cited by [48]), 85.1% [46] and 80% - 90% [48].

3.3.1. eCG

Fertility rate after AI depends on eCG dose [Hill *et al.*, 1998; cited by 47]: 200 IU—62.4%, 250 IU—72.9%, 300 IU—79.1% and 375 IU—>69.4%. Dendena [46] stated a fertility rate of 85.1% after the administration of 500 IU of eCG. In the present paper, the administration of 750 IU of eCG determined a fertility rate of 76.5%. Racial differences may partially explain the results [5] [12] [54]. Less prolific and less seasonal breeds tend to be more responsive to eCG administration [4] [53]. Different follicular populations present on the ovaries before progestagen treatments + eCG may also condition fertility rate [41] [54].

3.3.2. Semen Preservation Technic

Fertility rates tend to be smaller after AI with chilled semen than with fresh semen [5] [55]—56.7% (Fernandez-Abella *et al.*, 2003; cited by [48]) or 74% - 76% (Gergátz and Gyökér, 1997; cited by [48]). Dendena [45] found exactly the opposite (Fresh: 79.4% vs. Chilled: 90.9%). During semen chilling the sperm cells lose motility, suffer morphological changes and decay survive ability in the female genital tract, resulting in lower fertility rates and higher embryonic mortal-

ity [56] [57]. In the present work, fertility rate was not conditioned by semen preservation technic (Fresh: 81.3% vs. Chilled: 72.2%) ($\chi^2 = 2.3$; $P > 0.05$).

3.3.3. Semen Extender

Semen extenders are expected to increase extended semen volume, stabilize pH (buffer effect), keep adequate osmolality, provide energy to spermatozoa and protect them from possible bacteria and fungus infections, cold shock and preservation process [4] [5] [9] [11] [12] [58]-[63] resulting in osmotic *stress* and reactive oxygen species (ROS) [57] [60].

Semen extender did not affect fertility rate (Andromed®: 77.8% vs. OviXcell®: 75.0%) ($\chi^2 = 0.3$; $P > 0.05$). Though Andromed® was developed to preserve bull semen and OviXcell® to preserve ram semen their effects on fertility rate were identical. Dendena [46] also found no significant difference between semen extenders (Andromed® vs. INRA 96®) in fertility rate.

3.3.4. Color and Lubrication of Vagina and Cervical Viscosity

In cervical AI, semen is usually placed in the anterior portion of the cervix [63]. So, semen transportation through the cervical canal depends on cervical mucus volume and quality [64]. All Assaf ewes presented a Pink vaginal mucosa and a Good lubrication (100.0%). Cervical mucus has Liquid in all ewes (100.0%). These results are possibly related to short-term progestagens + eCG treatments and to the time of AI and reveals that ewes were well-fed, healthy and in an advanced stage of the follicular phase or early stage of the luteal phase. Dendena [46] reported that the majority of Churra Galega Bragançana Branca ewes presented a Pink color and highly lubricated vagina.

3.3.5. Cervical External *Os*

Assaf ewes presented all types of external *Os* in [24] classification table (**Table 4**). The same was found by [46] in Churra Galega Bragançana Branca ewes. *Os* types frequency variation was significant ($\chi^2 = 13.8$; $P \leq 0.01$). Nevertheless frequency distribution was uniform, but for Slit type (less frequent). Frequency distribution registered by [46] was quite different and may be due to genetics, age and parity factors [24] [54].

Table 4. Anatomical conformation of the cervical external *Os* (frequency) and its relation to fertility rate.

External *Os*	Frequency	Fertility rate
Duckbill	20.6%^{a} (7/34)	85.7%^{x} (6/7)
Slit	8.8%^{b} (3/34)	33.3%^{y} (1/3)
Rose	29.4%^{a,c,e} (10/34)	80.0%^{z,x} (8/10)
Papilla	23.5%^{a,d,e} (6/34)	100.0%^{w,z,v} (6/8)
Flap	17.6%^{a,b,f} (5/34)	88.9%^{v,x,y,w} (5/6)

$a \neq b$, for $P \leq 0.05$; $b \neq d$, for $P \leq 0.01$; $b \neq c$, for $P \leq 0.001$; $x \neq w$, for $P \leq 0.05$; $x \neq y$, $y \neq z$, $y \neq w$, $y \neq v$, for $P \leq 0.001$ (between lines).

External *Os* type influenced fertility rate (χ^2 = 93.5; P < 0.001) (**Table 4**). The same was registered by [45]. Pappila (100.0%), Flap (88.9%), Duckbill (85.7%) and Rose (80.0%) types were related to high fertility rates. On the opposite, Slit type was related to the lowest fertility rate. Dendena [46] found the highest fertility rates among ewes with Papilla and Slit types of the external *Os*.

3.3.6. Semen Deposition Place

Fertility rate is higher when semen is placed deeper in the cervix during cervical insemination [3] [65]. Spanish sheep breeds with low fertility rate (Assaf and Churra) present a more complex cervical canal, which impairs deep penetration [65]. Cervix distress or trauma may condition fertility rate by affecting semen transport and viability in the female genital tract, originated by an influx of immune cells to the cervical canal [7] [13] [66]. In the present study, semen was mainly placed after the first cervical fold (**Table 5**). Dendena [46] reported the same. Fertility rate was quite high (78.8%). The only vaginal inseminated ewe did not get pregnant (0.0%). This author found the same rate of fertility in Churra Galega Bragançana Branca ewes when semen was left in the vagina or after the first cervical fold (Vagina: 88.9% vs. 1º: 88.2%).

3.3.7. Cervical Outflow

Low fertility rates are related to semen cervical outflow in sheep [13] [14]. Cervical outflow should be avoided [15] [46], especially when it is plentiful [46]. In the present work, most ewes inseminated after the first fold did not present any cervical outflow (**Table 6**) and resulted in a high fertility rate (81.5%). Ewes with a light outflow presented a lower fertility rate (66.7%) (χ^2 = 81.9; P < 0.001).

4. Conclusions

- On the second fortnight of May, Assaf ewes were all cycling.
- Both short-term progestagens (FGA e MAP) + eCG were 100.0% efficient.
- FGA and MAP showed equal precision controlling first CL formation (FGA - 28.3 ± 12.7 days vs. MAP - 29.6 ± 10.5 days.

Table 5. Semen deposition place (frequency) and fertility rate.

Semen deposition place	Frequency	Fertility rate
Vagina	2.9%^{a} (1/34)	0.0%^{x} (0/1)
1st fold	97.1%^{b} (33/34)	78.8%^{y} (26/33)

a ≠ b for P ≤ 0.0001; x ≠ y for P ≤ 0.001 (between lines).

Table 6. Cervical outflow (frequency) and fertility rate.

Cervical outflow	Frequency	Fertility rate
No	27/33 (81.8%^{a})	22/27 (81.5%^{x})
Light	6/33 (18.2%^{b})	4/6 (66.7%^{y})

a ≠ b, for P ≤ 0.0001; x ≠ y for P ≤ 0.05 (between lines).

- Forty-one days after AI 76.5% of the Assaf ewes were pregnant.
- Fertility rate was significantly affected by external *Os* type, semen deposition place (Vagina: 0.0% vs. 1º: 78.8%) and seminal cervix outflow (No outflow: 81.5% vs. Light outflow: 66.7%).
- Fertility rate was not significantly influenced by vagina color (Pink: 100.0%) and cervical mucus viscosity (Liquid: 100%), semen extender (Andromed®: 77.8% vs. OviXcell®: 75.0%) or semen preservation technic (Fresh: 81.3% vs. Chilled: 72.2%).

Animal Rights

This experiment comply with the ARRIVE guidelines and have be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Conflict of Interest

The authors declare that they have no conflict of interest.

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