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Theriogenology

Theriogenology 65 (2006) 356–365

[www.journals.elsevierhealth.com/periodicals/the](http://www.journals.elsevierhealth.com/periodicals/the)

## The effect of melatonin implants during the seasonal anestrus on embryo production after superovulation in aged high-prolificacy Rasa Aragonesa ewes

F. Forcada<sup>a,\*</sup>, J.A. Abecia<sup>a</sup>, J.A. Cebrián-Pérez<sup>b</sup>,  
T. Muño-Blanco<sup>b</sup>, J.A. Valares<sup>a</sup>, I. Palacín<sup>a</sup>, A. Casao<sup>a</sup>

<sup>a</sup> *Department of Animal Production and Food Science, University of Zaragoza,  
177 Miguel Servet, 50013, Zaragoza, Spain*

<sup>b</sup> *Department of Biochemistry and Molecular and Cellular Biology, University of Zaragoza,  
177 Miguel Servet, 50013, Zaragoza, Spain*

Received 1 February 2005; received in revised form 13 May 2005; accepted 13 May 2005

### Abstract

The aim of this study was to assess the effect of melatonin implants administered in March on the ovarian cyclicity, ovulatory response and embryo production after repeated superovulation of selected high-prolificacy Rasa Aragonesa aged ewes. During the seasonal anestrus of two consecutive years, 113 superovulatory treatments have been performed. Ewes were treated (M) or not (C) with melatonin implants in March (day 0). All of them received intravaginal progestogen sponges on day 24 (recovery 1) and 80 (recovery 2) after melatonin implants insertion in year 1, and on day 28 and 77 in year 2. The intravaginal sponges were removed after 14 days. Superovulatory treatments consisted of eight doses in decreasing concentrations (2 mL × 2 and 1 mL × 6) of oFSH (Ovagen™) administered twice daily starting 72 h before sponge removal. Seven days after the onset of estrus, embryos were recovered by laparotomy. Melatonin increased cyclicity only in recovery 2 year 2 (83% versus 42%;  $P < 0.05$ ) but not in the other experimental periods. Among the 78% (88) ewes that ovulated and produced functional corpora lutea, melatonin implants tended to improve embryo viability in recovery 2 by increasing the number of blastocysts per superovulatory treatment ( $2.4 \pm 0.6$  versus  $1.1 \pm 0.4$ ;  $P = 0.09$ ), the rate of viability ( $67 \pm 9\%$  versus  $43 \pm 9\%$ ;  $P < 0.05$ ), and freezability ( $55 \pm 9\%$  versus  $33 \pm 8\%$ ;  $P < 0.05$ ). More specifically, melatonin induced a significant reduction of the number and rate of non-viable (degenerate and retarded) embryos in

\* Corresponding author. Tel.: +34 976 761600; fax: +34 976 761612.  
E-mail address: [forcada@unizar.es](mailto:forcada@unizar.es) (F. Forcada).

recovery 2 ( $0.4 \pm 0.1$  embryos versus  $1.3 \pm 0.3$  embryos and  $4 \pm 1\%$  versus  $22 \pm 6\%$ , respectively;  $P < 0.05$ ). Our results demonstrate that melatonin implants in March can improve at medium term (3 months after implantation) the viability of embryos collected from selected high-prolificacy Rasa Aragonesa aged ewes after superovulation.

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*Keywords:* Melatonin; Sheep; Embryo; Superovulation; Seasonal anestrus; Rasa Aragonesa

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## 1. Introduction

Multiple ovulation and embryo transfer (MOET) technology was developed in sheep to accelerate genetic improvement by increasing the number of offspring produced by each superior female, although the broader application of the technology is limited by the seasonality of breeding in this species. Embryo production *in vivo* (from donor ewes) is performed mainly during the reproductive season of the ewe, which limits the number of animals that an embryo transfer team can manage per year. Extending the application of the techniques to ewes during their non-breeding season would enhance labour efficiency and enable the sheep industry to benefit from the wider application of the technology.

Frequently, the conception rates and litter sizes of spontaneously ovulating ewes are higher during the peak of the breeding season compared to the beginning and end of the breeding season or during the seasonal anestrus period. Conception rate is known to decline after treatment by superovulation in anestrus sheep [1], possibly because of seasonal shifts in LH secretion and/or associated effects on follicular function [2]. The effect of season on embryo production *in vivo* seems to vary with latitude, and is small or non-existent in the Mediterranean area after superovulation using porcine [3] or ovine FSH [4].

The administration of melatonin is used widely to improve reproductive performance during anestrus in both highly seasonal [5] and Mediterranean ewes [6–8]. *In vivo* and *in vitro* melatonin stimulates luteal progesterone production in sheep [9,10]. Little is known, however, about the direct effect of melatonin on the embryo. McEvoy et al. [11] did not find differences in the number and quality of embryos of melatonin-treated and untreated donor Border Leicester  $\times$  Scottish Blackface ewes in anestrus.

Forcada et al. [12] tested the possibility of obtaining embryos of high genetic value from selected ewes at the end of their reproductive lives. The secretion of melatonin is reduced in senescence [13]. Although no information is available on the effect of ageing on the functioning of the reproductive system in sheep, the administration of melatonin to aged female rats increases GnRH synthesis [14] and pituitary responsiveness to GnRH [15] to levels exhibited by young, cyclic animals.

To discover new ways of obtaining embryos of high genetic value, in the Rasa Aragonesa breed, a local Spanish genotype that has a short anestrus period (<100 days) between May and July [16], we are investigating the use of the best females for a given character as donors at the end of their reproductive lives. The objective of the present study was to evaluate the effect of melatonin implants on embryo production and quality during the seasonal anestrus in aged ewes selected for prolificacy and superovulated twice before culling for non-reproductive reasons (e.g., age, teeth).

## 2. Materials and methods

### 2.1. Animals

The experiment, which was conducted at the experimental farm of the University of Zaragoza, Spain (latitude 41°40'N), met the requirements of the European Community Commission [17].

In the study, we used mature Rasa Aragonesa ewes older than 8 years (average  $10.3 \pm 0.3$  years) that had more than eight lambings in their life and were selected for prolificacy (mean litter size  $\geq 1.4$  lambs per lambing). The ewes were reared on farms registered by ANGRA (Spanish Association of Rasa Aragonesa Breeders) and, at the end of their reproductive life, brought to the experimental farm, where were housed in communal yards with uncovered area and fed a concentrate ration, lucerne hay, and barley straw at rates designed to provide 1.2 times their maintenance requirements. Fresh, clean water was available at all times. Both live weight and body condition score were recorded at the beginning of the experiments, at the time of implantation with melatonin.

### 2.2. Experimental design

The study was performed in the spring of two consecutive years. In 2002, we used 32 ewes. On 7 March (day 0), 17 ewes received a single 18-mg melatonin implant (Melovine, Ceva Santé Animale, Libourne, France) (group M) at the base of their left ear. The remaining 15 ewes were used as controls (group C). The implants are designed to maintain high plasma melatonin concentrations for at least 60 days, although we have previously reported that their functionality can be extended for more than 100 days [25].

On day 24, ewes received intravaginal sponges containing 30 mg fluorogestone acetate (FGA) (Chrono-gest; Intervet, Salamanca, Spain), which were inserted for 14 days. The superovulatory treatment was started 72 h before sponge removal and consisted of 176 NIH-FSH-S1 units of NIADDK-oFSH-17 (Ovagen<sup>TM</sup> ICP, Auckland, New Zealand) administered in eight i.m. doses of decreasing concentrations ( $2 \text{ mL} \times 2$  and  $1 \text{ mL} \times 6$ ) at 12-h intervals. Rams of proven fertility were placed with the ewes at the time of pessary withdrawal, and ewes were checked for estrus every 8 h using different males.

Seven days after the onset of estrus, embryos were collected (recovery 1) using mid-ventral laparotomy. Ewes were anesthetized using an i.m. injection of 0.4 mL 2% xylazine, and 10 mL sodium thiopental (20 mg/mL) (Thiobarbital, Braun Medical, Jaén, Spain) was administered by i.v. injection 5 min later. The ovarian response was assessed in terms of number of functional corpora lutea with a good morphological quality compatible with an active luteal phase. Uterine horns were exposed and flushed using a Foley catheter with prewarmed phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) and antibiotics (penicillin and streptomycin). To minimize the development of post-operative abdominal adhesions, reproductive tracts were flushed with a 2.5% heparin solution in saline before closure. All ova and embryos were examined under a stereomicroscope (20–40 $\times$  magnification) and classified according to their stage of development and morphology [18]. Compacted morulae and early, expanded, and hatched blastocysts were considered to be viable embryos. According to the

day on which the embryos were collected, and the good viability in vivo of vitrified ovine blastocysts [19–21] and compacted morulae [22], only selected embryos without imperfections and with a spherical/symmetrical shape [23] in these two stages of development (except hatched blastocysts) were deemed freezable. The same procedure was repeated on day 80 following melatonin implantation, when ewes received intravaginal sponges, again, and were treated with the same superovulatory protocol (recovery 2).

In 2003, we performed the same experiment using 26 ewes, half of which were implanted with melatonin on 9 March (day 0). Intravaginal sponges were inserted on day 28 (recovery 1) and day 77 (recovery 2).

In each year of the study, one melatonin-implanted ewe died between the first and second superovulatory treatments and was excluded from the study, but previous performances in recovery 1 were included in the analyses.

### 2.3. Blood sampling and progesterone assay

To evaluate previous cyclicity in each synchronization treatment using plasma progesterone concentrations, blood samples were collected from all ewes just before and 1 week prior to each sponge was inserted. To assay progesterone, we used solid-phase RIA kits based on polypropylene tubes coated with rabbit antibodies to progesterone. The radiolabeled tracer for the assay was  $^{125}\text{I}$  progesterone (DPC, Los Angeles, CA). The intra- and inter-assay coefficients of variation were 6.4% and 8.7%, respectively. Ewes were considered to be in anestrus if progesterone did not exceed 1 ng/mL in either of the two blood samples taken before the start of each superovulatory treatment [24].

### 2.4. Assessment of the response to the superovulatory treatment

The following information was recorded for each ewe: number of corpora lutea (CL), total number of recovered oocytes and embryos (RE), number of fertilized embryos (FE), number of non-viable embryos (both degenerated and retarded embryos) (NVE), number of blastocysts (B), number of viable embryos (compacted morulae and early, expanded and hatched blastocysts) (VE) and number of freezable embryos (compacted morulae and blastocysts except hatched blastocysts) (FRE). The rate of recovery was calculated by dividing, in every sheep, the total number of RE by the total number of CL. Rates of blastocysts and of fertilized, non-viable, viable and freezable embryos were obtained by dividing the number of B, FE, NVE, VE and FRE, respectively, by the total number of RE. All rates are expressed as percentages.

### 2.5. Statistical analysis

The proportions of cyclic ewes before the insertion of sponges were compared by chi-square. This test was also used to compare the percentages of females not ovulating or with regressed corpora lutea following superovulatory treatments. Analysis of variance revealed that year did not have a significant effect on ovulation rate and embryo production traits. Therefore, data from both years were pooled and the effects of melatonin and recovery

number on ovulation rate and embryo production were tested through a  $2 \times 2$  ANOVA. Statistical treatment of the data expressed as percentages was performed after transformation of the values for each individual percentage to the arcsine. Results are expressed as mean  $\pm$  S.E.M.

### 3. Results

Both M and C groups had similar live weights and body conditions at the time of implantation ( $55.3 \pm 1.7$  kg and  $2.6 \pm 0.1$  versus  $56.4 \pm 1.4$  kg and  $2.7 \pm 0.1$ , respectively). With the exception of the control group in year 2 recovery 2, percentages of cyclicity were higher than 50% (Fig. 1). In year 2 recovery 2, melatonin implants had a significant effect on cyclicity at medium term ( $P < 0.05$ ) (Fig. 1).

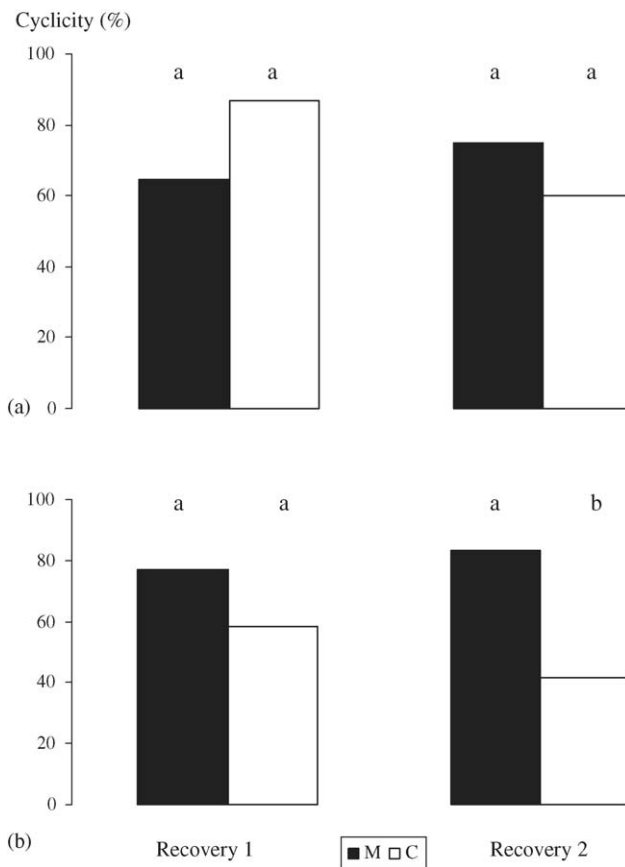


Fig. 1. Percentage of cyclicity in aged melatonin-treated (M) and control (C) Rasa Aragonesa ewes during the seasonal anestrus through plasma progesterone concentrations just before sponge insertion in 2002 (a) and 2003 (b). The synchronization-superovulation treatment began 24 and 28 days (recovery 1) and 77 and 80 days (recovery 2) after the insertion of melatonin implants in early March. Bars with different letter differ at  $P < 0.05$ .

One hundred and thirteen superovulatory treatments were performed, but only those of them which resulted in ovulation with functional corpora lutea, 88 (78%), were included in the analysis to test the effect of the melatonin treatment and number of recovery on embryo production and quality. All ewes showed estrous behaviour after sponge withdrawal. Most of them ovulated (90%), although we observed a high proportion (12%) of premature luteal regression. Both parameters were not influenced by the number or recovery or the treatment with melatonin.

Because there was not an effect of year on the response of oFSH treatment, data were pooled to test for an effect of melatonin implants at short and medium term on embryo production and quality parameters. Despite individual variability in all parameters in the response to the superovulatory treatment, the factorial  $2 \times 2$  analysis of variance revealed that melatonin implants reduced both the number and rate of non-viable embryos ( $P < 0.05$ ), increasing the rates of blastocysts and of viability ( $P < 0.05$ ). Freezability rate seemed to be also improved by melatonin ( $P = 0.05$ ). The number of recovery exhibited a tendency to influence the number of recovered ova/embryos per ovulating ewe ( $P = 0.06$ ) and the recovery rate ( $P = 0.07$ ), by decreasing both of them after the second superovulatory treatment.

More specifically, the effect of melatonin in the improvement of the embryo quality after superovulation seemed to be exerted at medium term, 3 months after implantation. Thus, implanted ewes exhibited a significantly higher rate of blastocysts and of viable and freezable embryos than controls in recovery 2 ( $P < 0.05$ ) (Table 1). Particularly and although ovulation and fertilization rates were not influenced by the hormone, melatonin

Table 1

A comparison of the embryo production (mean  $\pm$  S.E.M.) in melatonin-treated and control Rasa Aragonesa aged ewes during the seasonal anestrus

	Melatonin-implanted		Control	
	Recovery 1	Recovery 2	Recovery 1	Recovery 2
No. of ovulating ewes with functional corpora lutea	22	22	23	21
Ovulation rate	11.9 $\pm$ 1.7	12.5 $\pm$ 2.3	12.9 $\pm$ 1.6	11.1 $\pm$ 1.7
No. of recovered ova	7.7 $\pm$ 1.2	6.5 $\pm$ 1.1	9.0 $\pm$ 1.3 e	5.9 $\pm$ 0.9 f
Recovery rate (%)	67.0 $\pm$ 6.7	60.8 $\pm$ 7.0	65.4 $\pm$ 6.8 e	48.6 $\pm$ 7.0 f
No. of fertilized embryos	4.2 $\pm$ 1.0	4.4 $\pm$ 0.8	4.9 $\pm$ 1.2	4.1 $\pm$ 0.9
Fertilization rate (%)	58.6 $\pm$ 10.1	70.8 $\pm$ 8.9	51.7 $\pm$ 9.5	65.4 $\pm$ 9.2
No. of non-viable embryos	0.6 $\pm$ 0.3	0.4 $\pm$ 0.1 c	0.7 $\pm$ 0.2	1.3 $\pm$ 0.3 d
Rate of non-viable embryos (%)	7.2 $\pm$ 3.8	4.2 $\pm$ 1.7 c	9.3 $\pm$ 3.1 e	22.4 $\pm$ 6.7 d,f
No. of blastocysts	2.3 $\pm$ 0.8	2.4 $\pm$ 0.6 e	2.5 $\pm$ 0.7	1.1 $\pm$ 0.4 f
Rate of blastocysts (%)	29.7 $\pm$ 8.8	36.7 $\pm$ 8.4 c	24.1 $\pm$ 6.6	16.3 $\pm$ 5.6 d
No. of viable embryos <sup>a</sup>	3.6 $\pm$ 1.0	4.0 $\pm$ 0.8	4.2 $\pm$ 1.2	2.8 $\pm$ 0.8
Viability rate (%)	51.3 $\pm$ 10.6	66.6 $\pm$ 8.9 c	42.3 $\pm$ 9.4	42.9 $\pm$ 8.9 d
No. of freezable embryos <sup>b</sup>	3.1 $\pm$ 1.0	3.4 $\pm$ 0.7	3.6 $\pm$ 1.1	2.2 $\pm$ 0.7
Freezability rate (%)	43.7 $\pm$ 9.9	54.5 $\pm$ 9.0 c	37.1 $\pm$ 8.4	33.2 $\pm$ 7.6 d

The synchronization-superovulation treatment began 24 and 28 days (recovery 1) and 77 and 80 days (recovery 2) after the insertion of melatonin implants in early March. Within row, different letters (c, d) indicate differences of  $P < 0.05$ . Within row, different letters (e, f) indicate differences of  $P < 0.10$ .

<sup>a</sup> Compacted morulae and early, expanded and hatched blastocysts.

<sup>b</sup> Compacted morulae and blastocysts (except hatched blastocysts).

induced a significant reduction of the number and rate of non-viable (degenerate and retarded) embryos in recovery 2 ( $0.4 \pm 0.1$  embryos versus  $1.3 \pm 0.3$  embryos and  $4 \pm 1\%$  versus  $22 \pm 6\%$ , respectively;  $P < 0.05$ ) (Table 1).

#### 4. Discussion

Given the age of the ewes involved in our experiments and the absence of males, percentages of cyclicity before the first superovulatory treatment of each year were high, with an average of 72% in both years, and not influenced by melatonin. This reflects the limited sexual seasonality of the Rasa Aragonesa breed. However and although no significant differences were elucidated, ewes of C group seemed to reduce cyclicity before the second synchronization treatment at the end of May, because of the response to the prevalent photoperiod. In fact, we have previously reported that the deepest seasonal anestrus takes place between May and July in Rasa Aragonesa ewes [16]. Yet, at medium term, between 70 and 80 days after implantation, melatonin prevented from a drop of the cyclicity, although this effect was only significant in year 2 but not in year 1. The high cyclicity exhibited by C ewes could be one of the factors involved in this lack of significance in year 1. In previous studies, no differences in cyclicity between melatonin-treated and control Rasa Aragonesa ewes were found just before ram introduction, 35–40 days after implantation [8,25], although the improvement of reproductive parameters in response to the male effect was higher in implanted ewes. Vigué et al. [26] reported the efficacy of melatonin in inducing a significant increase in GnRH and LH pulsatility in anestrus ewes at medium term, after 74 days of treatment.

In our study, we detected premature luteal regression in 12% of the superovulated females. The premature demise of the corpora lutea after exogenous hormone stimulation is a poorly understood cause of embryo recovery failure and is reported mainly in superovulated ewes following estrus synchronization using a dual prostaglandin injection treatment [27,28]. Inadequate luteal function following GnRH-induced ovulation in anestrus ewes is known [29], although progestogen priming before ovulation induction eliminates premature luteolysis in those females [30,31]. In embryo donor ewes, the incidence of premature luteal regression after a progestogen protocol and superovulation is higher in the seasonal anestrus than in the breeding season, even at temperate latitudes [32,33]. However, the percentage of ewes with premature luteal regression found in the present study during the seasonal anestrus period was similar to that reported in the breeding season using the same breed and age of superovulated ewes [12].

Despite high variability in ovulation rate after superovulatory treatments in small ruminants, especially when they are not subjected to treatment with gonadotrophin-releasing hormone [34,35], the high ovulation rate obtained in our study (average higher than 12 corpora lutea) indicates a very good response to the gonadotrophin treatment in the selected Rasa Aragonesa ewes. This was achieved despite the season and the high average age of the females. At short and medium term, melatonin did not appear to affect ovulation rate. McEvoy et al. [11] reported that melatonin did not induce significant increases in ovulation rate following a superovulatory treatment. However, the efficacy of melatonin in improving the number of ovulating follicles occurs in seasonally [5,36] and Mediterranean

[7,8] non-superovulated ewes because of decreasing atresia [37]. The failure of melatonin to increase ovulation rate in our study suggests that FSH probably recruited all the follicles independently of such mechanisms [11].

A seasonal difference in the response to superovulatory treatments in terms of ovulation rate occurs in ewes from high latitudes [11]. However, no significant effect of season on ovulatory response and embryo production was found in Mediterranean sheep breeds superovulated either with porcine FSH [3] or ovine FSH [4], suggesting that exogenous gonadotrophin treatment obliterates the seasonal effect on ovulation rate that occurs in those ewes when ovulate spontaneously. The mean ovulation rate exhibited by the Rasa Aragonesa ewes in our experiment was similar to that observed in the same breed under the same superovulatory hormone regimen, although during the natural breeding season [12]. However, embryo production in our present study (3.6 and 2.2 freezable embryos per ovulating ewe for recovery 1 and 2 in control ewes) was lower than the embryo yield in the breeding season (4.1 and 4.5 freezable embryos, respectively) [12], showing the lower efficacy of aged ewes as embryo donors during the seasonal anestrus period. This efficacy seemed to be particularly impaired in recovery 2, probably because of the effect of photoperiod in deep anestrus.

The main goal of the present study was to test for an effect of melatonin treatment as a means of improving embryo production in aged ewes when used. Despite high variability the parameters we examined, our results indicate that melatonin has a positive effect at medium term on the viability of fertilized embryos, which results in an increased rates of viability and freezability in recovery 2 because of a significant decrease in the number and rate of fertilized, but non-viable, embryos (degenerated and retarded). Middle-aged females rats are known to exhibit improved function of the neuroendocrine-reproductive axis following melatonin treatment. More specifically, Li et al. [14] reported that melatonin induced an 18% increase in the concentration of mRNA encoding GnRH, completely reversing the effect of age. Furthermore, Diaz et al. [15] demonstrated that melatonin might also act upon the hypothalamus-pituitary axis to improve the LH response to GnRH during ageing by being able to restore this pituitary response to the levels observed in young cyclic rats. Although no similar studies have been conducted on sheep, it has been suggested that melatonin might be useful in improving the functionality of ewes that have a impaired reproduction due to the season or undernutrition. Vigué et al. [26] reported the efficacy at medium term of melatonin to induce a significant increase in GnRH and LH pulsatility in anestrus ewes after 74 days of treatment. The effect of melatonin in increasing ovulation rate was more evident in undernourished than in overfed ewes [7]. Moreover, the addition of melatonin to the culture medium appeared to reduce the *in vitro* secretion of prostaglandin F2 $\alpha$  by the endometrial tissue shortly before implantation, but only in undernourished ewes that exhibited a higher embryonic mortality [38]. To clarify the effect of melatonin as a means of improving reproductive efficiency in aged ewes, further study is required.

In conclusion, the results of our study indicate that exogenous melatonin can improve the viability of embryos collected from high-prolificacy Rasa Aragonesa aged ewes after superovulation in the seasonal anestrus period. However, the effect of melatonin occurs at medium term, 3 months after implantation, increasing the rates of blastocysts, viability and freezability of embryos because of a decreasing number and rate of non-viable embryos.

## Acknowledgements

This study was supported by grant AGL2001-1817 from C.I.C.Y.T. and A26 from D.G.A. (Spain).

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