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SHORT COMMUNICATION

Effectiveness of selected methods for manipulating the oestrus cycle and ovulation in mares during the breeding season: A retrospective clinical study

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ABSTRACT. Hormones used for the induction of oestrus and ovulation are widely applied in veterinary practice. The objectives of this study were to assess: (1) the effectiveness of cloprostenol in inducing oestrus, and (2) the influence of cloprostenol and human chorionic gonadotropin (hCG) on the incidence of multiple ovulations (MO) and multiple pregnancies (MP). The first group of mares (n = 77), which entered spontaneous oestrus, received hCG to induce ovulation, while the second group (n = 40) had oestrus induced with cloprostenol, followed by hCG injection. The pregnancy rate was 55.8% in the first group and 65% in the second group ($P < 0.073$). The frequency of MO was significantly higher in the second group (27.5%) compared to the first group (3.9%; $P < 0.0006$). However, this treatment did not affect the incidence of MP. Endometrial oedema scores at the time of hCG administration decreased significantly over time in both groups. These results suggest that hormonal manipulation of the oestrous cycle during the breeding season and ovulation induction can help ensure satisfactory pregnancy rates. Moreover, cloprostenol can increase the frequency of MO without affecting MP rates.

Introduction

Management of mares undergoing artificial insemination (AI) and other reproductive techniques requires the use of hormonal agents to induce oestrus and ovulation (Samper, 2008; Squires, 2008; Wojtysiak et al., 2020). Given the seasonal pattern of the oestrus cycle in mares, different methods are used for oestrus induction during the spring transition period compared to the breeding season. During peak reproductive activity, prostaglandin F2α or its analogues represent the most widely implemented pharmacological intervention for oestrus induction, administered during the dioestrus phase. Dinoprost is a natural form of prostaglandin F2α available on the market, while cloprostenol and luprostiol are the most widely used synthetic analogues in veterinary practice. The effectiveness of prostaglandin treatment, as measured by the time to ovulation, mainly depends on the diameter of the largest follicle present on the ovary at the time of drug administration. Research indicates that for follicles measuring less than 25 mm in diameter, the ovulation interval typically ranges from 6 to 12 days post-treatment (Coffman and Pinto, 2016), though considerable individual variation has been documented, with reported intervals spanning 2 to 16 days in clinical settings (Newcombe et al., 2008;

Cuervo-Arango and Newcombe, 2010). Mares with larger follicles at the time of prostaglandin administration generally show shorter intervals to ovulation. Most mares respond to a single prostaglandin treatment with luteolysis when administered from day 5 after ovulation, though some individuals may exhibit earlier responses (Coffman and Pinto, 2016). In addition, the effectiveness of the treatment depends on both the specific prostaglandin agent used (Kuhl et al., 2017) and its dose (Newcombe et al., 2008). Regardless of the information presented above, the effect of treatment can be more reliably predicted when the genital tract is examined prior to drug administration, rather than when prostaglandins are administered blindly. Therefore, the first objective of this study was to assess the effect of prostaglandins on oestrus induction when given after ovarian ultrasound assessment. Human chorionic gonadotropin (hCG) is another hormone commonly applied in equine reproduction. It has a relatively long half-life and mimics the action of luteinizing hormone (LH) (Squires, 2008). Administration of hCG during oestrus, when a dominant follicle is present, reliably induces ovulation within 24–48 h (Barbacini et al., 2000). While the recommended hCG doses range from 1500 to 6000 IU, successful ovulation induction has been achieved with as little as 750 IU (Davies Morel and Newcombe, 2008). Following hCG administration, the growth of the dominant follicle may cease and a reduction in endometrial oedema has been observed (Gastal et al., 2006; Cuervo-Arango and Newcombe, 2008; Dolezel et al., 2012).

Hormonal manipulation of the oestrous cycle and ovulation induction have also been associated with an increased frequency of double or even multiple ovulations (MO) (Veronesi et al., 2003, Ginther and Al-Mamun, 2009). In the present study, hCG was routinely administered during oestruses induced by prostaglandin administration, allowing us to compare the effect of hCG on ovulation in both spontaneous and prostaglandin-induced oestrus cycles. This experimental approach also facilitated assessment of whether prostaglandin treatment affected the frequency of MO. Accordingly, the second aim of this study was to evaluate the effects of prostaglandins and hCG on the induction of MO.

Material and methods

Data for analysis were obtained from clinical records collected over three consecutive breeding seasons (2021–2023) at an artificial insemination centre located in the Wielkopolska region of Poland. The population consisted exclusively of fertile Warmblood mares with no reported reproductive issues, as confirmed by their owners. Most mares were multiparous, and all had normal perineal conformation, with over 80% of the labial length positioned below the ischiatic arch of the pelvis.

Ultrasound examination of the reproductive tract was conducted using a Honda HS1600 ultrasound unit, equipped with a linear probe operating at a frequency of 5 MHz. During each examination, the diameter of the dominant follicle(s) and the endometrial oedema score were recorded. Endometrial oedema was evaluated based on the system described by Samper (2010) and modified by Rasmussen et al. (2015), where a score of 0 indicates no oedema, 1 indicates mild oedema, 2 indicates moderate oedema, 3 indicates strong oedema, and 4 indicates abnormal hyper oedema (Grabowska and Kozdrowski, 2022).

Mares were divided into two groups based on the oestrus status (spontaneous or induced). The first group (hCG group, n = 77, aged 8.04 ± 5.56 years) consisted of mares in spontaneous oestrus, either arriving at the insemination centre already in oestrus, or remaining there until oestrus occurred. In this group, ovulation was induced by intravenous (IV) administration of 1500 IU hCG (Chorulon®, Intervet, Holland) when the dominant follicle reached at least 4 cm in diameter with pronounced endometrial oedema. The second group (prostaglandin/human chorionic gonadotropin (PGF/hCG group), n = 40, aged 10.13 ± 5.26 years) consisted of mares with induced oestrus following intramuscular injection of 250 μg cloprostenol (Estrumate®, Intervet, Holland). Administration of cloprostenol was preceded by transrectal palpation and ultrasonography examination of the genital tract. The injection was only given when three specific criteria were met: absence of endometrial oedema (score 0), presence of either a corpus luteum or haemorrhagic anovulatory follicle on the ovary, and all follicle measuring ≤ 30 mm in diameter. Three to four days after the cloprostenol administration, a follow-up examination of the genital tract was conducted and, if necessary, continued daily until oestrus was detected. In this group, hCG was also administered in the same manner and under the same conditions as described for the hCG group.

In the hCG group, 67 first insemination cases and 10 second insemination cases during the breeding season were analysed, while in the PGF/hCG group, 33 cases of first inseminations and 7 cases of second inseminations were evaluated.

Mares inseminated with frozen/thawed semen (all mares from the hCG group, and six mares from the PGF/hCG group) were examined ultrasonographically after hCG treatment at 4–6 h intervals, beginning 12–24 h after hCG administration until ovulation was confirmed. Additional scans were performed 24 and 48 h after AI. Mares inseminated with cooled semen (34 mares from the PGF/hCG group) were subject to ultrasonographic monitoring after hCG treatment at 24h intervals (usually starting 12–24 h after hCG administration) until ovulation occurred, and again 24 and 48 h after AI. In both groups, the following parameters were recorded after hCG administration: changes in dominant follicle(s) diameter, alterations in endometrial oedema, and the time of ovulation. For the PGF/hCG group, additional intervals were documented, including the time from cloprostenol injection to oestrus detection, hCG administration, and subsequent ovulation. In both groups, the frequency of MO and multiple pregnancies (MP) were recorded and compared between the groups.

AI with cooled semen was performed approximately 24 h after hCG treatment, while AI with frozen/thawed semen was carried out 4–6 h after ovulation. Prior to AI, the tail was protected with a glove, and the perineum and vulva were washed with warm soapy water, disinfected with povidone, and dried with a paper towel. Cooled semen, obtained from various European centres from stallions with proven fertility, contained more than 500×10^6 spermatozoa with motility exceeding 75%. Frozen semen doses varied from 1 to 6 straws, with motility ranging between 20 and 60%, though total sperm counts were unspecified. Cooled semen was deposited into the uterine body, while frozen/thawed semen required transcervical placement near the uterine papilla using a sterile flexible catheter guided by rectal palpation. Pregnancy diagnosis was performed by ultrasound examination 14–16 days after ovulation.

All statistical analyses were performed using Statistica 13 (TIBCO Software Inc., 2017). Data normality was assessed with the Shapiro-Wilk test. Non-parametric tests were used for group comparisons (Mann-Whitney U test for two groups, Kruskal-Wallis test for multiple groups), with Bonferroni-adjusted post-hoc analyses. Categorical variables were analysed using contingency tables with the chi-square independence test. Correlations between variables were assessed using Spearman's rank correlation coefficient. A significance level of $P \leq 0.05$ was adopted.

Results

In the hCG group, 43 mares became pregnant (55.8% pregnancy rate), while in the PGF/hCG group, 26 mares were pregnant (65% pregnancy rate); however, this difference was not statistically significant ($P < 0.073$). The interval from cloprostenol administration to oestrus detection was 5.03 ± 1.94 days; from cloprostenol administration to hCG injection – 6.28 ± 2.01 days; and from cloprostenol administration to ovulation – 8.06 ± 2.14 days. At the first examination following cloprostenol injection, 57.5% (23/40) of the mares were in oestrus.

Most ovulations following hCG administration occurred within 48 h in both groups (hCG group – 97.4%, PGF/hCG group – 95.0%), with no significant differences between the groups ($P = 0.09$; Table 1).

Table 1. Timing of ovulation after hCG administration

Time from hCG to ovulation, h	hCG group	PGF/hCG group
0–24	6/77 (7.8%)	6/40(15.0%)
24–48	69/77 (89.6%)	32/40 (80.0%)
>48	2/77 (2.6%)	2/40 (5.0%)

hCG – human chorionic gonadotropin, PGF/hCG – prostaglandin/human chorionic gonadotropin

The frequency of MO was statistically higher in the PGF/hCG group, with 11 out of 40 mares (27.5%) having MO, compared to only 3 of 77 mares (3.9%) in the hCG group ($P < 0.0006$). However, hormonal treatment did not affect the frequency of MP, with one twin pregnancy (1.3%) in the hCG group and two twin pregnancies (4.88%) in the PGF/hCG group. Endometrial oedema scores at hCG administration and at ovulation showed no intergroup differences but demonstrated a significant decrease over time in both groups (Figure 1).

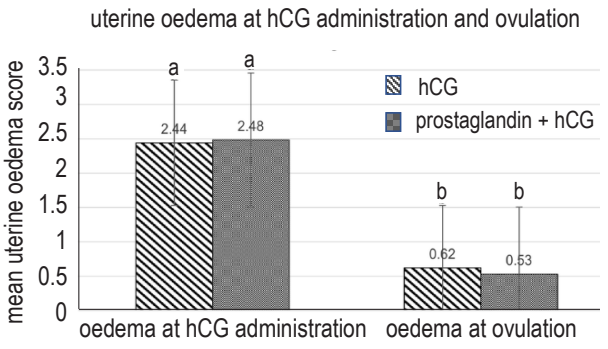

Figure 1. Endometrial oedema scores at human chorionic gonadotropin (hCG) administration and ovulation

data are presented as mean values ± SD. ab – means within a bar with different superscripts are significantly different at $P < 0.001$

Table 2. Spearman rank correlation coefficients (r_s) between pregnancy rates and mare characteristics

r_s value	Age of mares	Endometrial oedema at hCG administration	Endometrial oedema at ovulation
hCG group pregnancy rate	−0.074	0.228	−0.130
	(*P* = 0.521)	(*P* = 0.046)[a]	(*P* = 0.258)
PGF/hCG group pregnancy rate	−0.165	−0.015	0.021
	(*P* = 0.394)	(*P* = 0.928)	(*P* = 0.894)

hCG – human chorionic gonadotropin, PGF/hCG – prostaglandin/human chorionic gonadotropin; [a] statistically significant

Spearman rank correlation coefficient (r_s) revealed no significant associations between pregnancy rates and mare age or endometrial oedema at ovulation in either treatment group. However, a positive correlation was found in the hCG group between pregnancy rates and endometrial oedema scores at the time of hCG administration (Table 2).

Discussion

Typically, the pregnancy rate after AI with frozen semen is lower than with cooled semen (Katila, 2003). In this study, the numerically higher (though not statistically significant) pregnancy rate in the PGF/hCG group could be partially explained by the fact that most mares in this group were inseminated with cooled semen. However, some studies indicate that frozen/thawed semen can occasionally yield comparable or even better fertilisation results to cooled semen (Crowe et al., 2008). The present study demonstrated that with proper veterinary supervision and management, satisfactory and comparable pregnancy rates can be achieved through AI using either frozen/thawed or cooled semen.

Research confirms that induction of oestrus and ovulation does not negatively impact fertility in artificial insemination programmes or natural mating (Barbacini et al., 2000; Morris and Allen, 2002; Allen et al., 2007; Metcalf and Thompson, 2010). Thus, hormonal manipulation of the oestrus cycle during the breeding season or ovulation induction can provide similar reproductive efficacy comparable to spontaneous oestrus without hormonal intervention. The use of prostaglandins for oestrus induction simplifies reproductive management in mares, while ovulation induction with hCG improves the precision of determining the optimal insemination time.

The first examination for oestrus detection was carried out three to four days after cloprostenol administration, as oestrus typically begins within this timeframe (Samper, 2008). At that initial examination, more than 50% of mares were in oestrus. Previous research indicates that the interval from cloprostenol-induced luteolysis leads to oestrus within an average of 5.1 days and ovulation by 7.2 days post-treatment (Kuhl et al., 2017). In our study, ovulation induction, and actual ovulation occurred approx. one day later than in the latter study. Following luteolysis induced by prostaglandins, fertility rates are generally better when ovulation occurs more than eight days after hormone injection compared to ovulations occurring within six days or between six and eight days (Cuervo-Arango et al., 2015). Our findings align with this pattern, with a mean ovulation interval exceeding eight days and a correspondingly high fertility rate of 65%. These results corroborate earlier observations that sooner ovulation following prostaglandin administration yields reduced fertility (Cuervo-Arango and Newcombe, 2010).

Samper et al. (2002) demonstrated that inducing luteolysis with prostaglandins followed by intravenous administration of 2500 IU hCG resulted in ovulation rates of 83.3% within 48 h, 91.6% within 72 h, and 100% within 96 h. A treatment response to hCG is considered clinically satisfactory if ovulation occurs within 48 h of administration. This benchmark aligns with published studies reporting ovulation rates within this timeframe in 73.8% of cycles (Green et al., 2007), 78.4% of cycles (McCue et al., 2004), and 91% of cycles (Barbacini et al., 2000). In the present study, hCG was administered only when the endometrial oedema score was high, and more than 95.0% of ovulations occurred within 48 h after drug administration. These findings are fully consistent with the results obtained by Sieme et al. (2003), who used the same hCG dose. Additionally, the strategic administration of hCG during periods of pronounced endometrial oedema has been shown to ensure a ≥ 95% treatment response rate during the first two oestrous cycles (Samper, 1997).

Barbacini et al. (2000) have argued that repeated injections of hCG during the breeding season do not reduce the drug's efficacy, as indicated by the proportion of ovulations occurring up to 48 h after

hormone administration. However, McCue et al. (2004) and Green et al. (2007) showed that the efficacy of hCG tend to decline as the number of treatments increases during the breeding season. In our study, most observations were made after the first hCG treatment of the season, which may explain the high ovulation rates within two days in both groups. It is important to note that various studies employed different hCG doses and routes of administration, e.g. 1500 IU subcutaneous (SC) (Cuervo-Arango and Newcombe, 2008), 1500 IU (IV) (Kuhl et al., 2017), 2500 IU IV (Samper et al., 2002), 750 and 1500 IU (SC) (Davies Morel and Newcombe, 2008), 2000 IU (IV) (Barbacini et al., 2000), and 3000 IU (IV) (Dolezel et al., 2012). These variations may partly account for the varying outcomes reported. Our study employed 1500 IU, which proved effective despite being a relatively low dose. Smaller doses may reduce the risk of diminished efficacy with subsequent treatments later in the season in mares requiring multiple inseminations; however, this hypothesis should be further evaluated. Notably, Davies Morel and Newcombe (2008) demonstrated that 750 IU could be as effective as 1500 IU.

Decrease in endometrial oedema prior to ovulation is generally considered a positive prognostic factor for ovulation and pregnancy. Our observations revealed a significant reduction in endometrial oedema in both treatment groups following hCG administration. While uterine oedema typically diminishes as ovulation approaches (Gastal et al., 1998; Cuervo-Arango and Newcombe, 2008; Dolezel et al., 2012), induced cycles may result in slightly higher endometrial oedemas compared to spontaneous cycles (Cuervo-Arango and Newcombe, 2008). Conversely, Gastal et al. (2006) reported that endometrial oedema 24–36 h after hCG treatment was lower than in non-treated mares and reflected changes in systemic oestradiol levels. Dolezel et al. (2012) showed that the decrease in endometrial oedema was more pronounced in mares treated with hCG given when dominant follicle measured 35–40 mm compared to mares treated with hCG when the dominant follicle exceeded 40 mm, or in mares with spontaneous ovulation; however, the degree of endometrial oedema before ovulation did not differ significantly between these groups.

Twin or multiple pregnancies in horses are associated with serious complications as abortion, dystocia, and neonatal death, with MO being the direct reason of MP. The incidence of MP in mares typically ranges within a few percent, and is more common in Thoroughbreds, older mares, and certain genetic lines. Some studies have also indicated that hormonal manipulation of the oestrus cycle and ovulation can stimulate MO and twin pregnancies (Ginther, 1995; Veronesi et al., 2003; Allen et al., 2007). Veronesi et al. (2003) showed that hormonal manipulation of the oestrus cycle and induction of ovulation increased twin pregnancies from 6.5 to 16.6%. Comparisons of treatments showed that twin pregnancy rates increased to 17.4% after cloprostenol treatment, 13.1% after hCG treatment and 30.6% after combined cloprostenol and hCG administration (Veronesi et al., 2003). The frequency of MP in the present study was low compared to the discussed reports. This discrepancy may potentially be attributed to factors such as a low genetic predisposition for MP in the study population, or a high incidence of spontaneous embryonic reduction from twins to singletons occurring in most MO cases before the first pregnancy examination.

The frequency of double ovulations significantly increased following prostaglandin treatment reaching 17% compared to 3% in untreated controls (Ginther and Al-Mamun, 2009). Cuervo-Arango and Newcombe (2010) found that cloprostenol treatment increased multiple ovulations in mares ovulating later than 7 days post-treatment, though this did not translate to higher multiple pregnancy rates. It is likely that prostaglandin administration during the luteal phase of the oestrus cycle promotes the selection and growth of two dominant follicles more frequently than in spontaneous cycles. However, the underlying mechanisms remain unclear (Veronesi et al., 2003). Cuervo-Arango and Newcombe (2010) demonstrated that a longer interval between prostaglandin administration and ovulation was associated with a higher probability of MO. When prostaglandin is administered in the presence of a dominant follicle, ovulation typically occurs sooner and as a single event. However, when only small follicles are present at the time of treatment, the interval to ovulation is extended, increasing the likelihood of developing two dominant follicles. In the current study, cloprostenol was administered only when no follicles larger than 3 cm in diameter were present on the ovaries. Another study also demonstrated that prostaglandin treatment elevated MO rates without necessarily increasing MP incidence (Katila, 2003). Similarly, Lindeberg et al. (2002) have found that the administration of prostaglandins does not increase the frequency of twin pregnancies (Lindeberg et al., 2002). Our findings are fully consistent with these observations. Research by Cuervo-Arango et al. (2015) demonstrated that prostaglandin-induced

oestrus and the interval from treatment to ovulation did not significantly affect multiple ovulation rates. Several studies have failed to demonstrate any significant effect of hCG treatment on the incidence of multiple ovulation or multiple pregnancy (Davies Morel and Newcombe, 2008). Kuhl et al. (2017) reported comparable rates of double ovulations and twin pregnancies following hCG administration, whether oestrus occurred spontaneously (15.2 and 2.6%, respectively) or was induced by luprostiol (8.2 and 7.9%, respectively) or cloprostenol (14.9 and 8.1%, respectively), without significant differences between groups.

Conclusions

Administration of 1500 IU of human chorionic gonadotropin (hCG) during periods of pronounced endometrial oedema achieves a > 95% response rate in both spontaneous and induced oestrus cycles. Our findings also indicate that the induction of ovulation using hCG does not affect the frequency of multiple ovulations (MO). In contrast, treatment with prostaglandin**s,** followed by hCG application, significantly increases MO frequency, although this combination does not appear to influence the occurrence of multiple pregnancies. These results indicate that prostaglandins may promote the development of more than one dominant follicle, while hCG does not seem to stimulate the formation of additional follicles.

Conflict of interest

The Authors declare that there is no conflict of interest.

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