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Fixed-time artificial insemination in *Churra Galega Bragançana* ewes: DARIO tip effect

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Abstract

This work was carried out with the objective of studying the effect of the anti-reflux device for ovine insemination (DARIO) on the fertility rate in ewes of the native Portuguese breed *Churra Galega Bragançana* (CGB) artificially inseminated at fixed-time. Eighty-one ewes aged between one and five years were used. At the end of April 2021, ewes' reproductive control was carried out, using a short progestagen treatment (five days) + equine chorionic gonadotropin (eCG). Cervical artificial insemination (AI) was performed at fixed-time (55 hours after the end of the hormonal treatment), with cooled semen. All ewes were inseminated by the same inseminator: 39 ewes with the DARIO tip on the end of the sheath and 42 without this tip. The pregnancy diagnosis was made by ultrasonography 41 days after AI. Age and body condition (BC) did not affect the ewes' response to the applied hormonal treatment or the fertility rate. Approximately 95.1% of the ewes responded to the progestagen + eCG treatment. Forty-one days after AI, 86.4% of the ewes were pregnant. The use of the DARIO tip did not affect the fertility rate.

Keywords: Pregnancy; Reproduction; Sheep farming.

Introduction

In sheep, one of the main barriers to the diffusion of the artificial insemination (AI) technique is the anatomy of the cervix (SAYRE and LEWIS, 1997; SALAMON and MAXWELL, 2000; STELLFLUG et al., 2001; KERSHAW et al., 2005; MACÍAS et al., 2020; PERA, 2020), which hinders the deep deposition of the seminal dose in the female genital tract (SAYRE and LEWIS, 1997; STELLFLUG et al., 2001; KERSHAW et al., 2005; FERRA and SERENO, 2006; KAABI et al., 2006; MACÍAS et al., 2020). The size and the "Os" shape of the cervical canal condition the introduction of the AI gun (MCKUSICK et al., 1998; STELLFLUG et al., 2001; FERRA AND SERENO, 2006; KAABI et al., 2005; LEETHONGDEE et al., 2010; LIMA, 2010; CANDAPPA and BARTLEWSKI, 2011) and it can determine the occurrence of cervical semen reflux (STEYN, 2003; BICUDO, 2005; FERRA and SERENO, 2006).

Ewes that produce cervical reflux have lower fertility rates (CANDAPPA and BARTLEWSKI, 2011; MORRELL, 2011), therefore it should be avoided (CSEH and FAIGL, 2012; AMIRIDIS, 2012; DENDENA, 2017; FORNAZARI, 2018), particularly in abundance (DENDENA, 2017). In order to reduce cervical reflux after AI, the anti-reflux device for ovine insemination (DARIO) was created, which allows a light penetration of the cervical canal (MACÍAS et al., 2017), preventing the occurrence of the cervical reflux (HUMECO, 2020). The use of this tip increases AI costs by 4.31% per ewe (MACÍAS et al., 2017), however, it results in increased fertility rates (MACÍAS et al., 2017; 2020).

Therefore, this work was carried out with the objective of studying the effect of the anti-reflux device for ovine insemination (DARIO) on the fertility rate in ewes of the native Portuguese breed *Churra Galega Bragançana* (CGB) artificially inseminated at fixed-time.

Material and methods

This research was carried out in Bragança, Portugal, at Pinheiro Manso Farm (41° 48' 33''N, 6° 44' 3''W and altitude 670 meters), belonging to the Agrarian Higher School of the Polytechnic Institute of Bragança (ESA-IPB), between April 20 and June 15, 2021.

Animals

In this work, 81 ewes of the *Churra Galega Bragançana* (CGB) breed, aged between one and five years, were used. The last parturition had occurred approximately six months earlier. The ewes grazed natural grasslands and were supplemented, as a group, with natural grassland hay (*ad libitum*) and 300 to 350 g daily per ewe of a commercial concentrate feed. During thirty days after AI, the energy/protein intake of the diet was maintained. This experiment began with the determination of the body condition (BC) of the ewes, according to the classification table of Russel, Doney and Gunn (1969). Intervals of 0.25 points were considered.

Reproductive control treatment

On April 30, 2021, the ewes received a vaginal sponge impregnated with 20 mg of flugestone acetate (FGA) (Chrono-Gest®, Intervet, Portugal). At the same time, they were injected intramuscularly (i.m.) with 100 μg of cloprostenol (Estrumate®, MSD Animal Health, Portugal). The FGA treatment duration was five days.

Upon removal of the vaginal sponge (May 5, 2021), the ewes were injected i.m. with 500 IU equine chorionic gonadotropin (eCG) (Intergonan®, Intervet, Portugal).

Response to the reproductive control treatment

In order to identify the formation of the first *corpus luteum* (CL) after FGA + eCG treatment, five days after the eCG administration, peripheral blood samples were collected to determine the plasma levels of progesterone (P4) through the radioimmunoassay technique. For this purpose, a scintillation reader DPC® Gamma C12 (Bertholt Technologies, Bad Wildbad, Germany) and DiaSource® kits (ImmunoAssays, Louvain-la-Neuve, Belgium) were used. The average of the intra- and inter-assay coefficients of variation were, respectively, 7.8 and 15.1%.

It was considered that the first CL had been formed when the plasma P4 level exceeded 0.5 ng mL^{-1} for the first time.

Semen collection

Semen was collected with the aid of an electroejaculator (eProvac®, Minitüb, MT model, Tiefenbach, Germany). Ejaculates were collected from adult rams (three to seven years old) of the CGB breed, which had not ejaculated for the last three days. After collecting the ejaculates, the collection tubes were transported to the laboratory, where they were kept at 37°C in a refrigerated water bath (Neslab® RTE 221, Newington, US). In the same equipment, the seminal extender Andromed® (Minitüb, Tiefenbach, Germany) was previously stored.

Seminal analysis

The volume was measured through the graduation at the collection tubes. Sperm concentration and motility were measured with the aid of a computer assisted sperm analysis (CASA) (Androvision®, Minitüb, Tiefenbach, Germany). The percentage of live spermatozoa was determined using a trinocular phase contrast microscope (Motic BA-310, Barcelona, Spain), after diluting one drop of semen with two drops of eosin and preparing a smear (200 sperm were

counted). The ejaculates that were used in this work had volume ≥ 2.0 mL, sperm concentration ≥ 3.0 x 10^9 spermatozoa mL^{-1}, sperm motility ≥ 75% and percentage of live sperm ≥ 75%.

Seminal doses

After performing the seminal analysis, each ejaculate was initially diluted (1:1 proportion) with Andromed, and the volume was adjusted to the values indicated by the CASA program. Posteriorly, the diluted semen's temperature was reduced for approximately 90 minutes from 37°C to 15°C (Neslab® RTE 221, Newington, US). Finally, after 10 minutes of resting, it was aspirated into 0.25 mL straws, which were sealed with polyvinyl powder. There was an interval of 30 minutes between the end of the cooling process and the beginning of the AI. Each semen straw contained at least 350 x 10^6 spermatozoa.

Fixed-time AI

All ewes were inseminated (May 7, 2021), regardless if they were in estrus or not, 55 hours after eCG administration, by the same inseminator. For this purpose, he used an IMV® vaginal speculum (L'Aigle, France), Quicklock® AI gun (Minitube, Tiefenbach, Germany) and Minitub® straws (Tiefenbach, Germany). Thirty-nine ewes were inseminated after placing a DARIO tip (Humeco, Huesca, Spain) at the end of the AI gun (Figure 1). Forty-two ewes were inseminated without the DARIO tip. The insemination was carried out in the stable with the ewes standing up. To facilitate the observation of the cervix and for the convenience of the inseminator, two members of the team lifted the hind limbs of the ewes, keeping, however, the forelimbs always in contact with the ground.

The semen deposition was always carried out as deeply as possible, without, however, forcing the gun against the cervical canal.

Pregnancy diagnosis

Forty-one days after the AI (June 15), pregnancy was diagnosed by real-time ultrasonography, with the aid of a Mindray Z5Vet ultrasound and a 5.0-10.0 MHz multifrequency rectal probe.

Statistical analysis

In order to identify statistically significant differences between parameters, analysis of variance were performed (STEEL and TORRIE, 1980). The comparison between means was carried out according to the Bonferroni-Dunn test (DUNN, 1961). To compare frequencies, the chi-square test was used (χ^2) (SNEDECOR and COCHRAN, 1980).

Results and discussion

At the beginning of this work, the CGB ewes' age was on average, 3.2 ± 1.9 years old (coefficient of variation (CV) = 59.2%), and age can affect post-AI fertility rate (SHACKELL et al., 1990; ANEL et al., 2005; ESMAILIZADEH et al., 2009; FORCADA, 2010; SANTOLARIA et al., 2011; PALACÍN et al., 2012). In nulliparous and primiparous ewes, the fertility rate is normally lower than in adult ewes (HALBERT et al., 1990; WINDSOR, 1995; PALACÍN

Figure 1 – DARIO tips (left) and prepared AI guns with and without DARIO tip (right).

et al., 2012; FORNAZARI, 2018), because they tend to produce less cervical secretions and the sperm transport in their genital tract is compromised (PALACÍN et al., 2012). In adult ewes, the fertility rate tends to increase with age (WINDSOR, 1995; PALACÍN et al., 2012), up to five (SANTOLARIA et al., 2011; PALACÍN et al., 2012), six (SHACKELL et al., 1990) or seven years old (ESMAILIZADEH et al., 2009; SANTOLARIA et al., 2011).

In older ewes, the fertility rate tends to decrease due to an increased risk of reproductive problems and a decrease in the quality of ovulated oocytes (PALACÍN et al., 2012). In this study, age did not affect the fertility rate (probability (P) > 0.05), possibly because most studied ewes (71.6%) aged between two and five years old. CGB ewes had a mean BC of 3.7 ± 0.7 points (CV = 20.0%). According to Molina et al (1994), the BC at the insemination moment can condition the fertility rate. O'Brein (2002), Scaramuzzi and Martin (2008) and Karikari and Blasu (2009) affirmed that the ideal BC of ewes at insemination is 2.5-3.5 points. Palacios (2010) and Valentim et al. (2015) reported values of 3.0-4.0 points. In this study, BC did not influence the fertility rate (P > 0.05), probably because most of them (93.8%) had a BC of 2.5-4.5 points.

The difference between ewes' age inseminated with or without the DARIO tip (with DARIO tip: 3.2 ± 1.9 years; without DARIO tip: 4.0 ± 2.0 years) was not significant (P > 0 .05). Similarly, the difference in BC between ewes inseminated with or without DARIO tip (with DARIO tip: 3.7 ± 0.6 points; without DARIO tip: 3.7 ± 0.7 points) was not significant. (P > 0.05). These data reflected the random division of ewes by group.

P4 + eCG treatment response

Short progestagen treatments allow to avoid the negative effects that long progestagen treatments have on ovarian activity and on the survival and dynamic of sperm in the female genital tract (AZEVEDO et al., 2006; ABECIA et al., 2012; MATEUS, 2014; VALENTIM et al., 2015; SWELUM et al., 2018a,b). Although more expensive, they are very effective (MATEUS, 2014; CONRADI, 2018; FORNAZARI, 2018). Administration of eCG improves the estrus synchronization rate and the ovarian response, in addition to the ovulation anticipation (RITAR et al., 1984; OMONTESE et al., 2016). In this work, five days after the eCG administration, 95.1% (n = 77) of the ewes had P4 plasma levels above 0.5 ng mL-1, in other words, they responded to the hormonal treatment. Similar results were observed by Mateus (2014), Conradi (2018), Fornazari (2018) and Pera (2020).

Age and BC did not affect the response of ewes to the hormonal treatment (P > 0.05). These results can be explained by the fact that age and BC of most of them were compatible with good reproductive performance.

Fertility rate after AI

Forty-one days after AI, 86.4% (n = 70) of the ewes were pregnant. This rate was much higher than the average indicated in the bibliography for sheep: 40-60% (ANEL et al., 2005; MASOUDI et al., 2017), 50-65% (BARIL et al., 1993; AX et al., 2004; VALENTIM et al., 2009) and 65-75% (COGNIÉ, 1988). However, some authors referred equally high fertility values: 70-82% (DONOVAN et al., 2001; 2004), 74,5% (FORNAZARI, 2018), 82,2% (HILL et al., 1998; EHLING et al., 2003; KUKOVICS et al., 2011), 83,7% (PERA, 2020), 84,1% (CONRADI, 2018), 85,1% (DENDENA, 2017) and 80-90% (KUKOVICS et al., 2011).

Ewes that produce cervical reflux have lower fertility rates (CANDAPPA and BARTLEWSKI, 2011; MORRELL, 2011). According to information from the Humeco company, the DARIO tip allows a sufficient deep penetration of the cervical canal (MACÍAS et al., 2017;

2020), while blocking the entry of the "Os" cervix, preventing the cervical semen reflux and improving the fertility rate up to 12% (HUMECO, 2022). In the present study, the use of the DARIO tip did not improve the fertility rate (with DARIO tip: 84.6%; without DARIO tip: 88.1%) ($\chi^2 = 0.4$; $P > 0.05$). In fact, the DARIO tip can decrease the incidence of cervical reflux, however it prevents the deposition of the semen beyond the first cervical crease, which, in some cases, can determine a reduction in the success rate of AI.

In sheep, some cervical reflux is expected, since the internal volume of the cervix may be smaller than the seminal dose (0.25 mL) (CSEH, 2012; FAIGL and AMIRIDIS, 2012). In fact, it varies between 0.1-0.3 mL (ANEL et al., 2006). The DARIO tip does not overcome this problem. Effectively, 12.8% (n = 5) of the CGB ewes inseminated with this tip, had cervical reflux. Even so, four of them (80.0%) became pregnant and only one (20.0%) did not ($\chi^2 = 72.0$; $P \leq 0.001$). Mild cervical reflux is compatible with achieving a good fertility rate (DENDENA, 2017).

Conclusion

The use of the DARIO tip does not affect the fertility rate after AI in CGB ewes.

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