

Effect of prostaglandin F_{2α} administration on uterine polymorphonuclear neutrophil counts in Japanese heavy draft horses

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The objective of this study was to examine the effect of prostaglandin F_{2α} (dinoprost) and oxytocin administration on uterine polymorphonuclear neutrophil counts in the Japanese heavy draft mare. To compare polymorphonuclear neutrophil (PMN) counts in the endometrium, a total of 162 samples were collected from 54 estruses of 47 mares (before ovulation, day 0, and day 2) using a double-guarded cytology brush. Dinoprost (PG; 5 mg) was administered intramuscularly (i.m.) only once, on day 0, whereas oxytocin (OT; 20 U i.m.) was administered three times at 12-hr intervals starting on day 0. The plasma progesterone concentrations from days 0 to 14 were not different between the non-treatment (CON), PG treatment, and OT treatment groups. The PMN counts increased in all the groups from before administration to day 0 (CON, 0.90 to 3.55; PG, 1.20 to 8.45; and OT, 0.70 to 1.70; P=0.0014, 0.0046, and 0.0073, respectively). There was a significant decrease in PMNs from day 0 to day 2 only in the PG group (P=0.0073). The pregnancy rate was not different among the CON (12/18), PG (14/18), and OT (10/18) groups. The results of this study indicate that dinoprost can reduce uterine polymorphonuclear neutrophil counts.

Key words: *dinoprost, endometritis, heavy draft horse, polymorphonuclear neutrophil*

J. Equine Sci.
Vol. 32, No. 4
pp. 117–124, 2021

Persistent breeding-induced endometritis is a major cause of infertility in mares. Uterine contraction weakness results in a reduction of uterine clearance. For mares to conceive, uterine inflammation should be cleared within the first 5 days after breeding [31]. Uterine inflammation after breeding is a normal physiological reaction that is necessary for the discharge of excess semen, debris, or accumulations from the uterus to protect the conceptus from harmful inflammatory products and maintain pregnancy. This inflammation generally resolves within 24–48 hr [24, 35, 36].

Japanese heavy draft horses are mixed breeds of

Percheron, Breton, and Belgian draft horses. They weigh approximately 1,000 kg and are one of the largest breeds in the world [13]. Breeding mares have less chance to work and lack physical exercise because of the mechanization of agriculture. These factors subsequently cause uterine contraction weakness [9]. Uterine fluid accumulation, resulting from a decrease or delay in uterine clearance, is associated with prolonged inflammation and infertility [11, 19]. Mares in which spontaneous resolution of uterine infection does not occur for longer than 48 hr are considered persistently infected or in the process of becoming persistently infected [18]. Compared with medium-weight or lightweight breeds, draft horses have higher incidence rates of retained placenta and dystocia [26], which lead to lower pregnancy rates. In addition, a larger body is generally associated with a higher foal birth weight, which is believed to result in a higher mortality rate [15]. Furthermore, in comparison with other horse breeds, the semen of draft horses has lower motility and is more frequently morphologically abnormal [1]. As

Received: January 18, 2021

Accepted: July 27, 2021

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a result, the reproductive performance of the draft horse is low. In Japan, there exists a unique sport called Banei horse racing in which only heavy draft horses participate. To preserve this sport, the Japanese heavy draft horse breed, and Japanese culture, the reproduction of draft horses should be improved.

The polymorphonuclear neutrophil (PMN) count is one of the diagnostic indicators of uterine inflammation. The PMN is a type of white blood cell that is associated with the immune system against bacterial pathogens and inflammation [8]. Once equine spermatozoa or bacteria enter the uterus, they trigger a chemotactic signal, and PMNs are recruited at the surface of the endometrium, which results in the removal of pathogens and the resolution of inflammation [32, 35]. In general, the classification of inflammation for equine endometrial cytology is as follows: normal (no PMNs to rare PMNs/hpf), mild inflammation (1–2 PMNs/hpf), moderate inflammation (3–5 PMNs/hpf), and severe inflammation (>5 PMNs/hpf) [7]. If any intrauterine fluids or echogenic lines remain present for 12 hr or longer, the mare is considered to have persistent breeding-induced endometritis [3, 27, 34]. A double-guarded cytology brush can help in the collection of a sufficient number of cells from the endometrium and is easily available for use in practical field conditions [5, 23]. Among the three techniques for the collection of cells from the endometrium (cotton swab, cytology brush, and low-volume lavage), the cytology brush consistently provides the best samples for obtaining an optimal cell density and adequate diagnostic quality [2].

In general, oxytocin (OT) is widely used as a treatment for endometritis to enhance mechanical uterine clearance in light breed mares. In addition to OT, prostaglandin F_{2 α} (PG) is used, but PG is considered to have a negative effect on corpus luteum regression [14, 22]. The use of PG after or before ovulation is controversial with regard to uterine contraction and luteolysis. Administration of PG and its effect on the uterus of the heavy draft horse has not been previously reported, and no studies have been published regarding the uterine PMNs in heavy draft horses. The effect of ecbolics (OT and PG) on uterine PMN counts is also unknown in these horses. Thus, the objective of this study was to examine the influence of ecbolics on uterine PMNs in heavy draft horses.

Materials and Methods

Animals and treatment

We studied 47 Japanese heavy draft horse mares; the mares were crossbreeds of Percheron, Breton, and Belgian draft horses and ranged in age from 3 to 19 years. The mares were divided into three groups as follows: CON (no treated mares), PG (dinoprost treated mares), and OT (oxytocin

treated mares). The median ages of the mares in the groups were as follows: CON, 10 years; PG, 7 years; and OT, 7 years, respectively. The numbers of barren, maiden, foal, and slipped mares were as follows: nine, two, four, and three in the CON group; six, three, five, and four in the PG group; and four, three, nine, and two in the OT group, respectively. The numbers of mares in foal heat were as follows: three in the PG group, two in the OT group, and zero in the CON group. A total of 162 samples were collected from 54 estruses (47 with one estrus cycle and 7 with two estrus cycles in the breeding season) of healthy heavy draft horses from two private farms and one stud farm in Ashoro, Hokkaido, Japan, between February and July in 2018 and 2019. During the study, mares at these farms were free to eat a mixture of mostly Timothy grass dry hay. All mares were left outside and freely consumed mineral salts. Mares were examined every day or every other day early in estrus by palpation and ultrasonography per rectum. All examinations were conducted in the early morning and detected ovulation. The day of ovulation was considered day 0. All breeding was conducted by natural mating and finished before ovulation. The estruses were randomized into the following three groups: control (CON; n=18), PG (n=18), and OT (n=18). The mares in the PG group were administered 5 mg dinoprost intramuscularly (i.m.; Panacelan-Hi[®], Fujita Pharmaceutical Ltd., Tokyo, Japan) only once, on the day of ovulation (day 0). The mares in the OT group were given 20 U OT i.m. (Oxytocin for animal use, DS Pharma Animal Health Co., Ltd., Osaka, Japan) three times every 12 hr from day 0. The CON group received no treatment. Mares underwent an ultrasonography examination for pregnancy on days 14 and 28, and the number of corpora lutea in each mare was recorded.

The present study received animal welfare and ethics approval from the Tokachi Agricultural Mutual Aid Association. All treatments were conducted after obtaining informed consent from clients.

Cytology and intrauterine fluids

Samples were collected using a double-guarded uterine cytology brush (Minitüb GmbH, Tiefenbach, Germany) three times during the 54 estruses: before ovulation (pre; early to mid-estrus, from day –10 to day –1), on the day of ovulation (day 0), and after mating (day 2). To obtain the samples, the tail of the mare was wrapped, and the vulva and perineal region were scrubbed and rinsed. The area was then dried with sterilized gauze. While wearing a sterilized sleeve or surgical glove, the experimenter then introduced the brush into the uterus and rotated it for 10 to 15 sec [16]. The cytology brush was then smeared on a microscope glass slide. The slide was stained using a simple and quick staining solution (Diff-Quik[®], Sysmex Co., Kobe, Japan),

air dried, and examined by light microscopy (400× magnification) for the presence of PMNs. PMNs were counted in 10 fields, and the average number of inflammatory cells per sample was recorded.

At days 0 and 2, the amounts of any intrauterine fluids or echogenic lines were recorded. These accumulations were classified according to a previous study: Grade 1 (G1), no fluids or echogenic lines in the uterine lumen; Grade 2 (G2), small amounts of fluids (<0.5 cm in diameter) or echogenic lines in the intrauterine cavity; and Grade 3 (G3), obvious intrauterine fluids (≥ 0.5 cm in diameter) [3].

Blood samples

The day of ovulation was considered day 0. Blood samples were collected on days 0–2, 9, 14, and 28 from all mares. Plasma progesterone concentrations were assayed using an EIA analyzer (AIA-360, Tosoh Co., Tokyo, Japan). All plasma samples were preserved in a freezer for later analysis.

Statistical analysis

The median number of PMNs, day of mating before ovulation, age, and median number of matings per estrus cycle for mares were compared among the three groups (CON, PG, and OT) using the Kruskal–Wallis rank-sum test with the Bonferroni adjustment method. For each of the three time points (pre, day 0, and day 2), numbers of PMNs were compared using the pairwise Friedman test with the Bonferroni adjustment method. Plasma progesterone concentrations of pregnant mares were compared using the Kruskal–Wallis rank-sum test with the Bonferroni adjustment method. The reductions in the amounts of intrauterine fluids or echogenic lines from day 0 to day 2 among the three groups were compared using Fisher's exact test with the Bonferroni adjustment method. We compared the pregnancy rate on day 28 among the three groups using Fisher's exact test with the Bonferroni adjustment method. $P < 0.05$ was considered significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of the R commander designed to add statistical functions frequently used in biostatistics [10].

Results

The median PMN count in each group increased significantly from before ovulation to day 0 (CON, 0.90 to 3.55; PG, 1.20 to 8.45; and OT, 0.70 to 1.70; $P = 0.0014$, $P = 0.0046$, and $P = 0.0073$, respectively; Fig. 1). PMNs significantly decreased from day 0 to day 2 only in the PG group (8.45

to 0.85, $P = 0.0073$). No significant differences in PMN count were found between the groups (CON, PG, and OT: 0.9, 1.2 and 0.7 at pre; 3.55, 8.45, and 1.70 at day 0; 1.70, 0.85, and 1.00 at day 2, respectively; Fig. 2) at any of the time points (pre, day 0, or day 2).

No significant differences in the median ages of the mares were found among the groups.

In the CON, PG, and OT groups, 10 out of 18, 14 out of 18, and 11 out of 18 mares, respectively, had intrauterine fluids or echogenic lines on day 0. The rates of reduction of intrauterine accumulation from day 0 to day 2 in the groups were 5/10 for CON, 10/14 for PG, and 8/11 for OT, with no significant differences between the groups. The numbers of mares with an accumulation grade of G2 at day 0 and day 2 in the CON, PG, and OT group were as follows: 4, 5, and 8 on day 0 and 5, 3, and 4 on day 2, respectively. For G3, the numbers were 6, 9, and 3 on day 0 and 4, 3, and 2 on day 2, respectively. The median PMN counts of the mares with a G3 accumulation grade on day 2 in each group were 1.05, 2.8, and 5.95 in the CON, PG, and OT groups, respectively.

The average and median last breeding days before ovulation in the groups were as follows: CON, 1.83 (SD, 0.92) and 2; PG, 1.56 (0.92) and 1; and OT, 1.94 (0.87) and 2, respectively. No significant differences were found between the groups in terms of the median last breeding days before ovulation.

The numbers of mating instances per estrus cycle in the groups were as follows: CON, 1.50 (SD, 0.78; median, 1); PG, 1.66 (0.68; 2); and OT, 1.50 (0.70; 1). No significant difference was found between the groups in terms of the median number of mating instances per estrus cycle. The pregnancy rate did not significantly differ between the CON (12/18), PG (14/18), and OT (10/18) groups. The numbers of mares with multiple corpora lutea in the CON, PG, and OT groups were 5/12, 5/14, and 5/10, respectively.

The plasma progesterone concentrations of the pregnant mares were not different among the three groups from day 0 to day 14. On day 28 only, the OT group showed a significantly higher concentration than the PG and CON groups (Fig. 3).

Discussion

To the best of our knowledge, the present study is the first to demonstrate the effects of ecbolics on uterine PMN counts during breeding in Japanese heavy draft mares. Dinoprost is a naturally occurring PG with several pharmacologic effects on the female reproductive system, including stimulation of myometrial activity, inhibition of steroidogenesis by corpora lutea, and luteal regression.

The aim of this study was to examine the effect of prostaglandin F_{2 α} (dinoprost) and oxytocin administration on

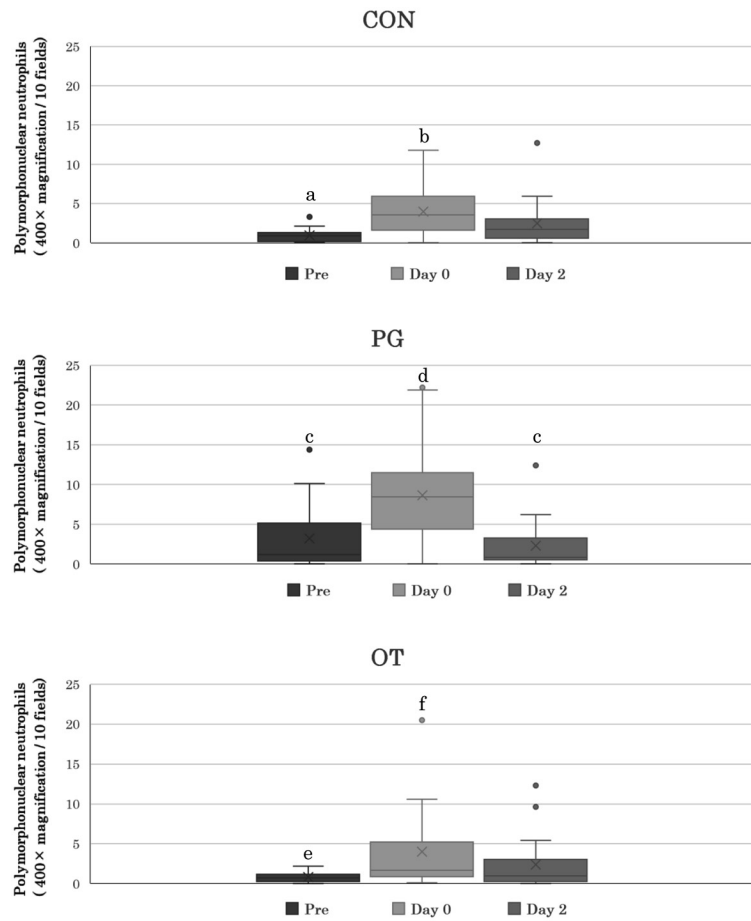


Fig. 1. Median numbers of the polymorphonuclear neutrophils (PMNs) of the groups. Different letters above the bars of each graph indicate significant differences. $P < 0.01$. The dots above the bars shows outliers. CON, control; PG, prostaglandin F_{2α}; OT, oxytocin.

uterine polymorphonuclear neutrophil counts in Japanese heavy draft mares.

The inflammatory reaction that occurs after breeding is a physiological response [6, 35]. Breeding attempts cause inflammation, and this inflammation could enhance uterine clearance [25]. The PMNs increased after breeding on day 0 in our study, which indicates that all the groups experienced endometrium inflammation.

The uterine PMN counts on day 0 in the PG group were higher than those in the other groups. It is possible that the mares in the PG group were bred an average of 1.56 days before ovulation, which is a shorter period than that in the other groups. Nevertheless, there was no significant difference in the PMN counts among the three groups.

The PMN count decreased from days 0 to 2 in all the groups, with no significant differences on days 0 and 2 between the groups; on the other hand, the PG group did show a significant decrease in the number of PMNs from days 0 to 2.

Uterine contraction is essential in removing accumulated fluid and harmful inflammatory products from the uterus [32]. In this study, the percentages of mares with intrauterine fluids or echogenic lines (G2 and G3) in the CON, PG, and OT groups on day 0 were 55.6% (10/18), 77.8% (14/18), and 61.1% (11/18), respectively. This result indicates that 64.8% (35/54) of the Japanese heavy draft mares had persistent intrauterine inflammation for more than 12 hr after breeding. The rate of reduction of intrauterine accumulation from day 0 to day 2 in each group was not statistically significant. However, in the PG and OT groups, approximately 70% of the mares showed reduced intrauterine accumulation as compared with only 50% of mares in the CON group. In addition, there were nine and three mares with an accumulation grade of G3 on day 0 in the PG and OT groups, respectively, whereas there were three and two mares on day 2 in the PG and OT groups, respectively. The percentages of mares with improved intrauterine fluid grades in the PG and OT groups were 66.7% (6/9) and 33.3% (1/3), respectively.

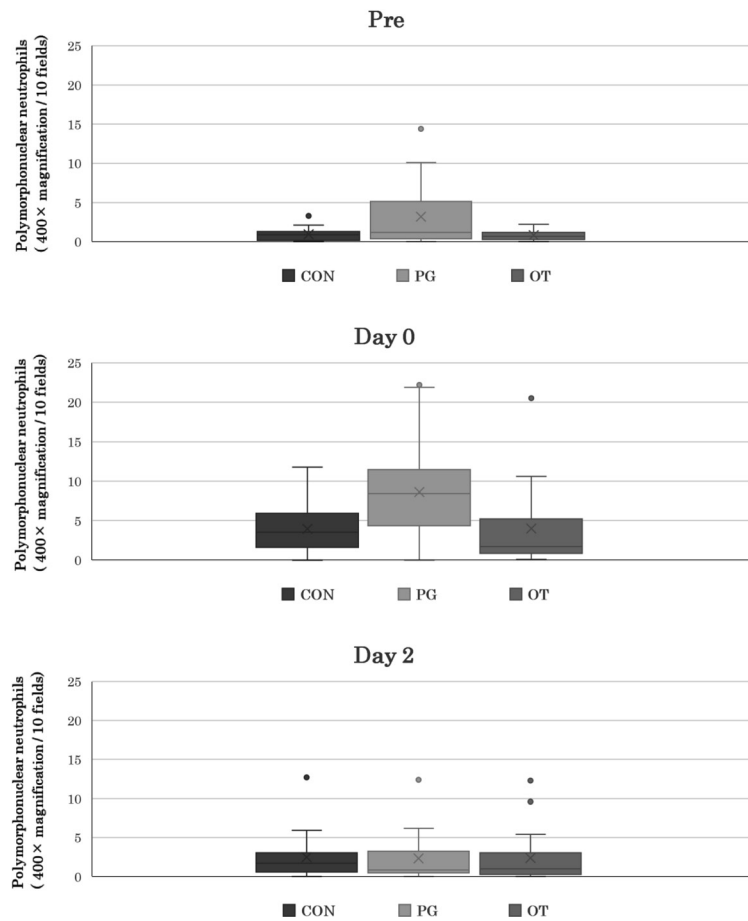


Fig. 2. Median numbers of the polymorphonuclear neutrophils (PMNs) at each time point for all three groups. There were no significant differences observed at any time point among the three groups. The dots above the bars shows outliers. CON, control; PG, prostaglandin F_{2 α} ; OT, oxytocin.

Moreover, in mares with an accumulation grade of G3 on day 2, the median PMN counts in the PG and OT groups were 2.8 and 5.95, respectively. Thus, the uterine accumulation grade was improved more in the PG group than in the OT group. Similarly, the number of uterine inflammatory cells reduced more in the PG group than in the OT group. This demonstrates that dinoprost administration was useful in cleaning contaminating products from the uterine lumen and reducing inflammation.

Ecbolic drugs such as OT and PG can stimulate uterine contractions and help in the excretion of any debris or accumulations from the uterus, which promotes uterine clearance [17]. In comparisons of PG and OT, the effect of PG on myometrial activity has been determined to be longer than that of OT [29, 30]. OT induces high-amplitude uterine contractions for approximately 30 min. PG also induces uterine contractions, but uterine clearance is considerably slower, with contractions lasting for approximately 5 hr [16, 34]. Moreover, PG is used to treat persistent corpora lutea.

In general, the administration of a single dose of 5 to 10 mg of dinoprost in the luteal phase can cause a mare to return to estrus within 2–5 days [4]. In clinical practice, for Japanese heavy draft mares, we use a single dose of 10 mg dinoprost to induce estrus. In this study, half the PG dose was used for estrus induction.

Previous studies of treatment with PG have reported conflicting results. One study concluded that PG administration after ovulation led to a low pregnancy rate, whereas another study concluded that the pregnancy rate was not decreased [22, 28]. The results of our study indicated that the conception rates in the three groups treated with PG were not decreased (CON, 66.7%; PG, 77.8%; and OT, 55.6%). The plasma progesterone concentrations from days 0 to 14 were not significantly different among the three groups. The reason for this is unclear. On day 28, the progesterone concentration in the OT group was higher than those in the PG and CON groups. OT administration in the luteal phase has been reported to disrupt luteolysis [33]. In this study,

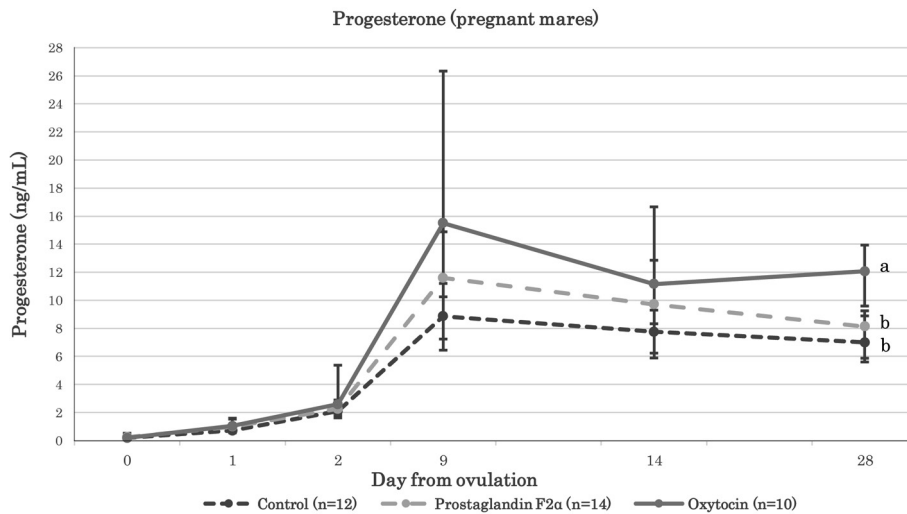


Fig. 3. Median values and 25th and 75th percentiles of plasma progesterone concentrations in pregnant mares. Different letters beside the plots for each group represent significant differences. $P < 0.01$.

OT was administered from day 0 to day 1, although some mares had multiple corpora lutea on day 28.

The multiple ovulation rates in light breed mares have been reported to range from 20.7% to 35.6% [21]. In contrast, in our study, the rates of incidence of multiple corpora lutea in mares on day 28 were 41.6%, 35.7%, and 50% in the CON, PG, and OT groups, respectively. Thus, the rate in the OT group was considerably higher than that previously reported. This might be the reason for the higher progesterone concentrations in the OT group than in the other groups.

In general, a serum progesterone concentration of 4 ng/ml is considered normal and adequate to maintain mare pregnancy in the first trimester [12, 20]. The median plasma progesterone concentrations in the pregnant mares in all the groups on day 28 in this study were higher than 6 ng/ml. Therefore, the results of the present study suggest that the pregnant mares in all the groups produced sufficient progesterone to maintain pregnancy.

In conclusion, the administration of dinoprost at ovulation, at half the dose for estrus induction, did not have a negative effect on the pregnancy rates and reduced the uterine polymorphonuclear neutrophil counts in the Japanese heavy draft horses in this study.

Acknowledgments

This work was supported by the Tokachi Federation of Agricultural Cooperatives. We thank Dr. Tetsu Saito for the great advisory support for studies on breeding Japanese heavy draft horses. The authors would like to thank Dr. M. Matsui and Dr. N. Yamagishi for advice on this research.

The authors also thank Mr. S. Kato, Ms. Y. Tachikawa, Mr. S. Ohno, Mr. N. Ohno, and Mr. K. Tomeda for their support of the field work. The authors would also like to thank their colleagues, Mr. M. Ide, Mr. Y. Shimizu, Mr. K. Hosono, Mr. M. Fukunaka, Mr. S. Togashi, Mr. S. Kawaguchi, Mr. M. Ootsuki, Mr. K. Mitsuhashi, Ms. Y. Nihei, the staff of the Tokachi Agricultural Mutual Aid Association, and all breeding farmers of heavy draft horses.

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