Small Ruminant Research 136 (2016) 54–58

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Superovulatory and embryo yielding in sheep using increased exposure time to progesterone associated with a GnRH agonist

O.O. Brasil[a,b], N.H. Moreira[a,b], G. Santos Júnior[a,b], B.D.M. Silva[b], A.S. Mariante[b], A.F. Ramos[b,*]

[a] Graduate Course on Animal Sciences—College of Agriculture and Veterinary, University of Brasilia, Brasilia, Brazil
[b] Embrapa Genetic Resources and Biotechnology, Brasília, Brazil

ARTICLE INFO

Article history:
Received 1 August 2015
Accepted 6 January 2016
Available online 11 January 2016

Keywords:
Biotechnology
Conservation
Reproduction
Superstimulation
Ewe

ABSTRACT

Response to superovulatory, embryo yields and quality, were evaluated after increasing exposure time to exogenous progesterone during superstimulation, with or without the addition of a GnRH agonist. Thirty-four ewes from the Santa Inês breed were synchronized with an intravaginal progesterone device (CIDR). The animals were randomly divided into three groups: exposure to progesterone for 14 days (Control, $n = 12$); 12 h extension of progesterone exposure (12hP4 group, $n = 11$); and 12 h extension, associated with the administration of 25 μg of gonadorelin acetate (12hP4GnRH group, $n = 11$). From the 12th to the 15th day of protocol, 133 mg of pFSH were administered through eight decreasing doses. Artificial inseminations were performed 36 and 48 h after device withdrawal, using frozen/thawed semen. The number of ewes on estrus and the time to onset of estrus after withdrawing device were similar among between all groups ($P > 0.05$). All groups presented high superstimulatory response (averages ranging from 14.33 to 16.18 follicles ≥4 mm) ($P > 0.05$). All groups also showed a large quantity of CL (averages ranging from 11 to 12) ($P > 0.05$). The longer exposure to progesterone no reduced degenerated embryo proportion (Control = 30%; 12hP4 = 7% and 12hP4GnRH = 10%; $P > 0.05$). The fertilization rate was significantly higher in 12hP4GnRH when compared to Control and to 12hP4 (77%, 34% and 41% respectively). These results suggest the association of longer progesterone exposure plus GnRH administration is an alternative to increase oocyte fertilization rates when fixed time insemination is used.

1. Introduction

Multiple ovulation and embryo transfer (MOET) technologies have substantial contributions to genetic improvement of sheep in several countries around the world. During the last three decades there has been considerable progress in the use of female germplasm through MOET. However, this technology applied to sheep has been of slow acceptance, mainly due to the oscillation of superovulatory (SOV) response (Cognie, 1999; Cognie and Baril, 2002). The main factors which influence variation in results are type of superstimulatory treatment, season, follicular condition, genetics and nutritional status of animals (Ammoun et al., 2006; Gonzalez-Bulnes et al., 2003). These factors can be directly or indirectly involved, thus influencing the oocyte/embryo quality and/or synchronization of multiple ovulations.

By the end of the superestimulatory treatment, oocytes at different stages of development are present in large follicles. Therefore, an attempt to permit such oocytes to acquire equal competence is necessary in order to achieve in higher production of good quality embryos. This could be achieved by a prolonged exposure to progesterone, with the aim to delay the LH peak and cause synchronized ovulation. Moreover, the use of the gonadotropin releasing hormone (GnRH) or its agonists, after FSH stimulation, provides better synchronization of ovulation, increasing embryo yields (Menchaca et al., 2009; Walker et al., 1986), in spite of controversial reports (Baril et al., 1996; Jabbour et al., 1996). We tested the hypotheses that prolonged exposure to progesterone (1) increase the synchronize of the ovulations, or (2) when used longer progesterone exposure plus GnRH further improves the ovulation synchronize, increasing the fertilization rate of the ewes inseminated with frozen/thawed semen in MOET programs.

Therefore, a longer progesterone exposure associated or not to ovulation induction, seems to be a viable alternative to increase

* Correspondence to: Parque Estação Biológica, Av. W5 Norte Final, Asa Norte, 70770–917 Brasília, Brazil. Fax: +55 61 33403669.
E-mail addresses: alexandre.floriani@embrapa.br, aleframos@yahoo.com.br (A.F. Ramos).

http://dx.doi.org/10.1016/j.smallrumres.2016.01.005
0921-4488/

oocyte viability and fertilization rate, especially when fixed time insemination with frozen/thawed semen is used. The aim of this study was to evaluate response to superovulatory, embryo yields and quality, using frozen/thawed semen, after an increased exposure time to exogenous progesterone during superovulation, with or without the addition of a GnRH agonist.

2. Materials and methods

This experiment was approved by the Animal Ethics Committee (CEUA) of the Institute of Biological Sciences at the University of Brasilia.

2.1. Experimental station and animals

This study was developed during the period of May to July, 2012 in Brasilia, located on the Central-West region of Brazil (latitude 15°52′S, longitude 48°00′W), with an altitude ranging from 1050 to 1250 m above sea level. This region presents a tropical rainy climate, with marked dry winters and rainy summers. Thirty-four ewes from the Santa Inês breed, with a body condition score of 2.9 ± 0.3 (range 1–5; Russel et al., 1969) and body weight of 47.4 ± 6.6 kg were used as embryo donors. Santa Inês breed has cyclical behavior during the year at this location. The animals were kept in a pasture of *Panicum maximum*, and had free access to water and mineral salt.

2.2. Treatments

All animals were synchronized with an insertion (Day 0) of a progesterone controlled-internal-drug-release device (Eazi-Breed CIDR—Controlled Internal Drug Release, Pfizer, New Zealand) for 14 days, with a new controlled-internal-drug-release introduced at Day 7. During the exchange of the CIDR, on Day 7, a luteolytic dosis of PGF2α (37.5 mg d-cloprostenol; Prolise, Tecnopec, ARSA SRL, Argentina; im) was administered. On Day 12, the superstimulation was induced using 133 mg of pFSH (Folltropin, Tecnopec, AHC Inc., Bioniche, Canada; im) that was administered through eight decreasing doses, twice a day, starting in the morning of Day 12 and finalizing in the afternoon of Day 15. At the time of CIDR removal, the ewes were randomly divided into three experimental groups: 1. Control ($n = 12$): progesterone device withdrawn at Day 14; 2. 12hP4 group ($n = 11$): progesterone device maintained for 12 more hours, until Day 14½; and 3. 12hP4GnRH group ($n = 11$) progesterone maintained for 12 more hours, until Day 14½ plus GnRH agonist (25 μg of Gonadorelin acetate; Gestran Plus, Tecnopec, ARSA SRL, Argentina; im) administered simultaneously with the last pFSH injection (Fig. 1).

2.3. Evaluation of estrus and artificial insemination

Estrus was observed at intervals of four hours counting from moment of CIDR withdrawal, until the moment of first insemination, with the aid of a teaser ram that had his pectoral region painted with a mixture of pigment powder and soybean oil. The ewes that were considered in estrus were those that had paint on their back and accepted mounting. All females were artificially inseminated with frozen/thawed semen via the laparoscopic method delineated by Maxwell and Butler (1984). The AI was preformed 36 and 48 h after CIDR withdrawal, using straws of 0.25 mL containing an insemination dosis of 100×10^6 spermatozoa, with parameters established by the Colégio Brasileiro de Reprodução Animal (1998). Half of each semen dose was introduced into each uterine horn by the laparoscopic method.

2.4. Superstimulatory response

Two ultrasound evaluations of ovarian activity were performed on all females, using the B-mode real-time scanner (Aloka Echo Camera SSD 500, Overseas Monitor Corp., Ltd., Richmond, BC, Canada) and an adapted 7.5 MHz transducer for transrectal exams. The first evaluation was preformed after the last FSH injection, to assess the superstimulatory response at the end of the gonadotropin treatment. The second evaluation was performed 12 h after the last AI, to assess the amount of large follicles that had not ovulated yet. All follicles larger than 4 mm were counted and drawn on maps.

2.5. Ovulatory response and embryo yield

On Day 7 after pessary removal, the number of ovulations was recorded by laparoscopic procedure and, immediately afterward, embryos were surgically recovered under general anesthesia. Ewes, deprived of food and water for 24 h, were im administered with xylazine (1.1 mg/10 kg; Rompun, Bayer, Brazil) and ketamine hydrochloride (20 mg/10 kg Ketamina, Agener, Brazil). Furthermore, local anesthesia was administered in the surgical field (0.2 mg of lidocaine; Lidovet, Bravet, Brazil). Ova/embryos were collected surgically after ventral laparotomy, using a paramedian incision (6 cm long) cranial to the udder to access the reproductive tract. Each uterine horn was flushed with 60 mL flushing media (DPBS, Cultilab, Brazil) which was collected via a 9 FG Foley catheter, inserted at the external bifurcation of the uterine horns. Embryos were recovered in a Petri dish, maintained in holding media (Holding plus, 0.4% BSA, Embriocare, Cultilab, Brazil), and morphologically evaluated under a stereomicroscope (×40 magnification), following the International Embryo Transfer Society (IETS) recommendations (Robertson and Nelson, 1999). The quality score of the embryos were: Grade 1 (excellent or good), Grade 2 (fair), Grade 3 (poor), and Grade 4 (dead or degenerated). Embryos graded as 1, 2 and 3 were considered viable and those were graded 1 and 2 were considered freezable embryos.

2.6. Indicators of superovulatory response

The following data were recorded for each ewe: number of corpora lutea (CL), total recovered oocytes/embryos (TR), viable embryos (VE), freezable embryos (FE), degenerated embryos (DGE), and total embryos (TE). The fertilization rate (FR) was obtained by dividing TE by TR; the freezable embryo rate (FER) was obtained by dividing FE by VE; while the degenerated rate (DGER) was obtained by dividing DGE by TE.

2.7. Statistical analysis

The data were analyzed using the SAS software (V9, SAS Institute Inc, Cary, NC, 2003). The parameters related to the amount of follicles during the last FSH injection and 12 h after AI, quantity of CL and total recovered structures were subjected to an analysis of variance (ANOVA) and the Duncan's test, to compare their means. Variables that did not a normal distribution, such as the number of viable and freezable embryos, have been evaluated using the Kruskal–Wallis test. Percentage of ewes exhibited estrus and embryo degeneration rate were analyzed by the Fisher exact test, while fertilization and freezable embryos rate were analyzed by the Chi-square test. Results are shown as Mean ± Standard Deviation or in percentage, and the differences were considered significant when $P < 0.05$.

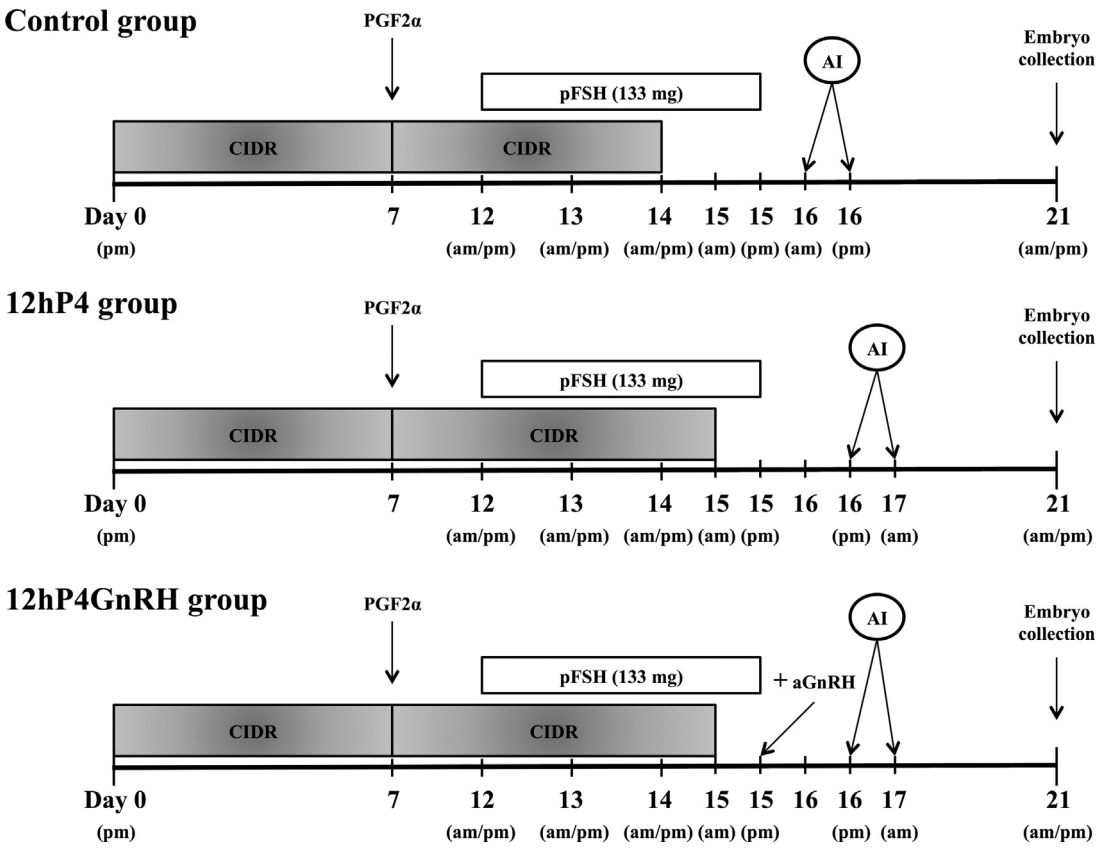

Fig. 1. Experimental groups. Ewes received an intravaginal progesterone (P4) device on Day 0 and a new progesterone device 7 day later (Day 7), along with a dose of PGF2α. All ewes received twice daily injections of FSH im over 4 days starting on Day 12. Ewes in the Control group had P4 devices removed on Day 14, whereas ewes in the 12hP4 and 12hP4GnRH groups had P4 devices removed in the morning of Day 15 (14,5 days), the last group received GnRH agonist (25 μg of gonadorelin acetate; im) simultaneously at the last pFSH injection. All ewes were inseminated 36 and 48 h after device withdrawal and ova/embryos were collected 5 days after the second insemination (Day 21) by a surgical technique.

Table 1
Effect of increased exposure time to progesterone associated or not with a GnRH agonist, on behavioral estrus, ovarian response and ovulatory response of Santa Inês donors.

	Control	G12hP4	G12hP4GnRH
Behavioral estrus			
Ewes in estrus	12/12 (100%)	11/11 (100%)	11/11 (100%)
CIDR withdrawal to estrus (h)	20.8 ± 4.6	17.4 ± 5.0	22.0 ± 6.0
Ovarian response			
Follicles (≥4 mm)[a]	14.33 ± 5.84	15.22 ± 5.87	16.18 ± 7.36
Follicles after AI (≥4 mm)[b]	5.50 ± 2.81	5.78 ± 1.86	5.00 ± 3.22
Corpora lutea	12.00 ± 6.48	11.11 ± 5.64	11.81 ± 7.60

Means did not differ ($P > 0.05$).
[a] Number of follicles ≥4 mm at last dose of FSH.
[b] Number of follicles ≥4 mm 12 h after last AI.

Table 2
Effect of increased exposure time to progesterone associated or not with a GnRH agonist, on embryo yield of Santa Inês donors.

	Control	G12hP4	G12hP4GnRH
Embryo yielding			
Total recovered structures	4.83 ± 3.86	6.89 ± 5.06	4.36 ± 3.26
Viable embryos	1.17 ± 1.70	2.55 ± 2.77	2.91 ± 2.59
Freezable embryos	0.92 ± 1.51	1.91 ± 2.63	2.27 ± 2.20
Fertilization (%)	20/58 (34)[b]	30/74 (41)[b]	37/48 (77)[a]
Freezable embryos (%)	11/14 (79)	20/24 (83)	25/32 (78)
Degenerated embryos (%)	06/20 (30)	02/26 (7)	05/37 (14)

[a] Values with different letters within a row indicate significant differences ($P < 0.05$).
[b] Values with different letters within a row indicate significant differences ($P < 0.05$).

3. Results

All groups presented high estrus rates, showing that the greater exposure time to progesterone, associated or not with GnRH had no effect ($P > 0.05$) on the number of ewes in estrus and neither on the onset of estrus after withdrawal of CIDR (Table 1).

All groups presented high superstimulatory (follicles larger than 4 mm) and superovulation (corpus luteum) responses ($P > 0.05$). The quantity of follicles of sizes ≥4 mm, obtained 12 h after the last AI was similar for all groups ($P > 0.05$) (Table 1).

Total number of collected structures, freezable embryos and proportion of freezable embryos, were similar among all groups ($P > 0.05$). The group that contained an ovulation inducer (12hP4GnRH) presented the highest fertilization rate ($P < 0.05$) when compared to the Control and 12hP4 groups. 12hP4 group with longer progesterone exposure, although it didn't have statistical difference, tended to reduce the degenerate embryos rate ($P = 0.06$) when compared to the control group (Table 2).

4. Discussion

The results obtained from this study show the treatments were effective in inducing estrus synchronization in all ewes of the Santa Inês breed. In all groups, the estrus behavior occurred from 12 to 32 h after removal of CIDR. The importance of the estrus evaluation is it association with the LH peak and ovulations (Gonzalez-Bulnes et al., 2002).

The high superstimulatory response detected in all groups during the last FSH injection proved that the 133 mg dosis of FSH was an effective stimulator for multiple follicular growth. However, it appears that the ovulation synchronization occurred differently among groups, whereas when analyzing the difference among the number of follicles observed between the two ultrasonographic examinations, the 12hP4GnRH group was the one that accounted a smaller difference when comparing the number of CL found (12hP4GnRH: 11.18 vs. 11.81; Control: 8.83 vs. 12.00; 12hP4 6.35 vs. 11.11), which suggests that the ovulations occurred between the two ultrasound evaluations, while in the others groups some ovulations have occurred after the second ultrasonography.

Another important information also supports this hypothesis is the high fertilization rate of the recovered structures in the 12hP4GnRH group, suggesting there was a greater synchronism between first and last ovulation, allowing the use of frozen/thawed semen. Increase progesterone exposure without ovulation induction (12hP4 group) was not enough to improve the synchronize of ovulations. So, association between longer progesterone exposure and GnRH is necessary to make the ovulations occur more synchronized.

The administration of GnRH during the end of superstimulation using FSH (Walker et al., 1986) results in a greater synchronism between first and last ovulation and reduces onset of ovulation. In another study (Quirke et al., 1979) GnRH led to ovulation in 44–46% of ewes in 24 h and all animals in 34 h, proving the beneficial effect of GnRH use on synchronization of the ovulation and therefore increasing fertilization rate. In these studies the use of GnRH improved synchronism of ovulation, without the need to increase the time of progesterone exposure. However, in an experiment conducted by our group using Santa Inês ewes, administration of GnRH without increasing the time of progesterone exposure not improved the synchronism of ovulation (Brasil et al., 2012). So we did not include this group in this experiment.

In a study in which a GnRH agonist was administered during the end of a superstimulation protocol, there has been an increase in the fertilization, which triggered an increase in the quantity of viable and freezable embryos (Menchaca et al., 2009). In the present study, although there was no difference in the amount of viable and freezable embryos, they were more than double in the 12P4GnRH group, when compared to the Control, possibly due to greater fertilization rates of oocytes when an inducer of ovulation was used.

When the treatment is not able to maintain physiological concentrations of progesterone, it is possible that there may be alterations in follicle growth patterns, and dominance of large estrogenic follicles (Leyva et al., 1998; Viñoles et al., 1999), as well as alterations in the fertilization process and development of good quality embryos (Gonzalez-Bulnes et al., 2005; Theodosiadou et al., 2004). In the present study, longer progesterone exposure plus GnRH, during the superstimulatory protocol with FSH, tended to reduce the degenerate embryos rate, promoting a possible beneficial effect on the oocytes and embryos.

At the end of the superstimulatory treatment, large follicles at different stages of development were found. It is known that for a healthy development of an embryo, it is necessary that the oocyte acquires competence, which occurs between the end of its growth and beginning of its maturation through structural and molecular changes (Dieleman et al., 2002; Hyttel et al., 1997). We believe the longer progesterone exposure inhibits for a time the peak of LH, and this permits those follicles containing incompetent oocytes to have an additional time for development. Furthermore, even if this causes some degree of follicle atresia on the already competent oocytes, it is not detrimental, since moderate degrees of follicular atresia do not affect the ability of the oocyte capacitation in vitro (Campbell et al., 1991), and a low degree of follicular atresia, could actually enhance embryonic development (Blondin and Sirard, 1995).

5. Conclusions

Use longer progesterone exposure associated with an administration of GnRH in MOET programs can be recommended to increase the fertilization rate, particularly when fixed time insemination with frozen/thawed semen is used.

References

Ammoun, I., Encinas, T., Veiga-Lopez, A., Ros, J.M., Contreras, I., Gonzalez-Añover, P., Cocero, M.J., McNeilly, A.S., Gonzalez-Bulnes, A., 2006. Effects of breed on kinetics of ovine FSH and ovarian response in superovulated sheep. Theriogenology 66, 896–905.

Baril, G., Pougnard, J.L., Freitas, V.J.F., Leboeuf, B., Saumande, J., 1996. A new method for controlling the precise time of occurrence of the preovulatory gonadotropin surge in superovulated goats. Theriogenology 45, 697–706.

Blondin, P., Sirard, M.A., 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol. Reprod. Dev. 41, 54–62.

Brasil O.O., Moreira N.H., Modesto M.R., Silva P.C.P., Silva B.D.M. and Ramos A.F., 2012. Uso de indutor da ovulação em protocolo de superovulação ovina. Anais do II Congresso Brasileiro de Recursos Genéticos, Belém-Pa, Brasil.

Campbell, B.K., Picton, H.M., Mann, G.E., Mcneilly, A.S., Baird, D.T., 1991. Effect of steroid and inhibin-free ovine follicular fluid on ovarian follicles and ovarian hormone secretion. J. Reprod. Fertil. 93, 81–96.

Cognie, Y., 1999. State of art in sheep and goat embryo transfer. Theriogenology 51, 105–116.

Cognie, Y., Baril, G., 2002. State of the art in sheep–goat embryo transfer. INRA Prod. Anim. 15, 199–207.

Colégio Brasileiro de Reprodução Animal, 1998. Manual para Exame Andrológico e Avaliação de Sêmen Animal, second ed. Belo Horizonte.

Dieleman, S.J., Hendriksen, P.J., Viuff, D., Thomsen, P.D., Hyttel, P., Knijn, H.M., Wrenzycki, C., Kruip, T.A., Niemann, H., Gadella, B.M., Bevers, M.M., Vos, P.L., 2002. Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre-implantation embryos. Theriogenology 57, 5–20.

Gonzalez-Bulnes, A., Garcia-Garcia, R.M., Santiago-Moreno, J., Domínguez, V., Lopez-Sebastian, A., Cocero, M.J., 2003. Reproductive season affects inhibitory effects from large follicles on response to superovulatory FSH treatment in ewes. Theriogenology 60, 281–288.

Gonzalez-Bulnes, A., Garcia-Garcia, R.M., Souza, C.J.H., Santiago-Moreno, J., Lopez-Sebastian, A., Cocero, M.J., Baird, D.T., 2002. Patterns of follicular growth in superovulated sheep and influence on endocrine and ovarian response. Reprod. Domest. Anim. 37, 357–361.

Gonzalez-Bulnes, A., Veiga-Lopez, A., Garcia, P., Garcia-Garcia, R.M., Ariznavarreta, C., Sanchez, M.A., 2005. Effects of progestagens and prostaglandin analogues on ovarian function and embryo viability in sheep. Theriogenology 63, 2523–2534.

Hyttel, P., Fair, T., Callesen, H., Greve, T., 1997. Oocyte growth capacitation and final maturation in cattle. Theriogenology 47, 23–32.

Jabbour, H.N., Ryan, J.P., Evans, G., Maxwell, W.M., 1996. Effects of season, GnRH administration and lupin supplementation on the ovarian and endocrine responses of merino ewes treated with PMSG and FSH-P to induce superovulation. Reprod. Fertil. Dev. 3, 699–707.

Leyva, V., Buckrell, B.C., Walton, J.S., 1998. Regulation of follicular activity and ovulation in ewes by exogenous progestagen. Theriogenology 50, 395–416.

Maxwell, W.M.C., Butler, L.G., 1984. Intra-uterine insemination of ewes with frozen semen. J. Agric. Sci. 102, 233–234.

Menchaca, A., Vilarinõ, M., Pinczak, A., Kmaid, S., Saldaña, J.M., 2009. Progesterone treatment, FSH plus eCG, GnRH administration, and Day 0 Protocol for MOET programs in sheep. Theriogenology 72, 477–483.

Quirke, J.R., Jennings, J.J., Hanrahan, J.P., Gosling, J.P., 1979. Oestrus, time of ovulation, ovulation rate and conception rate in progestagen-treated ewes given Gn-RH, Gn-RH analogues and gonadotrophins. J. Reprod. Fertil. 56, 479–488.

Robertson, I., Nelson, R., 1999. Certification and Identification of Embryos. In: Stringfellow, D.A., Seidel, S.M. (3th ed.), International Embryo Transfer Society, editors. Manual of the International Embryo Transfer Society: A Procedural Guide and General Information for the use of Embryo Transfer Technology, Emphasizing Sanitary Precautions. USA: The Society, Champaign, pp. 109-122.

Russel, A.J.F., Doneya, J.M., Gunn, R.G., 1969. Subjective assessment of body fat in live sheep. J. Agric. Sci. 72, 451–454.
Theodosiadou, E., Goulas, P., Kouskoura, T.H., Smokovitis, A., 2004. Oestrogen and progesterone concentrations in plasma and oviductal tissue of ewes exhibiting a natural or induced oestrus. Anim. Reprod. Sci. 80, 59–67.
Viñoles, C., Meikle, A., Forsberg, M., Rubianes, E., 1999. The effect of subluteal levels of exogenous progesterone on follicular dynamics and endocrine patterns during early luteal phase of the ewe. Theriogenology 51, 1351–1361.
Walker, S.K., Smith, D.H., Seamark, R.F., 1986. Timing of multiple ovulations in the ewe after treatment with FSH or PMSG with and without GnRH. J. Reprod. Fertil. 77, 135–142.