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Efficiency of Superovulatory Treatment with FSH-p for In Vivo Embryo Production in Dairy Ewes: Multiple Versus Single Dose Regimen

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Abstract: The experiment was designed to evaluate ovarian response and embryo production in relation to multiple (4 vs 6 vs ≥ 8) and single injection treatment with FSH-p. Fifty dairy ewes were synchronized (FGA, in vaginal pessary, for 9 days + $PGF_{2\alpha}$, 50 μg at 7th day) and subdivided into 5 superovulatory treatment groups: A) single FSH-p dose performed at the 24th h before pessary withdrawal (p.w.); B) single FSH-p dose at the 48th h before p.w.; C) 4 FSH-p doses, starting 24 h before p.w.; D) 6 FSH-p doses, starting 48 h before p.w.; E) ≥ 8 FSH-p doses, starting 60 h before p.w. until onset of estrus. All animals were treated with the same total dose of 250 IU FSH-p; Groups A) and B) were s.c. injected while Groups C), D) and E) were i.m. injected twice daily. Seven days after estrus, the ovarian response and embryo production were assessed. The results indicate that the increase in injections to ≥ 8 (Group E) does not improve superovulatory response, especially in comparison with the 6-dose regimen (Group D). The single dose treatment results in a similar ovarian response as the multiple injection regimen when performed at −24 h before p.w., but it decreases transferable embryo production when compared to Group D). Treatment D) induces a good ovarian response (8.8 ovulations/ ewe) and the highest number of transferable embryos (5/ ewe; 0.05>P<0.01).

Key words: FSH-p, superovulation, embryo production, dairy ewes

Introduction

In ewes, the use of porcine pituitary extract (FSH-p) to induce superovulation for embryo transfer has permitted a remarkable improvement in quantitative and qualitative embryo production (Martemucci *et al.,* 1988a, 1999; Cognié, 1999 and D'Alessandro *et al.*, 2000).

The efficiency of Follicle stimulating hormone is due fundamentally to a lower impact on ovarian functionality and endocrine pattern. When compared with PMSG, which has been widely used in the past (Mutiga and Baker, 1982; Armstrong and Evans, 1983 and Cogniè, 1999), FSH-p has resulted in a lower incidence of iperthrophic ovaries and anovulatory large follicles (Moor *et al.,* 1985; Martemucci *et al.,* 1988a; Jabbour and Evans, 1991 and Martemucci *et al.*, 1997), a high synchronization rate of ovulation time (Walker *et al.*, 1986), a low presence of prematurely activated oocytes (Moor *et al.*, 1984, 1985 and Driancourt e Fry, 1995), lower estrogenic activity of large unovulated follicles (Driancourt and Fry, 1992) and low circulating levels of oestradiol-17β (Jabbour and Evans, 1991).

The short half-life of FSH-p (110 min.; Fry *et al.*, 1987) requires frequent injections to stimulate follicular activity. Usually, superovulatory protocols consist of administration of several FSH-p doses during the last days of a progestative treatment to synchronize the occurrence of estrus, at regular intervals, over 2 to 4 days (Ruttle *et al.*, 1988; Martemucci *et al.*, 1988a; Rexroad and Powell, 1991 and D'Alessandro *et al.*, 1996a, b).

In cows, it has been shown that twice daily injections of pituitary extract induced a greater superovulatory response compared to once daily treatment (Looney *et al.*, 1981; Monniaux *et al.*, 1983 and Walsh *et al.*, 1993) and the increase in FSH-p injection frequency could lead to a decrease in the total dose of FSH required for superovulation (Looney *et al.*, 1981).

The main problems relating to the optimization of FSH-p treatment are the improvement of its efficiency and the possibility to simplify the protocol by reducing the number of injections, thereby reducing donor handling costs and the stress associated with a lower superovulatory response (Edwards *et al.*, 1987). In cows, it has been shown that a single subcutaneous injection of FSH-p induces a superovulatory response comparable to that of multiple intramuscular injections (Hockley *et al.*, 1992 and Yamamoto *et al.*, 1992).

The study was designed to determine the efficacy of different regimens of FSH-p treatment, as number and time of injection, on ovarian response and embryo production in dairy ewes.

Materials and Methods

The experiment was conducted in Southern Italy (41 °N latitude) during the autumn. Fifty adult, non-lactating Leccese breed ewes were considered. Superovulatory treatment was performed using a total dose of 250 IU of a porcine pituitary extract (FSH-p; Pluset, Laboratorios Calier, Madrid, Spain), having a FSH/LH ratio of 1:1, dissolved in saline. The ewes were synchronized for estrus by vaginal pessaries containing 30 mg fluorogestone acetate (FGA) (Chrono-gest; Intervet, Milan, Italy), left in situ for 9 days, associated to a i.m. injection of 50 μg $PGF_{2\alpha}$ (Cloprostenol, Estrumate, Schering-Plough) at 48 hours before sponge removal. The ewes were randomly allocated to five homogeneous groups (n = 10) corresponding to the experimental treatments. Regimens were as follows (Fig.1): (A) a single subcutaneous dose FSH-p 24 hours before pessary removal; (B) a single subcutaneous dose FSH-p 48 hours before pessary removal; (C) four doses FSH-p (100, 75, 50, 25 IU), given 12 h apart, beginning at 24 hours before pessary removal; (D) six doses FSH-p (71.5 - 71.5, 35.7-35.7, 17.8-17.8 IU), given 12 h apart, beginning at 48 hours before pessary removal; (E) eight or more doses FSH-p (65 - 65, 30-30, 20-20, 10-10 IU), given 12 h apart, beginning at 60 hours before hours pessary removal, continuing the administration of 10 IU FSH-p until the occurrence of estrus (W. Vivanco, personal communication). In Groups (A) and (B) the ewes were s.c. injected behind the shoulder. Estrous detection commenced 16 hours after the end of progestative treatment by teaser rams and the sexual receptive ewes were hand-mated every 6-8 hours onward until standing estrus. On the 7^{th} day after sponge removal, ovarian response (numbers of *corpora lutea* and follicles >4 mm) and embryo production were estimated by medium-ventral laparotomy of ewes previously anesthetized, flushing of uterine hbrns and recovery of ova (Martemucci *et al.*, 1988b). Ova recovered were observed first under a stereomicroscope (40X), then they were examined under a phase contast microscope (225X), considering cleavage as evidence for fertilization. Embryos were classified according to their stage of development in relation to estrus and to their morphological features. Only embryos graded as excellent and good were considered as transferable (Martemucci *et al.*, 1988a).

Statistical Analysis: Data were analyzed by least square analysis of variance using the GLM procedure of the SAS (SAS, 1999/2000). The *t*-test with the predicted difference (PDIFF) option of the GLM was used to compared least square means while Chi-square test was used for the percentage values (SAS, 1999/2000).

Results

Superovulatory treatment differing for time and number of FSH-p injections significantly affected ($P<0.01$) oestral response of ewes. Mean interval from pessary removal to onset of estrus was significantly ($P<0.01$) later in ewes treated with a single FSH-p dose 48 hours from the end of progestative treatment (Group B; 42.6 hours) (Table 1). Considering the multiple dose regimens of 4, 6 and ≥ 8 FSH-p injections (Groups C, D and E), the ovulatory response was similar, ranging from 8.0 to 5.1 ($P>0.05$; Table 1). However, the Group D) treated with 6 FSH-p doses had a tendentially higher ovulation rate (8 *corpora lutea*) and showed the highest proportion of superovulated ewes (90%), as well.

Whithin the single dose regimen, FSH-p injection time of 24 h from pessary removal (Group A) induced an ovulation rate of 5.3 ± 1.2, not different ($P>0.05$) among the other treatment groups. The advance of the single FSH-p injection to 48 from pessary removal (Group B), affected the lowest ovulation rate (2.2 ± 2.1) with significant difference compared to both 6-dose (Group D; $P<0.01$) and ≥ 8 FSH-p dose (Group E; $P<0.05$) regimens (Table 1).

No differences were found among the treatment groups in relation to the mean number of large unruptured follicles, which ranged from 0.2 to 1.3 for ewe. Total ovarian response, defined as the sum of *corpora lutea* plus unruptured follicles, was significantly influenced ($P<0.05$) by the regimen of superovulation. The highest value was observed in the 6 FSH-p dose group (8.8 ± 1.2), with significant differences compared to the single dose at 48 h before pessary removal ($P<0.01$) and 4-dose ($P>0.05$) groups (Table 1).

The mean number of ova recovered was significantly ($P<0.01$) influenced by the regimen of FSH-p treatment. The 6-dose group recordered the highest value of ova recovered (7.2 ± 0.9; Table 2) corresponding to the highest recovery rate (90.0 ± 12.3%); both values resulted significantly different compared to Group B) for $P<0.01$ and $P<0.05$, respectively.

Table 1: Estral and ovarian responses (mean ± ES) in dairy ewes treated with 250 IU FSH-p by a single dose given sc at 24 or 48 h before pessary removal (p.r.) and by multiple dose regimens (4, 6, ≥ 8 doses) 12 h apart

	1Dose (-24 h p.r.) Group A	1Dose (-48 h p.r.) Group B	4Doses Group C	6Doses Group D	≥ 8Doses Group E
N. treated ewes	10	10	10	10	10
% Superovulated ewes	80	30^{a}	80	90^{b}	80
Onset of estrus from p.r. (h)	29.0 ± 1.6^{B}	43.6 ± 1.6^{A}	24.8 ± 1.6^{B}	25.5 ± 1.6^{B}	28.8 ± 1.6^{B}
Ovulation rate	5.3 ± 1.2	2.2 ± 1.2^{Aa}	5.1 ± 1.2	8.0 ± 1.2^{B}	5.9 ± 1.2^{b}
Large Follicles	1.3 ± 0.4	1.1 ± 0.4	0.2 ± 0.4	0.8 ± 0.4	0.2 ± 0.4
Total Ovarian reponse	6.6 ± 1.2	3.3 ± 1.2^{A}	5.3 ± 1.2^{a}	8.8 ± 1.2^{Bb}	6.1 ± 1.2

Values with different letters in the row differ significantly: A, B ($P<0.01$); a, b ($P<0.05$).

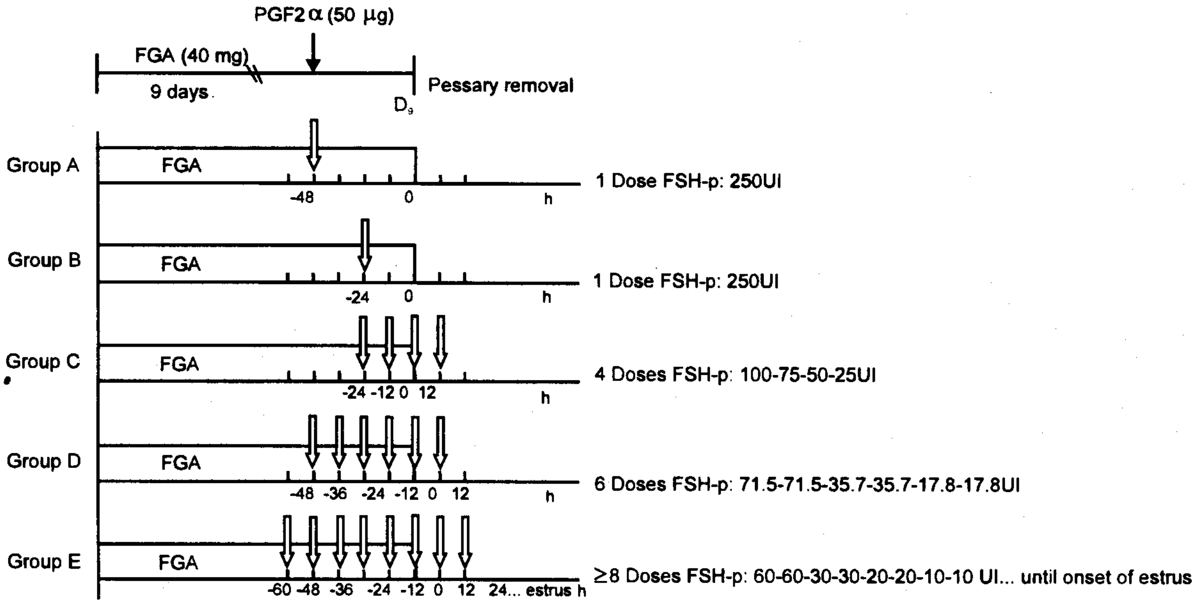

Fig.1: Protocols of superovulatory treatments with 250 IU FSH-p by a single dose given s.c. at 24 or 48 h before pessary removal (p.r.) and by multiple i.m. dose regimens (4.6≥8 does) 12 h apart

Table 2: Ova recovery and embryo production (mean ± ES) in dairy ewes treated with 250 IU FSH-p by a single dose given sc at 24 or 48 h before pessary removal (p.r.) and by multiple dose regimens (4, 6, ≥ 8 doses) 12 h apart

	1Dose (-24 h p.r.) Group A	1Dose (-48 h p.r.) Group B	4Doses Group C	6Doses Group D	≥8Doses Group E
Ova recovered	3.7±0.9	1.2 ± 0.9^{A}	3.5±0.9	7.2 ± 0.9^{B}	3.8±0.9
%	69.8±13.0	54.5 ± 14.7^{a}	68.6±13.0	90.0 ± 12.3^{b}	64.4±13.0
Empty zona	0.2 ± 0.4^{A}	0.1 ± 0.4^{A}	0.4 ± 0.4^{a}	1.6 ± 0.4^{Bb}	/
Unfertilized ova	1.2±0.4	0.3±0.4	0.7±.4	/	0.1±0.4
Fertilization rate (%)	51.6 ± 12.4^{A}	59.5 ± 14.0^{a}	65.5±12.3	77.5±11.7	96.8 ± 12.4^{Bb}
Embryos	2.3±0.8	0.8 ± 0.9^{Aa}	2.4±0.8	5.4 ± 0.8^{B}	3.7 ± 0.8^{b}
Transferable embryos	1.8 ± 0.9^{A}	0.7 ± 1.2^{A}	2.1 ± 0.8^{A}	5.0 ± 0.7^{Bb}	2.5 ± 0.8^{a}

Values with different letters in the row differ significantly: A, B (P<0.01); a, b (P<0.05).

Mean number of empty zona was influenced (P<0.01) by superovulatory treatment, but the values resulted low, with a range of 0.1 (Group A) to 1.6 (Group D) per ewe (Table 2). There were not significant differences among the treatment groups regarding number of unfertilized ova (range 0.1 to 1.2; P> 0.05).
Fertilization rate was highest under the ≥ 8 FSH-p dose regimen (Group E; 98.8±12.4%), with significant differences compared to the single dose at 24 h (P<0.01) as well as 48 h before pessary removal (P<0.05).
The highest embryo production was provided by the 6-dose regimen of Group D) (5.4±0.8 per ewe), significantly different in comparison with the single dose at 48 h (0.8±0.9; P<0.01). This last value also resulted lower than that of the ≥ 8-dose regimen (3.7±0.8; P<0.05). Group D) also had the highest production of transferable embryos (5.0±0.7 per ewe) with significant differences compared to the other treatment groups (0.05>P<0.01) (Table 2).

Discussion

In ewes, like in cows (Walsh *et al.*, 1993), the response to the treatment for ovarian hyperstimulation is due fundamentally to three factors: the used gonadotrophin, its biological activity and its regimen of administration. In general, the protocols widely used for porcin pituitary extract (FSH-p), are based on multiple injections, with intervals of 12 hours, over a 2 to 4 day period (Armstrong and Evans, 1983; Walker *et al.*, 1986; Martemucci *et al.*, 1988a; Rexroad and Powell, 1991 and D'Alessandro *et al.*, 1997), with the aim to maintain blood concentrations of the hormone, characterized by a short half life (110 min; Fry *et al.*, 1987), sufficient to induce ovarian stimulation. This is because the continuous administration of FSH would negatively influence the ovarian response (Sharpe *et al.*, 1986).

Several studies have focused on a simplification of the superovulatory treatment with FSH-p by reducing the number of injections. For this goal, various molecules capable of increasing the clearance of hormonal preparations, and therefore of extending their biological effect, have been used as vehicles for FSH-p, such as propylen glycole (Lopez-Sebastian *et al.*, 1993) or polyvinilpyrrolidone (sheep: Dattena *et al.*, 1994; D'Alessandro *et al.*, 2001; heifers: Takedomi *et al.*, 1995; rabbit: Kanayama *et al.*, 1994). In cows, it has been reported that a single bolus injection of FSH-p can be an alternative to a 5-d, twice-daily treatment protocol (Hill *et al.*, 1985), also when FSH-p is dissolved in saline (Hockley *et al.*, 1992 and Bo *et al.*, 1994).

In this study we wanted to evaluate the possibility of improving superovulatory response in dairy sheep by extending the duration of FSH-p treatment, until the appearance of estrus ($\geq$ 8 doses), adopting a protocol employed commercially in Australia (W. Vivanco, personal communication). Evaluated simultaneously was the possibility of reducing the number of hormonal injections to a single one, without the use of long acting molecules as the vehicle. In order to reduce the degradation rate of FSH-p, the single dose treatment was performed by subcutaneous injection behind the shoulder.

The increase in the number of FSH-p injections from 4 or 6 (Groups C, D) to more than 8, beginning the treatment 60 hours from the progestative pessary withdrawal (Group E), did not determine an improvement in either ovarian response or embryo production. Within multiple injection regimens, the protocol of 6 FSH-p beginning at the 48th hour before progestative pessary withdrawal provided an ovarian response and embryo production tendentially higher, but the highest ($0.05>P<0.01$) production of transferable embryos. Also, in other studies this regimen of treatment has conditioned a higher superovulatory response (Lopez-Sebastian, *et al.*, 1990 and Boland *et al.*, 1995); in goats the greater number of injections (7 vs 4) corresponds to an improvement in the ovarian response and embryo production (Martemucci *et al.*, 1992), as well. In this study, the treatment with 6 doses of FSH-p has provided a higher production of good quality embryos than that reported in other papers, with the same regimen of treatment (D'Alessandro *et al.*, 2001 and Cordeiro *et al.*, 2003) and also with a greater dose of gonadotrophin (525 UI FSH-p; D'Alessandro *et al.*, 1996a).

As far as the single dose regimen is concerned, the results show a remarkable infuence related to the time of treatment. FSH-p injection 48 hours from the pessary withdrawal has conditioned the worst response in the treated ewes in terms of induction of the superovulation, ovulation rate and quantitative and qualitative embryo production, and also resulted in a significant delay in the appearance of the estrus. This could probably be ascribed to interference caused by the massive dose of FSH-p (250 UI) on the physiological mechanisms regulating folliculogenesis, considering that the gonadotrophic treatment (in a single dose) coincided with the administration of $PGF_{2\alpha}$, recruitment of the follicles happens after luteolysis (Driancourt *et al.*, 1985) and the half life of FSH-p is very short (110 min; Fry *et al.*, 1987). The negative effect of 48 hours as hormonal administration time agrees with the results of a previous study (D'Alessandro *et al.*, 2001), where the superovulatory treatment in a single dose was carried out employing PVP (polyvinilpyrrolidone) as the vehicle for FSH-p.

In this study, the regimen in a single FSH-p injection 24 hours from pessary withdrawal has determined estral and ovarian responses and embryo production similar to those of the treatments in multiple doses; on the other hand, transferable embryo production was lower, especially in comparison with the 6-dose treatment. From these findings, it can be assumed that the hormonal treatment must satisfy precise physiological requirements in order to induce multiple ovulations, but such conditions are not the same as those required for the production of good quality embryos. It could be hypothesized that both the hormonal product and dose used in this study have provided an idoneous stimulus for follicular growth through the effect of the FSH fraction of the pituitary extract, but that the contemporary action of the LH fraction has negatively affected the quality of embryos. In cows, in fact, it has been demonstrated that hormonal products having high LH content negatively influence the quality of embryos (Monniaux *et al.*, 1984; Murphy *et al.*, 1984 and Herrler *et al.*, 1991) and a low LH content improves the superovulatory response in cows as well as in ewes (Chupin *et al.*, 1984; Murphy *et al.*, 1984; Donaldson and Ward, 1985 and Torres *et al.*, 1987).

Conclusion

Superovulatory treatment performed by $\geq$ 8 doses of FSH-p, beginning 60 hours before the end of progestative treatment until the appearance of estrus does not improve the embryo production in comparison with the 4- and 6-dose regimens. The single FSH-p dose protocol provides an ovarian response similar to those of multiple dose regimens only when it is performed 24 hours from pessary withdrawal. Compared to the other treatments, this protocol of superovulation gives a transferable embryo production lower only than that provided by the 6-dose FSH-p treatment. It can therefore be concluded that the treatment performed following 6 injections of FSH-p is effective to induce superovulation and provides a satisfactory production of good quality embryos (5 transferable embryos per ewe).

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