

## Effects of a single use of the GnRH analog buserelin on the induction of ovulation and endocrine profiles in heavy draft mares

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*We observed structural changes in the follicles and uterus of heavy draft mares during estrus and examined the effect of a single injection of the gonadotropin-releasing hormone analog buserelin on ovulation and endocrine profiles. Twenty-two heavy draft mares were divided into a buserelin-treated group (n=8) and a control group (n=14). Mares were given an intramuscular injection of 40 µg buserelin when they presented signs of estrus to a teaser stallion, had ≥45 mm diameter follicles, and presented decreased uterine edema compared with the previous examination. The follicles and uterus were monitored using transrectal ultrasound imaging and measurement of blood levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), progesterone, and estradiol-17β. The ovulation rates within 48 hr was significantly higher in the treated group (100%, 8/8) than in the control group (57.1%, 8/14; P=0.051). The mean ± SEM time before confirmation of ovulation was 29 ± 9 hr for the treated group and 59 ± 7 hr for the control group. There were no significant differences in mating frequency, double ovulation rate, or fertility rate between the two groups. One to two days after administering buserelin, LH and FSH temporarily increased, and in the control group, LH was high during ovulation, whereas FSH temporarily increased with the growth of the follicle. These results indicate that a single injection of 40 µg buserelin when follicles are at least 45 mm in diameter and uterine edema is decreased is effective for inducing ovulation.*

**Key words:** GnRH analog buserelin, heavy draft mares, ovulation, single injection, uterine edema

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Horses are a seasonally polyestrous species with a limited breeding season. Tokachi, Hokkaido, is known as a producing area for heavy draft horses with a breeding season that is limited to March to June, and the success or

failure of conception greatly affects productivity. To effectively produce heavy draft horses, conception by one mating or by means of artificial insemination is ideal. However, there are no reports on the effective induction of ovulation in heavy draft mares.

Generally, human chorionic gonadotrophin (hCG) is used to induce ovulation in horses. For a follicle that is ≥35 mm in diameter, 1,500–6,000 IU hCG is administered. A strong ovulation effect, with ovulation occurring within 72 hr, has been reported in many studies [5, 8, 13, 20, 21]. In contrast to the relatively low cost and strong ovulation effect of hCG, there are reports on the risk of twin pregnancy

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**Fig. 1.** Japanese heavy draft mare. The Japanese heavy draft mare is acknowledged as the largest horse in the world, weighing an average of approximately a ton.

due to multiple ovulation [27] and decreased reactivity due to antibody production [30, 33]. Therefore, effective induction of ovulation has been attempted by implanting the gonadotropin-releasing hormone (GnRH) analog deslorelin acetate (Ovuplant<sup>TM</sup>) [4, 7, 16, 19]. However, if conception does not occur, the implant could lead to anestrus due to pituitary downregulation or a prolonged interval between ovulations. Therefore, removal of the implant is recommended once ovulation is confirmed [7, 16]. Furthermore, deslorelin is not approved for use in horses in Japan. Therefore, induction of ovulation has been attempted with buserelin administration [1, 2, 14, 35], and cases of ovulation after a single injection have been reported [35]. On the other hand, although a single injection of GnRH analog induces a temporary increase in luteinizing hormone (LH), it is reported to be insufficient to maintain the LH surge [10, 20, 26, 29]. Therefore, we examined the ovulation effect of a single injection of buserelin and its impact on reproductive performance to clarify structural changes in follicles and the uterus in addition to the endocrine profiles of heavy draft mares during estrus. Before investigating the use of buserelin in detail, a preliminary test was conducted to investigate the dosage and timing of its administration.

## Materials and Methods

### *Subjects*

To examine the administration period and dose of buserelin for effective ovulation in heavy draft mares, a preliminary test was done on 102 heavy draft mares with normal estrous cycles at two ranches in Obihiro, Hokkaido, from April to June in 2002 and 2003. Next, to elucidate the structural changes in the follicles and uterus during estrus and conduct endocrinological examinations, we tested a total of 22 heavy draft mares including 16 with normal estrous cycles at a ranch in Obihiro, Hokkaido. The mean  $\pm$  SD age of the subjects in this study was  $8.9 \pm 4.7$  (range, 3 to 17) years. The foaling numbers of the mares ranged from 0 to 10 births (3 mares had never previously foaled, 19 mares had previously foaled), with the mean number of births being  $3.2 \pm 2.8$ . One subject in the control group was observed 15 days after a delivery, whereas the treated group ( $n=8$ ) and other mares in the control group ( $n=13$ ) were observed from 29 days after delivery onward. Nonpregnant mares ( $n=4$ ) in estrus were also observed. The test was implemented using a crossover design for the treated and control groups based on the total number of estrous cycles (Fig. 1).

### Preliminary test to identify the optimum dose of buserelin

Mares that presented signs of estrus to a teaser stallion during April to June in 2002 and 2003 and had follicles of  $\geq 45$  mm in diameter confirmed over 1 to 3 days via transrectal ultrasonography were intramuscularly administered 40 (n=31) or 20  $\mu\text{g}$  (n=21) buserelin (Estmal, Kawasaki Mitaka Pharmaceuticals, Tokyo, Japan). Ovulation rates within 48 hr were compared with that of the control group (n=50) observed under the same conditions. The ovulation rates were 56% (28/50), 93.5% (28/31), and 71.4% (15/21), in the control, 40  $\mu\text{g}$  buserelin-treated, and 20  $\mu\text{g}$  buserelin-treated groups, respectively. The ovulation rate of the 40  $\mu\text{g}$  buserelin-treated group within 48 hr was significantly higher than that of the control group ( $P < 0.01$ ) (Fig. 2). Therefore, we used a buserelin dose of 40  $\mu\text{g}$  for the present test.

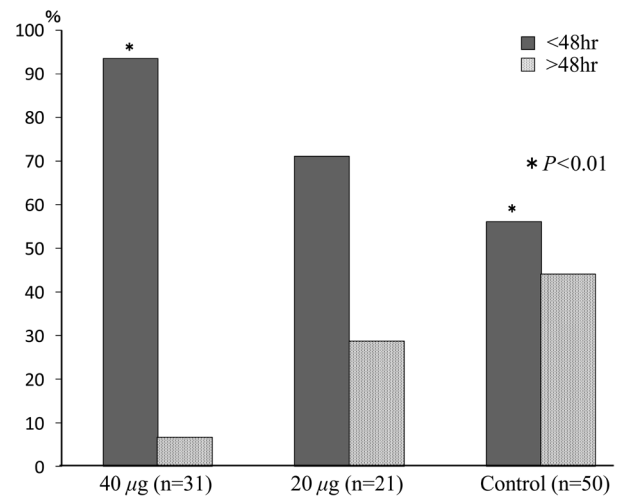
### Buserelin administration test

The structures of the follicles and uterus were rectally examined by connecting a 5-MHz linear rectal probe (UST-588-5, Aloka, Tokyo, Japan) to an ultrasonography (SSD-500, Aloka). Follicle size was determined by calculating the average of two lines of measurement from a frozen ultrasound image as follows; (major axis + minor axis)/2. The progress of the treated group (n=8), which received an intramuscular injection of 40  $\mu\text{g}$  buserelin when signs of estrus were presented to a teaser stallion, follicles of  $\geq 45$  mm in diameter were confirmed, and the degree of a previously recorded cross section of uterine edema was reduced, was examined, and the control group (n=14), which was left untreated, was examined until ovulation. Transrectal ultrasonography of the follicles (ovaries) and uterus was performed on the treated group every 6 hr after buserelin administration until ovulation, after which it was performed every 12 hr up to 48 hr after ovulation. For the control group, the follicles (ovaries) and uterus were observed every other day until 2 days after ovulation. Blood was sampled from both groups during ultrasonography through jugular veins with heparin-sodium Vacutainer blood collection tubes. After centrifugation, plasma was frozen and stored at  $-30^\circ\text{C}$  and subsequently used to measure hormone levels.

### Hormone measurements

The plasma concentrations of FSH and LH were determined by homologous double-antibody equine RIA methods as described previously [17]. The intra- and inter-assay coefficients of variation were 4.9% and 12.2% for FSH and 12.56% and 15.06% for LH, respectively.

The plasma concentrations of estradiol-17 $\beta$  and progesterone were measured by double-antibody RIAs using 125I-labeled radioligands [34] after steroid extraction and defatting using hexane and acetonitrile as described



**Fig. 2.** Comparison of the ovulation rate of the control mares and those administered 20  $\mu\text{g}$  and 40  $\mu\text{g}$  buserelin. A dose of 40 or 20  $\mu\text{g}$  buserelin was intramuscularly administered when mares presented signs of estrus to a teaser stallion and dominant follicles reached  $>45$  mm in diameter. The ovulation rate within 48 hr in the control group was evaluated under the same conditions. The ovulation rate within 48 hr after administration of 40  $\mu\text{g}$  buserelin was significantly higher than that of the control group. \* $P < 0.01$  for comparison between the 40  $\mu\text{g}$  buserelin group and control group.

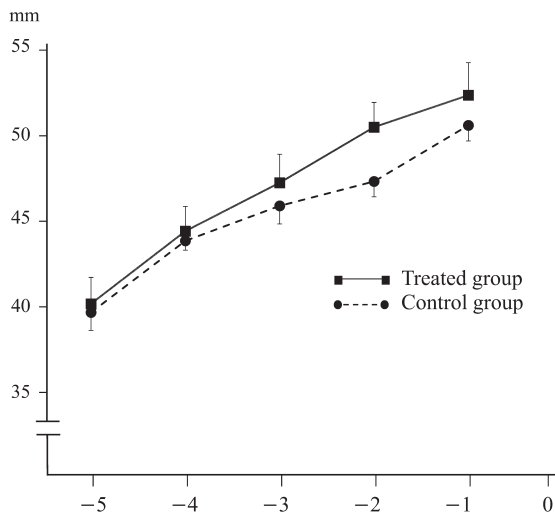
previously [22]. Antisera against estradiol-17 $\beta$  (GDN 244) and progesterone (GDN 337, kindly provided by Dr. G.D. Niswender, Colorado State University, Fort Collins, CO, U.S.A.) were used in each assay. The intra- and inter-assay coefficients of variation were 4.8 and 5.8% for estradiol and 3.5 and 13.4% for progesterone, respectively.

### Frequency of mating, double-ovulation rate and fertility rate

During the testing period, subjects naturally mated with stallions. When a second ovulation was confirmed within 48 hr of the first ovulation, it was considered a double ovulation, and the rates of double ovulation were compared. On days 14 and 21 after ovulation, pregnancy diagnosis was performed through transrectal ultrasonography, and fertility rates were compared between the groups.

### Statistical analysis

The data are shown as the mean  $\pm$  SEM. The results were subjected to ANOVA for repeated measures to determine the effect of the dominant follicular size measured. Following a significant effect by ANOVA. Significant difference was analyzed between the control and treated group. The differences in ovulation rate and fertility rate between groups were analyzed by Fisher's exact test. The number of mating



**Fig. 3.** The changes in mean follicle diameter from 5 days before to 1 day before ovulation. The mean follicle diameters (major axis + minor axis/2) from 5 days before (–5) to 1 day before (–1) ovulation are shown. Day 0 is the day of ovulation.

was tested by Welch's *t*-test. The occurrence of double ovulation was determined by  $\chi^2$  test. All data were analyzed using the StatView computer software package. A value of  $P < 0.05$  was considered to be significant.

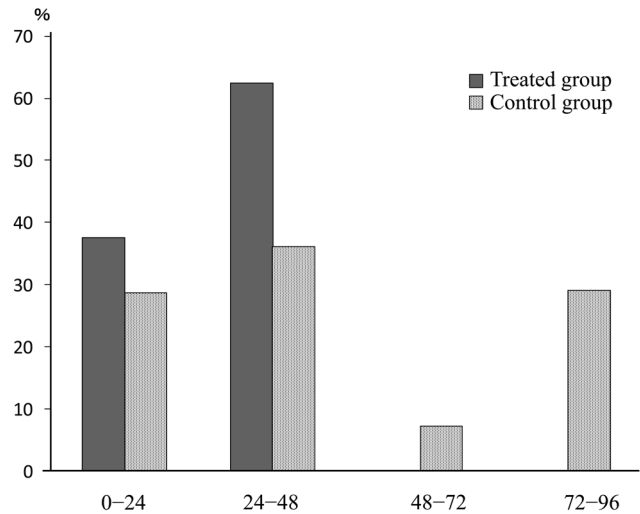
## Results

### *The follicle diameter and shape until ovulation*

Changes in the mean follicle diameter from 1 to 5 days before ovulation are shown in Fig. 3. In the control group, follicle diameter was  $45.7 \pm 1.1$  mm ( $n=9$ ) 3 days before ovulation and increased to  $50.3 \pm 1$  mm ( $n=14$ ) the day before ovulation. In the treated group, follicle diameter was  $47.0 \pm 1.6$  mm ( $n=7$ ) 3 days before ovulation and increased to  $52.1 \pm 1.9$  mm ( $n=8$ ) the day before ovulation. Follicle diameters increased as ovulation neared in both the treated and control groups, with no differences in follicle diameter accompanying growth observed between the two groups. In six of eight subjects (75%) in the treated group, follicles changed from spherical to conical and pear-shape within 6 to 24 hr before ovulation. In two subjects (25%), the follicles remained spherical until ovulation, presenting no clear structural change.

### *Interval to ovulation*

The ovulation rates for subject mares at 24 hr intervals are shown in Fig. 4. The mean interval to ovulation was  $29 \pm 9$  (range, 6–48) hr for the treated group ( $n=8$ ), whereas it was  $59 \pm 7$  (range, 12 to 120) hr for the control group



**Fig. 4.** Interval between treatment and ovulation (hr). The time of buserelin administration was designated 0 hr for the treated group, and the time at which the follicle diameter was  $>45$  mm and uterine edema was decreased was designated as 0 hr for the control group.

( $n=14$ ). The ovulation rate in the treated group within 48 hr was 100% (8/8), whereas it was 57.1% (8/14) in the control group; thus, the treated group presented a higher rate ( $P=0.051$ ). The treated group could be divided into the following two groups according to the interval to ovulation:  $\leq 6$  hr in 37.5% (3/8) of the mares and 42 to 48 hr in 62.5% (5/8) of the mares. In the control group, only one mare ovulated after 48 to 72 hr (7.1%), and five mares ovulated after at least 72 hr (35.7%).

### *Frequency of mating, double ovulation rate, and fertility rate.*

The mean frequency of mating per estrous cycle was  $1.6 \pm 0.2$  (range, 1–3) for the control group ( $n=14$ ) and  $1.8 \pm 0.2$  (range, 1–2) for the treated group ( $n=8$ ); thus, there was no significant difference between the two groups. In the control group, three mares mated three times. No mare in the treated group mated more than twice.

The double ovulation rate within 48 hr for the treated group was 37.5% (3/8), which was higher than that of the control group (28.6%, 4/14), but the difference was not significant.

The fertility rate was 50% for both the treated and control groups (4/8 and 7/14, respectively), presenting no difference between the two groups. No twin pregnancies were confirmed in either group.

Results for mating frequency, multiple ovulation, and fertility rates are shown in Table 1.

**Table 1.** Frequency of mating, double-ovulation rate, and fertility rate

	n	Frequency of mating*	Double-ovulation rate (%)	Fertility rate (%)
Treated group	8	1.8 (1–2)	37.5	50
Control group	14	1.6 (1–3)	28.6	50

\* Mean (range).

### Endocrine profiles

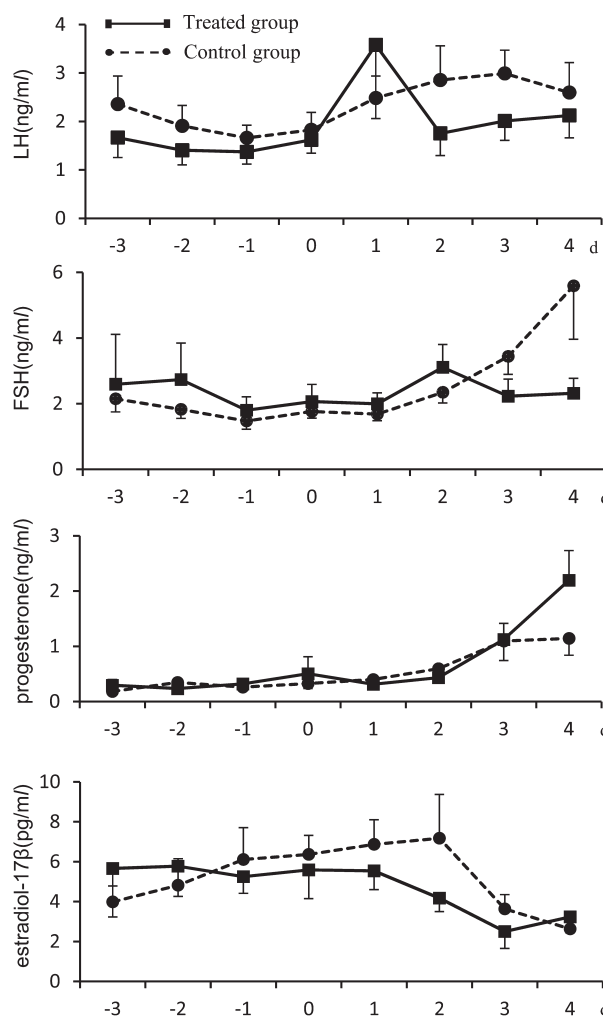
The day of buserelin administration was set as day 0 for the treated group, whereas the day on which a follicle of  $\geq 45$  mm in diameter and reduced uterine edema were confirmed in the treated group was set as day 0 for the control group. The changes in LH, FSH, progesterone, and estradiol-17 $\beta$  levels from day -3 to 4 are shown in Fig. 5. In the treated group, one value per day was analyzed. Either the value measured at the same time as the first day or an average of values around that time was used.

In the control group, the LH levels gradually increased from  $1.8 \pm 0.4$  ng/ml on day 0 (n=13) to  $3.0 \pm 0.5$  ng/ml on day 3 (n=11) and then decreased back to  $2.6 \pm 0.6$  ng/ml on day 4 (n=9). The LH levels in the treated group were similar to those in the control group (n=8):  $1.6 \pm 0.3$  ng/ml at the time of buserelin administration followed by a temporary increase to  $3.6 \pm 1.5$  ng/ml and then a decrease to  $1.7 \pm 0.5$  ng/ml on day 2.

The FSH levels in the control group fluctuated around 2.0 ng/ml from day -3 to day 1, started increasing on day 2, and reached  $5.6 \pm 1.6$  ng/ml on day 4 (n=9). In contrast, in the treated group, the FSH level was  $2.1 \pm 0.5$  ng/ml on day 0 (n=8), temporarily increased to  $3.1 \pm 0.7$  ng/ml on day 2 (n=8), and then decreased to  $2.3 \pm 0.5$  ng/ml on day 4 (n=6). The last two values in the treated group were lower than those in the control group, but the differences were not significant.

The baseline P<sub>4</sub> levels fluctuated but stayed below 0.5 ng/ml from day -3 to day 1 and then increased from day 2 to day 3 in both the treated and control groups. On day 4, the P<sub>4</sub> levels increased to  $2.2 \pm 0.5$  and  $1.1 \pm 0.3$  ng/ml in the treated (n=6) and control (n=9) groups, respectively. In the mares in the treated group that ovulated within 48 hr, the P<sub>4</sub> level was higher, but the difference from that of the control group was not significant.

In the control group, the E<sub>2</sub> levels increased from day -3 to day 2, reaching a peak of  $7.2 \pm 2.2$  pg/ml (n=14), and then decreased to  $3.6 \pm 0.7$  pg/ml on day 3 (n=12). In the treated group, the E<sub>2</sub> level was  $5.6 \pm 1.4$  on day 0 (n=8) and decreased to  $2.5 \pm 0.8$  pg/ml on day 3 (n=6). In both groups, the E<sub>2</sub> levels tended to decrease around the time ovulation was confirmed.



**Fig. 5.** Mean ( $\pm$  SEM) plasma concentrations of LH, FSH, progesterone, and estradiol-17 $\beta$ . Day 0 for the treated group was the day of buserelin administration, and for the control group, it was when follicles of  $>45$  mm in diameter and a decrease in uterine edema were confirmed. For the treated group, values measured at the same time as the first day or averages of values taken around the same time were used as the date each day for analysis.

## Discussion

In this study, a single administration of buserelin (40  $\mu\text{g}$ ) was very effective for inducing ovulation in heavy draft mares. Furthermore, by avoiding antibody production due to repeated administration of hCG [30, 33] and through procedures to remove GnRH analog or deslorelin acetate implants [16], this method appears to offer high clinical applicability. The timing of administration during the growth of the dominant follicle ( $\geq 45$  mm) and decrease in endogenous estrogen (and thus uterine edema) may explain its efficacy in inducing ovulation.

The diameter of dominant follicles and uterine edema findings are considered effective indicators to predict ovulation timing in horses [3, 21, 28, 32]. The mean maximum diameter of the ovulatory follicle is usually between 40 and 45 mm, but there are variations depending on season and type of horse [32]. In this study, the follicle diameter before ovulation in the Japanese heavy draft mares (Fig. 1), which are acknowledged as the largest horses in the world, was  $\geq 50$  mm, with some reaching 60 mm, values that are larger than the mean follicle diameters previously reported for horses. These results are consistent with a previous report of follicle diameter in heavy draft mares before ovulation [15].

It has been reported that preovulatory follicles exhibited a pronounced change in shape from a spherical to a conical or pear-shaped structure in 84% of preovulatory periods, whereas in the remaining 16%, the follicle retained a spherical shape [28]. In the treated group that was observed every 6 hr, two mares presented spherical follicles 6 hr before ovulation (25%), whereas six mares presented conical or pear-shape follicles 6 to 24 hr before ovulation (75%). However, changes in shape also occurred on days  $-7$  (3%),  $-6$  (5%),  $-5$  (8%),  $-4$  (13%), and  $-3$  (13%) [28], so predicting the timing of ovulation based on follicle shape alone is not appropriate.

Uterine edema findings become most clear approximately 3 days before ovulation, but decrease 1 to 2 days before ovulation, thus uterine edema is considered an effective indicator for the timing of ovulation [3, 11]. However, uterine edema findings were found in 64% of anovulatory cycles during the breeding transition period [36], and significant uterine edema findings are also observed in endometritis [31]. Therefore, determining the timing of ovulation based on uterine edema findings is not appropriate. In the present study, we chose the timing for buserelin administration as the time at which follicles were  $\geq 45$  mm in diameter and uterine edema was decreased, and the ovulation rate within 48 hr was 100%. Furthermore, the treated group could be divided into the following two subgroups: the 37.5% (3/8) of mares that ovulated within 36 hr and the 62.5% (5/8) of

mares that ovulated within 36 to 48 hr. The group for which ovulation was confirmed within 36 hr likely had endogenous hormone dynamics similar to ovulation at the time of administration. The tendency for ovulation to occur within 36 to 48 hr may have been due to an increase in LH and FSH caused by buserelin administration speeding up follicle maturation, with ovulation resulting from a temporary LH surge induced by buserelin administration rather than by endogenous GnRH.

The rate of double ovulation is high in horses, particularly in thoroughbreds at 37.2%, and twin pregnancies have been confirmed in 16.2% of such horses during early pregnancy diagnosis (13–16 days after ovulation) [25]. The rate of double ovulation in this study was higher in the treated group, but the fertility rate was 50% (4/8 and 7/14) in both groups, and no twin pregnancies were confirmed.

In heavy draft horse production, where frequent mating has become the norm, decreasing the frequency of mating is a challenge. During the present study, horses in both groups mated naturally according to the judgment of the owners, and there was no significant difference in the frequency of mating. However, in the control group, three mares mated three times during one estrus. In contrast, in the treated group, no mares mated more than twice. Avoiding frequent mating lowers the risk of breeding-induced endometritis and is effective in reducing the burden on stallions.

Regarding the changes in endocrine profiles induced by a single injection of buserelin, differences in LH and FSH levels were noticeable between the groups. The LH level in the control group gradually increased 3 days before ovulation, peaking 1 to 2 days after ovulation, which was similar to previously reported LH secretory patterns in horses [18, 23]. In contrast, in the treated group, the LH level temporarily increased 6 to 24 hr after buserelin administration and then fluctuated at low values. We hypothesize that buserelin administration promoted LH secretion, leading to the desensitization of gonadotropic cells or negative feedback on LH secretion.

FSH levels increase around the time of ovulation, which is known to be an endocrine profile [6], and they did so in our control group. However, in our treated group, a temporary increase was observed 1 to 2 days after administration, and FSH fluctuated at low values after ovulation. Regarding the stimulation of FSH secretion, the effect of buserelin is assumed to be similar to its effect on LH secretion.  $P_4$  levels are thought to fluctuate around baseline values until ovulation and then increase 24 to 36 hr after ovulation, peaking at 5 to 7 days after ovulation [18, 24]. Both of our groups presented similar fluctuations. In the treated group, ovulation occurred within 48 hr and the  $P_4$  level on day 4 was high. This indicates that there was a physiological effect on the corpus luteum after buserelin administration.

E<sub>2</sub> levels decreased as ovulation neared, presenting a secretory pattern similar to that previously reported [12, 18]. A connection between hCG administration before ovulation and a decrease in E<sub>2</sub> and cessation of follicle growth has been suggested [9]. E<sub>2</sub> is secreted from the granular layer of large follicles and is closely associated with the development and maturation of follicles. During the period of decreasing uterine edema findings, E<sub>2</sub> starts to decrease, so it could possibly be used as an indicator for maturity of the follicle near ovulation.

In conclusion, the present study clearly demonstrated that the treatment with 40 µg buserelin when follicles are ≥45 mm in diameter may be used as an effective management tool to shorten the interval to ovulation in heavy draft mares and for improving reproductive efficiency in the case of reproductive failure. In addition, reducing the mating frequency by inducing ovulation reduces the risk of breeding-induced endometritis in mares and could be an effective breeding management technique for heavy draft horses.

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