

**Original Article**

## **Comparison between two progesterone sources and two oestradiol formulations in a Heatsynch protocol for postpartum cycling dairy cows in pasture**

**Maarten F.A. Andringa<sup>1</sup>, Frank J.C.M. Van Eerdenburg<sup>1</sup>, Elisa Fernández<sup>2</sup>, Sofía García<sup>2</sup>, Daniel Cavestany<sup>2,\*</sup>**

<sup>1</sup>*Department of Herd Health and Reproduction, Faculty of Veterinary Medicine, University of Utrecht, Utrecht 80125, The Netherlands*

<sup>2</sup>*Department of Reproduction, Faculty of Veterinary, University of Uruguay, Montevideo 11600, Uruguay*

**To compare an injectable progesterone (MAD-4) with an intravaginal device (IPD), and natural O17 with synthetic oestradiol (OB) in a synchronisation protocol, 51 cows were divided into four groups. Each group was treated with one of the two sources of progesterone and one of the two oestradiol formulations. Oestrus behaviour, follicle diameter, and pregnancy rates were evaluated. Oestrus behaviour ( $p = 0.902$ ), numbers of cows in oestrus ( $p = 0.917$ ), follicle diameter ( $p = 0.416$ ), and pregnancy rates ( $p = 0.873$ ) were similar among the four groups. More cows in the group treated with the IPD and OB scored  $> 200$  oestrus behaviour points compared to the other groups ( $p = 0.038$ ). A longer interval between the end of treatment and oestrus was observed among cows treated with MAD-4 than cows given the IPD ( $p = 0.030$ ), but no differences were found between animals receiving the two oestradiol formulations (OB and O17). While the use of MAD-4 requires further testing, similar responses to natural oestradiol observed in the present study could allow the use of this formulation in reproductive protocols because it is not associated with the potential human health risks of OB.**

**Keywords:** dairy cows, oestrus behaviour, synchronisation

### **Introduction**

In seasonal pasture-based breeding systems, it is important for cows to become pregnant at a specified time of the year to match milk production with the availability of nutrients [12]. Oestrus synchronisation is an effective management tool [20]. However, a great variety of protocols is available [17,19].

Many of these include intravaginal progesterone-releasing devices used in both cycling and anoestrus cows to reduce premature oestrus, improve ovulation synchronisation, and increase pregnancy rates [3,4,21]. These devices maintain progesterone blood levels above 1 ng/mL while placed in the vagina [7,22] but have the disadvantages of high costs, the labour required for insertion and removal, and the environmental pollution caused by discarded and lost devices containing high hormonal levels. Furthermore, placing a device in the vagina also results in localized vaginitis in 50~65% of cows [8,33], and while this does not affect pregnancy rates [33,34] it does impact animal welfare. Many protocols use oestradiol to synchronise ovulation, increase the number of animals that show oestrus signs, and elevate pregnancy rates [14]. The types of oestradiol that are commonly used are synthetic oestradiol salts (benzoate, cypionate, or valerate).

The purpose of the present study was to compare a new injectable progesterone formulation (which circumvents the use of silicon devices) and natural oestradiol versus the synthetic salts. For this, effects of a subcutaneous injectable progesterone preparation were compared to those of an intravaginal progesterone-releasing device (IPD) using a modified Heatsynch protocol. The injectable progesterone has slow elimination time so that progesterone levels  $> 1$  ng/mL are maintained for at least 48 h [6] and the decline in progesterone levels is similar to that observed at the end of a normal oestrus cycle. This can represent an advantage over intravaginal devices with which progesterone levels suddenly drop after removal of the device. The effects of one synthetic oestradiol salt (OB: oestradiol benzoate) were also compared to those of

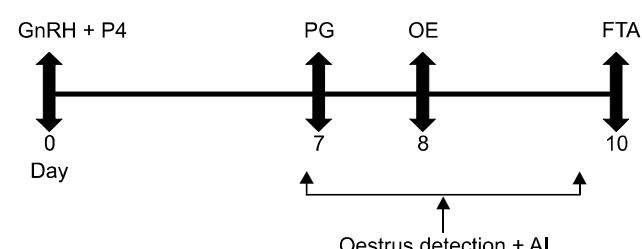
\*Corresponding author: Tel/Fax: +59826280130; E-mail: daniel.cavestany@gmail.com

natural oestradiol 17 $\beta$  (O17). Variables used to measure responses were oestrus symptoms, diameter of the preovulatory follicle, and pregnancy rates.

## Materials and Methods

All animal experiments were approved by the Animal Experimentation Committee of the University of Uruguay. The trial was conducted at the experimental dairy farm of the National Agricultural Research Institute (INIA) in Uruguay that maintains a herd of approximately 280 lactating Holstein cows. From these, 56 that were not bred but were cycling and lacked histories of reproductive disorders were selected for the trial. Parity of the cows ranged from two to six lactations with a body condition score of  $2.47 \pm 0.03$  points (scale of 1 to 5) based on the scale described by Edmondson *et al.* [10], 104.1  $\pm$  7.8 days postpartum, and average milk production of  $21.8 \pm 0.7$  L/day [mean  $\pm$  standard error (SE)]. The average milk production of the herd was 6,500 kg/lactation. During the experimental procedure the cows were kept in a group separated from the rest of the herd to facilitate oestrus detection, and were milked twice a day at 400 and 1,600 h. The animals were fed a mixture of improved pastures (ryegrass and white clover; 50%), corn silage (30%), and commercial concentrates (20%). Half of the concentrates was administered at the milking parlour and the other half was mixed with the silage provided in the pasture at 1,000 h. A modified Heatsynch protocol [17] was used for oestrus synchronisation (Fig. 1). The cows were divided into four groups; 16 received the intravaginal device and 12 were treated with each of the parenteral progesterone formulations. The number of cows per group was determined according to availability of the drugs. Four cows had to be eliminated from the study before the end of the synchronisation protocol due to mastitis, lameness, and other problems. Ultimately, the final number of animals used was 51. Descriptions of the treatments are presented below (Fig. 1).

**Group A:** IPD + OB ( $n = 15$ ); Day 0: administration of 8  $\mu$ g of a synthetic analogue of gonadotropin-releasing hormone



**Fig. 1.** Schemes of the oestrus synchronisation protocols. The cows were divided into four groups that received different progesterone sources or oestradiol formulations. OE: oestradiol, FTAI: fixed time artificial insemination.

(GnRH, Buserelin; Laboratory Rio de Janeiro, Argentina) and insertion of an intravaginal progesterone-releasing device (IPD) with 500 mg of natural progesterone (OB, Cronipres; Laboratory Biogenesis Bago, Uruguay). Day 7: administration of 500  $\mu$ g of a synthetic analogue of prostaglandin F2 $\alpha$  (PG, D-cloprostenol; Laboratory Rio de Janeiro, Argentina) and withdrawal of the IPD. Day 8: administration of 1 mg of OB (Estradiol 10; Laboratory Rio de Janeiro, Argentina).

**Group B:** IPD + O17 ( $n = 16$ ); Day 0: administration of GnRH and IPD (similar to Group A). Day 7: administration of PG and withdrawal of the IPD. Day 8: administration of 1 mg of Estradiol 17 $\beta$  (O17; Laboratory Rio de Janeiro, Argentina).

**Group C:** MAD-4 + OB ( $n = 10$ ); Day 0: administration of GnRH (similar to Groups A and B) and subcutaneous administration of 200 mg of natural progesterone (MAD-4, 4-pregnano-3-20-dione; Laboratory Rio de Janeiro, Argentina). Day 7: administration of PG. Day 8: administration of 1 mg of OB (similar to Group A).

**Group D:** MAD-4 + O17 ( $n = 10$ ); Day 0: administration of GnRH (similar to Groups A, B, and C) and subcutaneous administration of 200 mg natural progesterone (similar to Group C). Day 7: administration of PG. Day 8: administration of O17 (similar to Group B).

GnRH and PG came diluted in water while OB and O17 were diluted in linseed oil.

Oestrus detection was performed three times a day for a period of 30 min each at 700, 1,300, and 1,900 h. Detection started at 1,900 h on Day 7 and continued until 1,900 h on Day 10. Cows that came into heat were artificially inseminated using frozen semen of proved fertility 12 h later. To identify individual cows, large numbers were painted on the sides of the animals. The observations were carried out by three individuals to ensure that every symptom was detected and oestrus behaviour (Table 1) was recorded according to the scale developed by van Eerdenburg *et al.* [31]. All symptoms of each cow seen

**Table 1.** Scale for scoring oestrous behaviour\*

| Oestrous sign                                 | Points |
|---|--------|
| Mucous vaginal discharge                      | 3      |
| Cajoling (flehmen)                            | 3      |
| Restlessness                                  | 5      |
| Being mounted but not standing                | 10     |
| Sniffing vagina of another cow                | 10     |
| Resting with chin on another cow              | 15     |
| Mounting (or attempting to mount) another cow | 35     |
| Mounting head side of another cow             | 45     |
| Standing heat                                 | 100    |

\*van Eerdenburg *et al.* [31]

during the observation period were noted, and a cow that exceeded 50 points in a 24-h period was considered to be in oestrus.

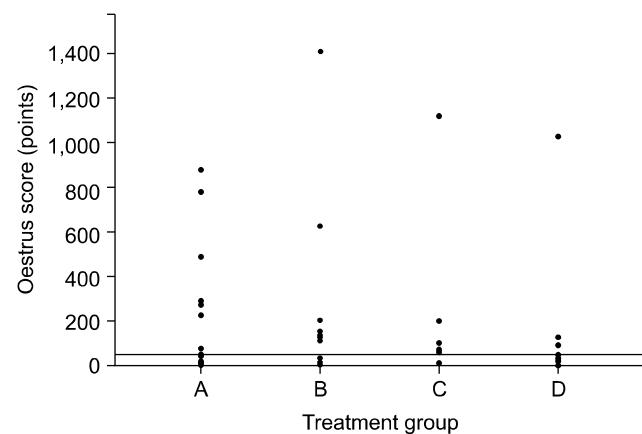
On Day 8 of the experiment, the diameter of the largest ovarian follicle was measured by transrectal ultrasonography using an Aloka SSD-500 (Aloka, Japan) with a 5-MHz linear probe. On Day 10 (52 h after PG administration) all cows not considered to be in heat underwent fixed time artificially insemination (FTAI) [12,21,29]. Pregnancy was diagnosed by transrectal ultrasonography 30 days after FTAI or from 31 to 35 days for cows inseminated after going into heat.

For statistical analysis of continuous variables (oestrus scores, time interval from treatment to oestrus, and follicle diameter), a general linear model was used with treatment as the main effect. Mean values were compared according to least significant difference with a cut-off point of 0.05. No interactions were tested. A chi-square test was used to evaluate the discrete variable pregnancy rates. Pearson correlations were also tested. All statistical analyses were performed with SPSS (SPSS 16.0.2; IBM, USA). Oestrus scores are presented as the mean  $\pm$  standard deviation (SD). Time intervals from the end of treatment to oestrus as well as follicle diameter are presented as the least square mean  $\pm$  SE.

## Results

### Oestrus behaviour scores

No differences in oestrus behaviour were observed between the groups ( $p = 0.611$ ). During the observation days, 24 of the 51 cows (47%) scored more than 50 points within a 24-h period. These cows were considered to be in



**Fig. 2.** Oestrus behaviour scores for the four treatment groups. The highest 24-h score of each cow is presented. Cows with scores above the 50-point threshold were considered to be in oestrus. Mean scores were  $273 \pm 282$  for group A,  $261 \pm 350$  for group B,  $202 \pm 372$  for group C, and  $182 \pm 311$  for group D. Differences between the groups were insignificant ( $p = 0.902$ ).

oestrus according to van Eerdenburg *et al.* [31] and were inseminated. Some oestrus signs were detected in 18 (35%) other cows, but scores for these animals did not reach 50 points within a 24-hour period and they were therefore not inseminated. Nine cows (18%) showed no oestrus behaviour during the observation period. Standing heat was noted in 10 of the 42 cows (24%) that also showed other oestrus signs. Mean oestrus behaviour scores of the cows that showed oestrus signs were  $273 \pm 282$  for group A,  $261 \pm 350$  for group B,  $202 \pm 372$  for group C, and  $182 \pm 311$  for group D ( $p = 0.90$ ). Distribution of behaviours is shown in Fig. 2.

### Oestrus

The oestrus scores were grouped into three categories: < 50 points, 50–199 points, and  $\geq 200$  points. All cows in the last two categories were considered to be in oestrus. There was no significant difference in the number of animals in oestrus between the groups A, B, C, and D ( $p = 0.92$ ). Differences in categories of oestrus scores between the groups were also insignificant ( $p = 0.49$ ) but there were significantly more cows with  $\geq 200$  points in group A than in group D ( $p = 0.04$ ; Table 2).

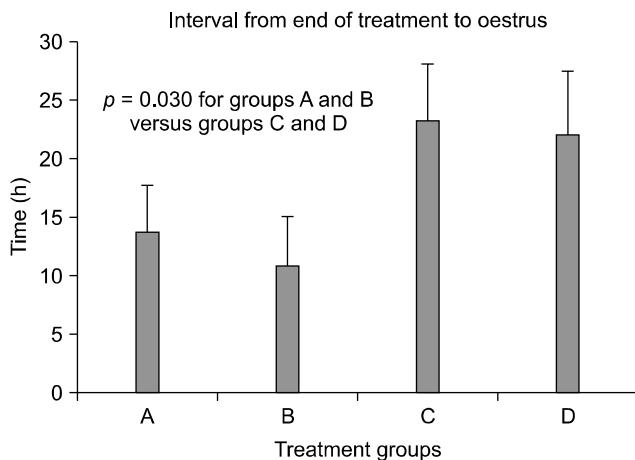
### Time interval from end of treatment to oestrus

The beginning of oestrus was defined as the time when a cow had a total of 50 oestrous behaviour points. The first cow initially showed oestrus behaviour 4 h after the end of treatment (Day 8) and the last cow displayed this behaviour 52 h after treatment cessation. Time intervals between the end of treatment and the beginning of oestrus are presented in Fig. 3. The mean time intervals between treatment and oestrus for each group were  $13.9 \pm 3.9$  h for group A,  $10.9 \pm 4.2$  h for group B,  $23.2 \pm 4.9$  h for group C, and  $22.0 \pm 5.5$  h for group D ( $p = 0.122$ ). When the effect of progesterone

**Table 2.** Number of cows with different ranges of oestrous behaviour scores in each treatment group and the percentage of cows in oestrus

| Group | Number | Score |          |      | Oestrus (%) <sup>‡</sup> |
|-------|--------|-------|----------|------|--------------------------|
|       |        | < 50  | 50 ~ 199 | 200+ |                          |
| A     | 15     | 7     | 1        | 7*   | 53.33                    |
| B     | 16     | 9     | 4        | 3*   | 43.75                    |
| C     | 10     | 5     | 3        | 2*   | 50.00                    |
| D     | 10     | 6     | 3        | 1†   | 40.00                    |

\*.<sup>†</sup>Values with different superscript symbols within the same column are significantly different ( $p = 0.04$ ). <sup>‡</sup>Oestrus was defined as acquiring a score  $> 50$  points within a 24-h period. Differences in the number of cows in oestrus between the groups were not significant ( $p = 0.917$ ).



**Fig. 3.** Time intervals from the end of treatment to oestrus. Overall differences between the groups were not significant ( $p = 0.122$ ) but groups treated with MAD-4 (C and D) had longer intervals to oestrus than the groups treated with an intravaginal progesterone-releasing device.

source was separately analysed, cows that received MAD-4 (groups C and D) had a longer interval ( $22.7 \pm 3.5$  h) to oestrus than the animals treated with the IPD (groups A and B,  $12.4 \pm 2.7$  h;  $p = 0.030$ ).

### Follicle diameter

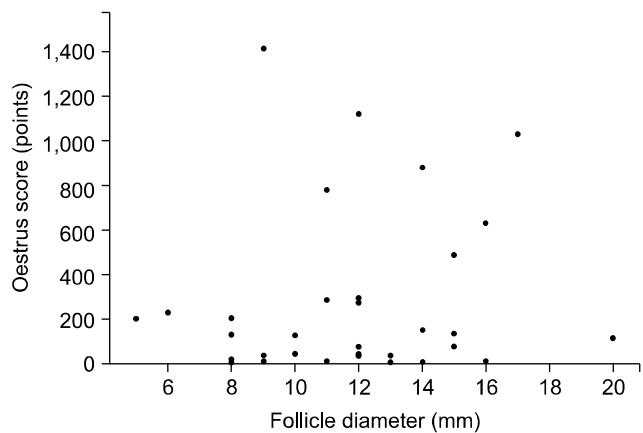
The mean follicle diameter measured on Day 8 ( $n = 47$ ) was  $19.5 \pm 1.3$  mm. There was no significant difference in follicle diameter between the groups ( $p = 0.84$ ). No correlation between follicle diameter and oestrus score ( $p = 0.37$ ) was observed as shown in Fig. 4.

### Pregnancy

Fifteen out of 51 (24.9%) cows were pregnant. There were no numerical differences in pregnancy rates between the cows inseminated at the time of oestrus detection (cows with more than 50 points) and those inseminated at a fixed time, nor among the different treatment groups. However, these results are difficult to interpret since there were fewer than five pregnant cows in each group.

### Discussion

Mean oestrus scores measured in the current study were higher than those reported earlier in confined dairy cows in Hungary [32], but were similar to those reported by Roelofs *et al.* [27] for confined cattle in the Netherlands. There was no treatment-specific effect on the percentage of animals with oestrus signs ( $p = 0.917$ ) between Days 7 and 10. However, oestrus was detected in twice as many cows using the scale established by van Eerdenburg *et al.* [31] rather than standing behaviour. Using less strict criteria or a more traditional  $2 \times 2$  day oestrus detection system, these cows



**Fig. 4.** Relationship between follicle size and oestrus score. No correlation was observed (Pearson correlation = 0.156,  $p = 0.370$ ). There was no difference in follicle diameter between the groups.

would be not considered in heat and would thus not be inseminated. This represents one advantage of an FTAI protocol given that this technique avoids oestrus detection and potential associated errors. The percentage of animals detected in oestrus was substantially lower than expected and also lower than numbers found in a previous report [32]. Furthermore, the percentage of animals shown to be in standing heat was lower than that described in earlier studies [2,11,15,18,24,25,27,28,31]. For Heatsynch protocols, at least 20% of dairy cows are in standing heat between the time of PG administration and FTAI [5]. Among the reasons that might have caused this low rate of oestrus, the most likely is stress caused by the number of times that the animals had to be kept in the separation enclosure for hormone treatments, ultrasonography, and insemination. As described by Dobson *et al.* [9], stress compromises oestrus expression and this probably caused of the low number of cows in standing heat observed in the present trial.

Although the overall pregnancy rates obtained in the current investigation were similar to those in previous reports [3,4,17,21] indicating that stress did not alter ovulation or other reproductive parameters, it is worth noting that experimental trials which require increased manipulation of animals may face a similar problem. However, Roelofs *et al.* [26] reported that repeated ultrasonography does not have any effect on oestrus expression, periovulatory hormone profiles, or ovulation time. In their experiment, the cows were also exposed to frequent manipulation and human handling. Thus, these animals were more used to human contact and there were fewer changes in their daily routine probably due to different management conditions along with housing in a free stall barn.

Another possible important fact could have been the deprivation of food and water in the separation enclosure

that led to nutritional stress. Underfeeding and deficient energy intake are negatively correlated with reproductive functions such as ovarian activity, oestrus expression, and ovulation rate [1]. Because the cows were unable to eat in the separation enclosure, they spent a substantial amount of time grazing when they returned to the pasture instead of showing oestrus behaviour; this could have influenced the oestrus detection rate.

The type of progesterone used affected the time interval from treatment to oestrus with longer intervals observed for animals with MAD-4 than those given the IPD ( $p = 0.030$ ). The spread of the intervals among the groups with the IPD was also smaller than that for the groups treated with MAD-4. After removal of the IPD, the animals came in oestrus within a shorter period of time. No differences in time intervals were found between the groups treated with OB and O17, indicating that the type of oestradiol did not affect the onset of oestrus. The length of the intervals could have been more precise if oestrus detection was performed four times a day instead of three times. In the present study, there was an interval of 12 h between the evening and morning detection. However, it would have been impossible to detect oestrus at night given the conditions under which the present study was carried out. No lights were placed in the pasture because this could have influenced the behaviour of the cows that were used to darkness during the night.

No differences in follicle diameter were observed between the treatment groups. The mean diameter of the dominant follicle was similar to that from previous reports measured under similar conditions [5], and were indicative of fertility as confirmed by the pregnancy results. No correlation was found between follicle size and oestrus score ( $p = 0.370$ ); this concurs with previous findings from a study by van Eerdenburg *et al.* [32]. Pregnancy rates did not differ between the treatment groups ( $p = 0.873$ ) similar to the findings of López-Gatius *et al.* [16] who reported that synchronisation does not significantly affect pregnancy rates. Other experimental studies on synchronisation protocols reported pregnancy rates from 30% to 40% [3,13,20,23,30,32].

No differences in oestrus behaviour, standing oestrus, follicle diameter, or pregnancy rates were found between animals receiving an IPD or subcutaneous injection. A subcutaneous progesterone injection might therefore be a suitable replacement for the IPD because it causes less environmental pollution, is easier to administer, does not have to be removed, and is associated with comparable pregnancy rates. However, more research with a greater number of cows is needed to more accurately determine the effects of subcutaneous progesterone injection on the precision of oestrus synchronisation and ovulation. There were no differences between animals treated with OB or O17. Oestradiol salts are banned in many countries

because of their potential negative effects on human health. Natural oestradiol might be a safe alternative for these potentially dangerous compounds.

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