

Effect of oestrus expression and eCG treatment on reproductive performance in dairy cows subjected to Progesterone plus Ovsynch protocol

Efecto de la expresión del estro y del tratamiento con eCG sobre el desempeño reproductivo en vacas lecheras sometidas a un protocolo de Progesterona más Ovsynch

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ABSTRACT

The objectives were to evaluate the effects of equine chorionic gonadotropin on oestrus expression and fertility in dairy cows undergoing a timed artificial insemination protocol. Holstein cows (n = 534) were subjected to a 7-day progesterone-based Ovsynch protocol. The equine chorionic gonadotropin group received 500 IU equine chorionic gonadotropin at progesterone device removal, whereas the control group received no additional treatment. Oestrus expression was monitored using an electronic neck-mounted activity system and recorded during the voluntary waiting period-oestrus expression and during synchronization prior to timed artificial insemination (pre timed artificial insemination-oestrus expression). The overall pre timed artificial insemination-oestrus expression rate during synchronization was 36.3 %. Administration of equine chorionic gonadotropin did not ($P > 0.05$) influence the pre timed artificial insemination-oestrus expression (36.0 vs. 36.7 %) and pregnancy per artificial insemination at day 30 (36.7 vs. 36.7 %). In addition, pregnancy loss, calving rate, and twinning rate did not differ between equine chorionic gonadotropin and control groups ($P > 0.05$). During the voluntary waiting period, 59.0 % of cows expressed oestrus. Pregnancy per artificial insemination was greater (41.3 % vs. 32.4 %) in voluntary waiting period-oestrus expression positive cows than voluntary waiting period- oestrus expression negative cows ($P = 0.04$) regardless of group (equine chorionic gonadotropin and control). However, there was no significant difference in pre timed artificial insemination-oestrus expression between equine chorionic gonadotropin and control groups in voluntary waiting period-oestrus expression negative ($P = 0.33$) and voluntary waiting period- oestrus expression positive cows ($P = 0.91$). Similarly, treatment with equine chorionic gonadotropin did not affect pregnancy per artificial insemination, either in voluntary waiting period-oestrus expression-negative cows ($P = 0.39$) or in voluntary waiting period-oestrus expression-positive cows ($P = 0.69$). In conclusion, oestrus expression during the voluntary waiting period-oestrus expression and prior to timed artificial insemination-oestrus expression had a strong association with higher fertility. However, equine chorionic gonadotropin did not enhance fertility outcomes in high-producing dairy herds using sex-sorted semen within an optimised synchronization protocol, regardless of parity or cyclicity status.

Key words: Dairy cows; equine chorionic gonadotropin; fertility; oestrus expression

RESUMEN

Los objetivos de este estudio fueron evaluar los efectos de la gonadotropina coriónica equina sobre la expresión del estro y la fertilidad en vacas lecheras sometidas a un protocolo de inseminación artificial a tiempo fijo. Vacas Holstein (n = 534) fueron sometidas a un protocolo Ovsynch de 7 días basado en progesterona. El grupo gonadotropina coriónica equina recibió 500 UI de gonadotropina coriónica equina al momento de retirar el dispositivo de progesterona, mientras que el grupo control no recibió tratamiento adicional. La expresión del estro se monitoreó mediante un sistema electrónico de actividad montado en el cuello y se registró durante el periodo de espera voluntario (periodo de espera voluntario-expresión del estro) y durante la sincronización antes de la inseminación artificial a tiempo fijo (Pre-inseminación artificial a tiempo fijo-expresión del estro). La tasa global de expresión del estro Pre-inseminación artificial a tiempo fijo-expresión del estro durante la sincronización fue del 36,3 %. La administración de gonadotropina coriónica equina no influyó ($P > 0,05$) en la expresión del estro Pre-inseminación artificial a tiempo fijo-expresión del estro ni en la preñez por inseminación artificial al día 30. Asimismo, la pérdida de gestación, la tasa de parto y la tasa de gemelaridad no difirieron entre los grupos gonadotropina coriónica equina y control ($P > 0,05$). Durante el periodo de espera voluntario, el 59,0 % de las vacas expresó estro. La preñez por inseminación artificial fue mayor en las vacas positivas a periodo de espera voluntario-expresión del estro en comparación con las vacas negativas a periodo de espera voluntario-expresión del estro (41,3 vs. 32,4 %; $P = 0,04$), independientemente del grupo de tratamiento. Sin embargo, no se observaron diferencias significativas en la expresión del estro Preinseminación artificial a tiempo fijo-expresión del estro entre los grupos gonadotropina coriónica equina y control tanto en vacas periodo de espera voluntario-expresión del estro negativas ($P = 0,33$) como en vacas periodo de espera voluntario-expresión del estro positivas ($P = 0,91$). De manera similar, el tratamiento con gonadotropina coriónica equina no afectó la preñez por inseminación artificial ni en vacas periodo de espera voluntario-expresión del estro negativas ($P = 0,39$) ni en vacas periodo de espera voluntario-expresión del estro positivas ($P = 0,69$). En conclusión, la expresión del estro durante el periodo de espera voluntario (periodo de espera voluntario-expresión del estro) y antes de la inseminación artificial a tiempo fijo (Pre-inseminación artificial a tiempo fijo-expresión del estro) se asoció fuertemente con una mayor fertilidad. Sin embargo, la gonadotropina coriónica equina no mejoró los resultados reproductivos en hatos lecheros de alta producción que utilizan semen sexado dentro de un protocolo de sincronización optimizado, independientemente de la paridad o del estatus cíclico.

Palabras clave: Vacas lecheras; gonadotropina coriónica equina; fertilidad; expresión del estro

INTRODUCTION

Intensive genetic selection of modern dairy cows for higher milk yield makes it difficult to maintain postpartum health and subsequent fertility [1]. As dairy herd productivity increases, new approaches are needed to improve herd management and ensure the efficient use of available resources [2].

The increasing scale of herds and milk yields has rendered traditional oestrous detection more challenging. Previous studies reported that monitoring the oestrus expression of cows (*Bos taurus*) during the voluntary waiting period (VWP-OE, before the time of insemination has yet arrived) will be a strategic predictor for postpartum health and higher fertility [1, 3]. However, timed artificial insemination (TAI) protocols have still been in the dairy industry, eliminating the need for oestrous detection [4].

Recent studies in dairy cows emphasize the critical role of oestrous expression (OE) at the near time of timed artificial insemination (TAI) in determining pregnancy per AI [4, 5, 6, 7, 8, 9]. Increased estradiol concentrations not only impact oestrous expression [10, 11] but also influence sperm transport, fertilization, and create an uterine environment affecting embryo development and pregnancy establishment [12, 13, 14]. Although increasing circulating estrogen levels by using additional oestradiol preparation in synchronization protocols [10, 15, 16, 17] seems to be the most logical option, oestradiol preparations in cattle are restricted in the European countries [18].

Equine chorionic gonadotropin (eCG), a glycoprotein hormone with approximately a 40-hour half-life, is secreted by the endometrial cups of pregnant mares (*Equus caballus*) and exhibits both FSH and LH-like activities in cattle [19]. Treatment with eCG during late dioestrous/prooestrous stages accelerates the development of the dominant follicle, increases ovulatory follicle size, and elevates P4 concentrations in the subsequent cycle [20].

In progesterone-based TAI protocols, eCG is often co-administered with the removal of a progesterone-releasing intravaginal device (PRID) due to its affinity for FSH and LH receptors [21, 22]. The administration of eCG concurrent with removing the progesterone implant has demonstrated enhancements in preovulatory follicle development, consequently leading to an augmented pregnancy per AI in beef cattle [23, 24, 25, 26].

However, previous reports demonstrated that using eCG in TAI protocols increased [27, 28, 29] or did not change [30, 31, 32, 33, 34] the pregnancy rate in dairy cattle. The primary objective of this study was to investigate the effect of oestrus expression at the near time of AI on pregnancy per AI in dairy cows undergoing progesterone plus Ovsynch protocol. The secondary objective of this study was to investigate the effect of eCG on oestrus expression and fertility the first and subsequent services in dairy cows undergoing progesterone plus Ovsynch protocol.

MATERIALS AND METHODS

All procedures for the maintenance and use of animals were approved by the Balıkesir University Animal Care and Use Committee (Approval No: 2022/1-4).

Herd management

The study was conducted on a commercial dairy farm located in the northwestern region of Türkiye, with an average herd size of 1,900 lactating cows. The annual replacement rate of the herd was approximately 30 %. The lactation number (mean \pm Standard Error of Mean [SEM]) of multiparous cows was 2.2 ± 0.06 .

The voluntary waiting period was ~ 90 d for primiparous cows and ~ 60 d for multiparous cows. Milking was carried out three times per day at 8-hour (h) intervals, and mean daily milk yield per cow ranged between 40 and 43 kg throughout the lactation period. Milk yield on the day of insemination and the corresponding week (± 3 d) was recorded via the herd management program. According to milk yield and days in milk (DIM), cows were grouped in free-stall barns and fed a total mixed ration based on NRC recommendations [35], with *ad libitum* access to water.

The farm had an automated activity monitoring system (Dataflow II™, Allflex, Israel) necessary for data collection. All cows were fitted with a neck collar containing an electronic identification tag. The system utilized a wireless receiver box to record average activity every 2 h and calculate an activity index (0–100). Oestrus was defined as an activity index > 35 , consistent with previously validated thresholds [36]. Oestrus expression was defined in two different stage in this study. First, spontaneous oestrus expression was evaluated from calving until the initiation of the synchronization protocol (VWP-OE). Cows exhibit oestrus during this period were classified as oestrus-expressing, and those not showing oestrus were classified as anoestrous. Second, protocol-induced oestrus expression was assessed from PRID removal until the time of timed artificial insemination (preTAI-OE) during synchronization. These two definitions were analyzed independently according to the objectives of the study.

Experiment

Experiment was designed as a randomized controlled trial to assess the effect of eCG supplementation on oestrus expression and fertility. 534 Holstein-Friesian cows (primiparous, $n = 153$; multiparous, $n = 381$) were enrolled between April 2022 and December 2023. DIM was 82.8 ± 0.3 in primiparous cows and 64.3 ± 0.5 in multiparous cows. Cows were randomly allocated by odd versus even ear tag numbers into a control group ($n = 259$) and an eCG group ($n = 275$). Cows in the eCG group received 500 IU eCG (Folligon®, MSD Animal Health) intramuscularly at the time of progesterone device removal (d 7), while the control group did not.

Synchronization protocol

In both experiments, cows were synchronized using a 7-day progesterone-based Ovsynch protocol prior to the first TAI. At the start of the protocol (day 0), an intramuscular injection of gonadotropin releasing hormone (GnRH, 0.1 mg gonadorelin acetate; Gestavet GnRH®, Hipra, Spain) was administered together with the insertion of a PRID containing 1.55 g P4

(PRID-DELTA®, Ceva, France). On day 7, the intravaginal device was withdrawn and cows were treated intramuscularly with cloprostenol (0.15 mg d-cloprostenol; Gestavet Prost®, Hipra, Spain). A second GnRH dose (0.1 mg) was given 56 h after device removal, followed by TAI using sexed semen 16 h later. Sexed semen was also used for the second and third inseminations, whereas conventional semen was applied from the fourth to the eighth services.

Ultrasonographic examination

A subsample of cows ($n = 75$) was examined to determine preovulatory follicle size at TAI. Ovarian structures were evaluated by transrectal ultrasonography using a 7.5 MHz linear-array probe (Ibex Pro®, E.I. Medical Imaging, Loveland, CO). The largest follicle was measured in two perpendicular diameters, and the mean was recorded as follicle size. All examinations were performed by the same experienced operator to minimize variation.

Pregnancy diagnoses were performed with the same system on days 31 ± 3 and 60 ± 3 post-AI. Pregnancy per artificial insemination (P/AI) was defined as the number of pregnant cows divided by the number of inseminated cows at each diagnosis. Pregnancy loss was calculated from the initial diagnosis of pregnancy ($d 31 \pm 3$) to confirmation of pregnancy ($d 60 \pm 3$). Pregnancy losses were reported for $d 30-60$ and $d 30-calving$. Calving rate was defined as calved cows divided by enrolled cows, and twinning rate as twin calvings divided by total calvings.

Statistical analysis

Data were analyzed using SPSS 27.0 (IBM, Chicago, IL, USA). The primary outcomes included two different measures of oestrus expression: (1) VWP-OE (Voluntary Waiting Period Oestrus Expression), defined as oestrus observed from calving until the initiation of the synchronization protocol, and (2) preTAI-OE, defined as oestrus observed from PRID removal until timed artificial insemination (TAI). Additional outcomes were P/

AI, calving rate, twinning rate, pregnancy loss ($d 30-60$ and $d 60-calving$), total pregnancy loss, return to oestrus, second-service P/AI, days open, number of AIs per pregnancy, and overall P/AI.

Categorical variables (e.g., VWP-OE, preTAI-OE, P/AI, calving rate) were compared using chi-square or Fisher's exact test. Continuous variables (DIM, milk yield etc.) were analyzed by Student's t-test or Mann-Whitney U-test depending on distribution. Logistic regression was used to evaluate associations between oestrus expression, eCG treatment, and P/AI, with parity included as a fixed effect. Survival analysis (Kaplan-Meier with log-rank test) was used for d open. Fixed effects were considered significant at $P \leq 0.05$. Results are presented as mean \pm S.E.M. for continuous outcomes and as percentages for categorical outcomes.

RESULTS AND DISCUSSION

In this study, milk yield (mean \pm S.E.M.) at the d of TAI was 44.2 ± 1.5 kg and 46.2 ± 1.1 kg for primiparous cows in the eCG and control groups, respectively, and 53.4 ± 1.3 kg and 54.1 ± 1.1 kg for multiparous cows (TABLE I).

Similarly, average weekly milk yield during the two weeks preceding TAI did not differ between treatments in primiparous cows (45.1 ± 1.4 vs. 46.5 ± 1.2 kg/day) or multiparous cows (52.7 ± 1.3 vs. 53.3 ± 1.2 kg/d). Mean milk yield during the first 100 DIM was also comparable between the eCG and control groups in primiparous (41.6 ± 1.2 vs. 42.3 ± 1.0 kg/d) and multiparous cows (49.8 ± 1.1 vs. 50.5 ± 1.0 kg/d).

Likewise, 305-d mature-equivalent milk yield did not differ between treatments, with primiparous cows producing $10,420 \pm 215$ kg and $10,570 \pm 208$ kg, and multiparous cows producing $11,960 \pm 240$ kg and $12,030 \pm 225$ kg in the eCG and control groups, respectively. Overall, milk production parameters at all evaluated time points were similar between treatments.

TABLE I

Comparison of oestrus expression, pregnancy per artificial insemination, pregnancy loss, calving rate and twinning rate between equine chorionic gonadotropin and control groups according to parity in dairy cows subjected to Progesterone plus Ovsynch protocol

Variables	Primiparous		Multiparous		p value		
	eCG	Control	eCG	Control	Parity	Treatment	Parity \times Treatment
Oestrus expression at preTAI; % (n/n)	40.3 (31/77)	46.1 (35/76)	34.3 (68/198)	32.8 (60/183)	0.04	0.64	0.42
P/AI on d 30; % (n/n)	41.6 (32/77)	44.7 (34/76)	34.8 (69/198)	33.3 (61/183)	0.05	0.86	0.61
Pregnancy loss 30 to 60 d; % (n/n)	nd (0/32)	nd (0/34)	2.9% (1/69)	8.2 (1/61)	0.31	0.95	0.95
P/AI on d 60; % (n/n)	41.6 (32/77)	44.7 (34/76)	34.3 (68/198)	32.8 (60/183)	0.04	0.86	0.61
Pregnancy loss 30 d to calving; % (n/n)	3.1 (1/32)	nd (0/34)	7.3 (5/68)	6.6 (4/60)	0.11	0.56	0.73
Total Pregnancy loss; % (n/n)	3.1 (1/32)	nd (0/34)	8.7 (6/69)	8.1 (5/61)	0.04	0.50	0.88
Calving rate; % (n/n)	40.3 (31/77)	44.7 (34/76)	32.3 (64/198)	31.1 (57/183)	0.02	0.53	0.72
Twinning rate; % (n/n)	nd (0/31)	nd (0/34)	4.7 (3/64)	5.3 (3/57)	0.07	0.92	0.92

Oestrus expression at preTAI: Pre-Timed Artificial Insemination Oestrus Expression (preTAI-OE), defined as oestrus detected by the automated activity monitoring system from PRID removal until TAI during synchronization.

P/AI: Pregnancy per artificial insemination.

eCG: Cows received 500 IU eCG on days 7 in TAI protocol.

Control: Cows did not additional hormone in TAI protocol.

nd: not detected.

Calving rate calculated by divided the number of calving cows to enrolled cows to study.

Twinning rate was also calculated by divided the calving cows that had twin calves to calving cows.

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The overall preTAI-OE rate during synchronization was 36.3 % (194/534) in cows. Primiparous cows had a higher preTAI-OE rate than multiparous cows (43.1 % vs. 33.6 %, $P = 0.04$) regardless of treatment group (eCG and control). In primiparous cows, preTAI-OE was 40.3 % in the eCG group and 46.1 % in the control group, while in multiparous cows the corresponding rates were 34.3 % and 32.8 %, respectively. Administration of eCG did not affect preTAI-OE ($P = 0.64$), and no significant parity \times treatment interaction was detected for preTAI-OE ($P = 0.42$, TABLE I).

The first objective of this study was to determine whether the expression of oestrus preTAI-OE was associated with improved P/AI in lactating dairy cows synchronised with a progesterone-based Ovsynch protocol. In this study, preTAI-OE was 36.3 % during the synchronization, a proportion that is greater than the approximately 20 % reported in earlier studies using conventional Ovsynch [37, 38], and consistent with more recent studies reporting oestrus expression rates ranging between 30 % and 40 % [4, 7, 39]. Differences among studies appear to be strongly influenced by the type of insemination protocol used. Protocols incorporating presynchronization, such as Double-Ovsynch, typically yield higher oestrus expression than protocols that rely solely on Ovsynch. For example, Laplacette *et al.* [4] and Lauber and Fricke [39] observed oestrus expression rates of 36.1 and 29.1 %, respectively, in cows enrolled in a Double-Ovsynch protocol.

These findings indicate that milk yield was not a confounding factor affecting reproductive outcomes. Although high-producing cows typically exhibit enhanced metabolic clearance of ovarian steroids, leading to reduced circulating estradiol and progesterone [11], the hormonal synchronization protocol used (PRID + Ovsynch) may have minimised such metabolic effects by providing sufficient endocrine control during the periovulatory period.

Additionally, supplementation of progesterone device in this study may also have contributed to the preTAI-OE. Progesterone supplementation provides a controlled luteal-phase environment that supports the emergence of a new follicular wave and enhances the uniformity of follicular development [5]. Higher circulating progesterone prior to luteolysis, particularly 3–4 d before TAI, has been associated with enhanced preovulatory estradiol (E2) production and stronger behavioural expression of oestrus [40].

Despite this, the reproductive physiology of high-producing dairy cows presents unique metabolic challenges that can modulate the hormonal responsiveness expected during synchronization. Elevated hepatic blood flow and intensified metabolic activity in high-yielding cows accelerate the clearance of ovarian steroids, reducing circulating concentrations of both progesterone and estradiol [11]. This increased metabolic clearance rate may attenuate the anticipated stimulatory effects of progesterone on follicular E2 synthesis, thereby reducing the manifestation of oestrus despite adequate follicular development.

Additionally, the altered endocrine environment may limit the potential for exogenous gonadotropins, such as eCG, to augment follicular development or increase E2 production [22]. In this study, preovulatory follicle size at insemination was not affected ($P = 0.25$) by eCG treatment. In primiparous cows, follicle size was 14.50 ± 0.44 mm in the eCG group and $14.88 \pm$

0.55 mm in the control group. In multiparous cows, follicle size was 15.41 ± 0.33 mm in the eCG group and 14.68 ± 0.34 mm in the control group. These metabolic constraints likely contributed to the absence of an eCG effect on preovulatory follicle size in this study.

Oestrus expression at preTAI remains an important determinant of fertility in TAI programs. High-producing dairy cows often exhibit diminished LH pulsatility and reduced circulating estradiol, both of which weaken the behavioural signs of oestrus [11]. Reduced intensity or duration of oestrus is particularly consequential because robust oestrus expression is tightly linked to improved P/AI [7, 8]. Adequate preovulatory E2 promotes the onset of behavioural oestrus and optimises the uterine environment, sperm transport, and early embryonic development [12, 14]. In agreement with previous findings, cows that expressed oestrus before TAI had higher P/AI than those that did not in this study. Several recent studies corroborate this relationship, reporting similar associations between oestrus expression and improved fertility under synchronised breeding programs [7, 8, 9, 39, 40, 41]. Reduced detection of oestrus by automated activity monitoring systems has been associated with lower fertility in TAI programs [40]. Although TAI protocols such as Ovsynch were designed to reduce dependence on heat detection [42], the presence of pre-TAI oestrus expression continues to serve as a reliable indicator of optimal endocrine status and improved reproductive potential.

Regarding the first TAI, P/AI was higher in primiparous cows compared with multiparous cows both on d 30 (43.1 % vs. 34.1 %; $P = 0.05$) and d 60 (43.1 % vs. 33.6 %; $P = 0.04$). However, P/AI did not differ between eCG and control groups on either d 30 ($P = 0.86$) or d 60 ($P = 0.86$). The second major objective of this study was to assess whether administration of 500 IU eCG at the time of progesterone device removal influenced reproductive outcomes in cows synchronised with a progesterone-based Ovsynch protocol. The administration of eCG did not influence preTAI-OE or P/AI in this study.

Previous studies have produced inconsistent results regarding the benefits of eCG in dairy cows. While some studies have reported no significant effect of eCG on oestrus expression or fertility [34, 43], others have noted improvements in anoestrous cows or in specific subgroups [27, 29]. For example, Bryan *et al.* [27] observed improved fertility only in anoestrous cows older than five years, and Bryan *et al.* [28] reported enhanced P/AI in anovulatory cows treated with eCG during progesterone-based synchronization. Garcia-Ispierto *et al.* [29] found increased fertility at 120 d in milk in cows without luteal activity prior to treatment, suggesting a potential benefit of eCG in cows with compromised ovarian cyclicity.

The inconsistent response to eCG between dairy and beef cattle has been widely discussed. Many studies have documented positive effects of eCG in beef cows, particularly in postpartum anoestrous animals [23, 24, 25, 26, 44, 45, 46]. Several mechanistic differences appear to underpin this disparity. Compared with dairy cows, beef cattle have lower metabolic demands, more stable endocrine profiles, and slower metabolic clearance of ovarian steroids.

These physiological conditions support higher circulating estradiol levels following eCG-induced follicular stimulation and allow for a more pronounced LH surge [23, 24]. In contrast, in high-producing dairy cows, negative energy balance, reduced

IGF-1 concentrations, and impaired LH pulsatility limit follicular responsiveness to gonadotropins [47]. These factors likely explain why eCG commonly enhances follicular activity and fertility in beef cattle but yields limited benefits in high-yielding dairy herds.

Pregnancy loss between d 30 and 60 was not observed in primiparous cows in either group, while multiparous cows exhibited rates of 2.9 % in the eCG group and 8.2 % in the control group. Pregnancy loss from day 30 to calving was 3.1 % in primiparous cows in the eCG group, whereas no losses were recorded in the control group. In multiparous cows, the corresponding values were 7.3 % in the eCG group and 6.6 % in the control group. None of these differences were statistically significant ($P > 0.05$). Overall, pregnancy loss from days 30–60 ($P = 0.95$), days 30–calving ($P = 0.56$), and total pregnancy loss ($P = 0.50$) was not influenced by eCG treatment (TABLE I).

Consistent with previous studies, eCG treatment did not affect pregnancy loss in this study. Pregnancy loss between d 30 and 60 of gestation was low, and no differences were noted between the treatment groups, consistent with reports indicating that eCG does not influence pregnancy loss during this stage [43, 48]. Since pregnancy losses are relatively uncommon in this window [21], a significant treatment effect would not be expected. The lack of differences in pregnancy loss from day 60 to calving further supports the conclusion that supplementation with eCG in this protocol does not influence late gestational outcomes. The role of eCG is generally more prominent in the early post-ovulatory period, when its actions on follicular development and luteal function may be more biologically relevant.

No twinning cases were observed in primiparous cows. In multiparous cows, the twinning rate was 4.7% in the eCG group and 5.3 % in the control group, with no significant difference between groups ($P = 0.92$). Therefore, parity had a significant effect on P/AI, calving rate, and twinning rate, while no treatment \times parity interaction was observed (TABLE I).

Finally, the absence of a treatment effect on twinning rate is noteworthy. Twinning is an undesirable outcome associated with increased pregnancy loss [49], postpartum disorders, and reduced longevity in dairy cows [50, 51, 52, 53]. Although doses exceeding 1000 IU of eCG can induce multiple ovulations [22], the 500 IU dose used in this study is generally considered insufficient to increase twinning risk. This aligns with previous findings in dairy cattle [54], and our results further demonstrate that the administration of eCG at this dose does not increase the likelihood of twin pregnancies, even when ovulation number is not directly assessed.

Overall, during the voluntary waiting period, the proportion of anestrus (VWP-OE negative) cows was found to be 41 % (219/534). The presence or absence of oestrus expression during VWP did not affect ($P = 0.17$) preTAI-OE during the TAI protocol (33.9 vs. 39.7 %). However, VWP-OE positive cows had greater (41.3 vs. 32.4 %) P/AI after the first AI compared with VWP-OE negative cows ($P = 0.04$, TABLE II).

TABLE II
Oestrus expression near timed artificial insemination and pregnancy per artificial insemination relative to oestrus expression during voluntary waiting period in dairy cows

Variables	VWP-OE Positive	VWP-OE Negative	P value
Number of animals; (n/n)	59.0 ^a (315/534)	41.0 ^b (219/534)	< 0.01
Oestrus expression at preTAI; % (n/n)	33.9 (107/315)	39.7 (87/219)	0.17
P/AI on day 30; % (n/n)	41.3 ^a (125/303)	32.4 ^b (71/219)	0.04

a,b: Percentages with different superscripts within a row are different ($P < 0.05$). VWP-OE positive: Cows expressed oestrus during voluntary waiting period. VWP-OE negative: Cows did not expressed oestrus during voluntary waiting period. WP-OE: Voluntary Waiting Period Oestrus Expression, defined as oestrus detected by the automated activity monitoring system from calving until initiation of the synchronization protocol Oestrus expression at preTAI (preTAI-OE): defined as oestrus detected by the automated activity monitoring system from PRID removal until TAI during synchronization
P/AI: Pregnancy per artificial insemination

During the voluntary waiting period, 59.0 % of cows expressed oestrus (VWP-OE positive) in this study (TABLE II). Higher percentage of anoestrous cows may reflect the physiological demands imposed by early lactation. High-producing cows undergo substantial metabolic stress during this period, requiring large amounts of energy to support milk synthesis. Failure to adapt to this metabolic load may delay the resumption of ovarian cyclicity and prolong the period of anoestrus. Low circulating concentrations of estradiol resulting from accelerated steroid metabolism [11] may further limit the expression of oestrus and subsequent fertility [12, 14].

Nevertheless, cows that expressed oestrus during the VWP exhibited approximately 7 % higher P/AI after first service, consistent with previous studies reporting oestrus expression rates of 50–55 % within 60 d in milk [3, 55] and even higher rates of 65–70 % in more recent cohorts [1, 56].

Differences in parity distribution and the shorter duration of monitoring in multiparous cows may partly explain the lower oestrus expression observed in some animals [3]. Variability in environmental conditions, management practices, and metabolic adaptation could also contribute to divergent findings across studies [40]. Increasing evidence suggests that automated oestrus detection during the VWP can serve as a useful predictor of reproductive potential, further highlighting the value of early oestrus expression [2, 3].

There was no significant difference in preTAI-OE between eCG and control groups in VWP-OE negative ($P = 0.33$) and VWP-OE positive cows ($P = 0.91$). Similarly, treatment of eCG did not affect P/AI, either in VWP-OE–negative cows ($P = 0.39$) or in VWP-OE–positive cows ($P = 0.69$, TABLE III). It is possible that the optimised synchronization protocol used here provided adequate endocrine control to mitigate the negative effects of high metabolic load on fertility. While eCG can support follicular growth and enhance luteal function, its effects are typically short-lived [22] and may not extend into subsequent cycles. Moreover, sex-sorted semen inherently has lower fertilisation potential [35], which may diminish any subtle benefit of eCG.

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TABLE III
Distribution of anoestrus, oestrus expression, oestrus frequency during the voluntary waiting period, and pregnancy per artificial insemination by parity and equine chorionic gonadotropin treatment

	Primiparous		Multiparous		P value		
	eCG (n = 77)	Control (n = 76)	eCG (n = 198)	Control (n = 183)	Parity	eCG	eCG x Parity
Oestrus expression at pre-TAI in anestrus cows; % (n/n)	40.0 (14/35)	53.3 (16/30)	36.7 (33/90)	37.5 (24/64)	0.19	0.33	0.39
P/AI in anestrus cows % (n/n)	40.0 (14/35)	33.3 (10/30)	30.3 (27/90)	31.2 (20/64)	0.70	0.39	0.57
Oestrus expression at preTAI in cows expressed oestrus during VWP % (n/n)	40.5 (17/42)	41.3 (19/46)	32.4 (35/108)	30.2 (36/119)	0.11	0.91	0.80
P/AI in cows expressed oestrus during VWP % (n/n)	42.9 (18/42)	50.0 (23/46)	38.9 (42/108)	34.5 (41/119)	0.08	0.69	0.26

Oestrus expression at preTAI (preTAI-OE): defined as oestrus detected by the automated activity monitoring system from PRID removal until TAI during synchronization. Anestrus cows: Cows did not expressed oestrus during voluntary waiting period (VWP-OE negative). Expressed oestrus during VWP: Cows expressed oestrus during voluntary waiting period (VWP-OE positive). P/AI: Pregnancy per artificial insemination

The rate of cows returning to oestrus after the first TAI was 47.3 % (160/338). This parameter did not differ between groups (P = 0.92) or parities (P = 0.96). The P/AI after the second service was 27.5 %, with no differences detected between treatment groups (P = 0.58) or parities (P = 0.43). The number of AI per pregnancy ranged from 2.04 to 2.49. Overall P/AI did not differ between treatment groups (P = 0.15).

The addition of eCG to the first TAI protocol did not influence return-to-oestrus rates, P/AI at the second service, number of AI per pregnancy, or days open compared with the control group (P > 0.05, TABLE IV). Despite expectations that high milk yield and the use of sex-sorted semen could negatively impact fertility [35], no adverse effect was detected on days open or AI per pregnancy.

TABLE IV
Return rate to oestrus after the first TAI, pregnancy per artificial insemination after the second service, the number of artificial insemination per pregnancy, and open days between equine chorionic gonadotropin and control groups in cows

	Primiparous		Multiparous		P value		
	eCG	Control	eCG	Control	Parity	Treatment	Parity x Treatment
Return rate to oestrus; % (n/n)	46.7 (21/45)	47.6 (20/42)	47.3 (61/129)	47.5 (58/122)	0.96	0.92	0.96
Second service P/AI; % (n/n)	37.8 (17/45)	23.8 (10/42)	22.5 (29/129)	30.3 (37/122)	0.43	0.58	0.05
Open days	128.97 ± 5.69	139.64 ± 6.54	125.76 ± 4.87	121.88 ± 4.43	0.07	0.55	0.21
Number of AI per pregnancy	2.04 ± 0.15	2.42 ± 0.19	2.49 ± 0.13	2.46 ± 0.13	0.12	0.28	0.21
Overall P/AI; % (n/n)	87.0 (67/77)	97.4 (74/76)	81.8 (162/198)	81.4 (149/183)	<0.01	0.15	0.12

Return rate to oestrus refers to cows expressed oestrus after the first insemination. Open days refers to the interval between calving and the establishment of a confirmed pregnancy

The optimised progesterone-based synchronization may have provided adequate endocrine support to standardise responses, thereby masking any potential long-term benefits of eCG. Since eCG's luteotropic effects are short-lived and typically do not extend beyond the immediate post-ovulatory period

[22], its inability to improve second-service fertility or days open is consistent with physiological expectations. Finally, in the present study, neither P/AI nor other fertility parameters such as d open, the number of AI per pregnancy, or responses at the second service differed between the eCG and control groups.

CONCLUSION

The present study demonstrates that oestrus expression during the voluntary waiting period (VWP-OE) and during synchronization prior to timed artificial insemination (preTAI-OE) remains a strong indicator of enhanced fertility in high-producing dairy cows, even within synchronised breeding programs. Although eCG offers well-documented benefits in beef cattle and certain subsets of dairy cows, its administration at 500 IU did not improve preTAI-OE, P/AI, or other reproductive outcomes in this high-yielding herd managed under an optimised progesterone-based Ovsynch protocol.

The standardisation of management practices, consistent monitoring, and robust hormonal control provided by the protocol may have stabilised reproductive outcomes sufficiently to eliminate measurable differences between groups. Finally, these findings suggest that the usefulness of eCG in dairy cows may be limited value in well-managed herds characterised by high production levels and efficient reproductive management.

Conflict of Interest

All authors declare that they have no conflict of interest.

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