Response to estrus induction with abortion treatment in microminipigs on different days after insemination

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Abstract. In microminipigs, estrus induction with abortion treatment, which is typically performed between 25 and 40 days after mating, is not always successful. Thus, the authors hypothesized that it may be more difficult to induce estrus by treating microminipigs approximately 40 days after mating. Accordingly, in this study, estrus induction was performed with abortion treatment in four microminipigs as follows: 0.3 mg of cloprostenol, a prostaglandin F2-alpha analog, was administered (day 0); after 24 h, 0.15 mg of cloprostenol and 250 IU of equine chorionic gonadotrophin were administered intramuscularly and simultaneously (day 1); after 96 h, 120 IU of human chorionic gonadotropin was injected intramuscularly (day 4). These treatments were compared at two different stages of pregnancy: early treatment (26.5 ± 0.7 days) and late treatment (38.3 ± 0.8 days). In the early treatment, all four microminipigs exhibited estrus on day 5, whereas in the late treatment, estrus was observed clearly in only two pigs on day 6 and slightly in 1 pig on day 10, whereas it was unclear in 1 pig. These results suggest that it is difficult to induce estrus with abortion treatment in microminipigs at approximately 40 days after mating.

Key words: Abortion treatment, Estrus induction, Microminipigs

Estrus induction with abortion treatment, involving single or dual administration of prostaglandin F2-alpha (PGF2α) during the 25–40 day period after mating, is a technique commonly used in domestic pigs [1]. In contrast to difficulties in regression treatment of the corpus luteum in both pregnant and pseudopregnant pigs readily responds to PGF2α, and estrus is induced promptly approximately 7 days after the treatment [2, 3]. By adopting this method, synchronized estrus can be accomplished in donor and recipient pigs, which can then be prepared for somatic cell nuclear transfer, embryo collection in specific stages, and other scientific purposes [4]. However, miniature pigs are different from domestic pigs not only in size, but also in physiology; therefore, techniques developed for domestic pigs are not always adaptable to miniature pigs.

Miniature pigs are expected to become a novel laboratory animal for translational research [5, 6]. One reason for this is that the pig species is similar to humans anatomically and physiologically [4–6]. Another reason is that miniature pigs are smaller and easier to handle than domestic pigs, which can weigh up to 200–300 kg in adulthood [7–9]. Microminipigs weigh approximately 10 kg at 9 months of age, approximately 20–30 kg in adulthood [8, 9], and are approximately one-half the size of other miniature pigs and comparable to beagle dogs in size [7].

We have observed that estrus induction in microminipigs with abortion treatment between 25 and 40 days after mating was not always successful; consequently, we hypothesized that it is more difficult to induce estrus in microminipigs at approximately 40 days after mating via treatment. To support the aforementioned hypothesis, we performed estrus induction with abortion treatment on different days of pregnancy, that is, serial administration of cloprostenol (the day of administration was recorded as day 0), cloprostenol, and equine chorionic gonadotrophin (eCG) on day 1 and human chorionic gonadotropin (hCG) on day 4; early treatment (26.5 ± 0.7 days after insemination); and late treatment (38.3 ± 0.8 days after insemination) in microminipigs (A–D).

Using the early treatment protocol, all four microminipigs exhibited estrus on day 5 (Table 1). Estrus was clearly exhibited in pigs A, C, and D and was weak in pig B. In addition, fetus-like delivery was observed in pig D on day 1 (three fetuses). In the late treatment protocol, estrus was clearly observed in pigs A and C on day 6 and only slightly in pig B on day 10, whereas it was unclear in pig D. Fetus-like delivery was not confirmed after the treatment in pigs A or C; however, two fetuses and one fetus were confirmed on day 2 in pigs B and D, respectively.

In pigs receiving early treatment, the average number of follicles and their diameters —confirmed using magnetic resonance imaging (MRI)— were within normal range [10]. The numbers of follicles...
in pigs A–D were three, five, 10, and four, respectively; the follicle diameters in pigs A–D were 4.3 ± 0.5 mm, 3.8 ± 0.2 mm, 5.1 ± 0.3 mm, and 4.4 ± 0.0 mm, respectively. Ovulation was confirmed in all pigs on day 6.

In pigs receiving late treatment, the average number of follicles was small; however, their diameters were within the normal range [9]. The numbers of follicles in pigs A–C were one, one, and three, respectively; their diameters were 4.2 mm, 4.6 mm, and 4.2 ± 0.2 mm, respectively. In pig D, no follicle was observed from day −3 to day 10. Ovulation was confirmed on day 7 in pigs A and C, and on day 11 in pig B. There were no differences in the average number of follicles or follicle diameters between pigs receiving early and late treatment.

Changes in plasma progesterone and estradiol concentrations between pigs receiving early and late treatments were compared. After administration of cloprostenol, plasma progesterone concentrations rapidly decreased and then increased from day 6 onward in both treatments (Fig. 1). The plasma progesterone concentrations in pigs receiving the early and late treatments on day 0 were 22.4 ± 7.7 ng/ml and 11.5 ± 6.5 ng/ml, respectively; the difference, however, was not statistically significant. The plasma estradiol concentration in microminipigs receiving the early treatment appeared to have increased on day 4 (61.1 ± 42.1 pg/ml), but it was not statistically different from that in microminipigs receiving the late treatment (11.1 ± 3.1 pg/ml). Our results suggested that the responsiveness to estrus induction treatment decreased as the duration of pregnancy increased, and estrus induction was not as effective in microminipigs as it is in domestic pigs when performed at approximately 40 days of pregnancy. We do not have sufficient evidence to ascertain the reason for the lower responsiveness in microminipigs to estrus induction with abortion treatment at approximately 40 days of pregnancy; therefore, additional studies are needed in this regard. However, we could conclude that because the regression of the corpus luteum responded to cloprostenol and the decrease in plasma progesterone concentration was not different between the early and late treatments a difference in estrus

| Group       | Pig | Duration of pregnancy (days) | Delivery (day) | Induced estrus (day) | Estrus behavior | Ovulation confirmed (day) | Number of follicles | Follicular diameter (mm) |
|-------------|-----|-------------------------------|----------------|----------------------|-----------------|---------------------------|---------------------|--------------------------|
| Early treatment | A   | 25                            | -              | 5                    | Clear           | 6                          | 3                   | 4.3 ± 0.5               |
|              | B   | 27                            | -              | 5                    | Weak            | 6                          | 5                   | 3.8 ± 0.2               |
|              | C   | 28                            | -              | 5                    | Clear           | 6                          | 10                  | 5.1 ± 0.3               |
|              | D   | 26                            | 1 (3)          | 5                    | Clear           | 6                          | 4                   | 4.4 ± 0.0               |
| Late treatment | A   | 37                            | -              | 6                    | Clear           | 7                          | 1                   | 4.2                    |
|              | B   | 40                            | 2 (2)          | 10                   | Clear           | 11                         | 1                   | 4.6                    |
|              | C   | 39                            | -              | 6                    | Slight          | 7                          | 3                   | 4.2 ± 0.2               |
|              | D   | 37                            | 2 (1)          | -                    | Unclear         | -                          | -                   | -                       |

1 Day on which a fetus or fetus-like delivery was observed. The number of fetuses delivered is in parentheses. 2 Days after administration of cloprostenol. 3 Total number of follicles counted using magnetic resonance imaging immediately before ovulation. 4 Data are shown as mean ± standard error of the mean (SEM).

Fig. 1. Changes in plasma progesterone and estradiol concentrations after the administration of cloprostenol in microminipigs (n = 4) receiving early (●) and late (○) treatment. The data are expressed as mean ± standard error of the mean (SEM). There were no statistically significant differences in plasma progesterone and estradiol concentrations from day −1 to day 10.
induction maybe induced by lower responsiveness to eCG or hCG, without any influence on follicular development as the duration of pregnancy increased.

Our results also suggested that estrus was synchronized approximately 5 days after the first administration of cloprostenol, when the treatment was performed on earlier days of pregnancy. Although a modification of the protocol may be needed to determine the exact duration of the treatment for estrus induction and appropriate reagent doses, our results may help establish estrus synchronization protocols in microminipigs.

Microminipigs are expected to become a novel experimental animal model; therefore, more experimental techniques and tools are necessary. Veterinary techniques involving animal reproduction have been developed in domestic pigs, and some are adaptable to microminipigs. However, microminipigs are not only different in size but also different in physiology from domestic pigs. Consequently, the modification of techniques used in domestic pigs, as well as the development of new experimental techniques for microminipigs, are needed to expand their use as experimental animals and promote translational research.

Methods

Animals

The experiment was approved by the Committee for Animal Research and Welfare of Gifu University (Gifu, Japan; #15090). Four female microminipigs (pigs A–D) were purchased from Fuji Micra Inc. (Fujinomiya, Shizuoka, Japan); only pig C had experienced labor once, whereas the others had not. The average ages of the microminipigs receiving early and late treatments were 19.8 ± 0.9 months and 19.6 ± 2.2 months, respectively. The average body weights of the microminipigs receiving early and late treatments were 19.0 ± 1.6 kg and 18.3 ± 1.5 kg, respectively.

The animals were housed in a controlled room (4 m × 2.8 m pen, with > 1 m² space per animal) at a temperature of 24°C (range, 21–27°C), humidity 70–80%, and a 12-h light/12-h dark cycle starting at 0600 h. The pigs were fed the recommended volume per body weight of Harb Kodakara 74 (Marubeni Nisshin Feed, Tokyo, Japan) and provided free access to water.

Preparation of pregnant pigs: estrus detection, ovulation confirmation via MRI, artificial insemination, and pregnancy determination

Estrus was detected via behavioral observations, examination for the presence of vulval swelling, and back-pressure testing. Soon after estrus detection, ovarian MRI was performed to confirm the presence of follicles. When follicles were present, artificial insemination was performed in sedated pigs. Estrus confirmation and MRI were performed again on the next day, and artificial insemination was repeated if follicles remained. The pregnancy date was set as the day that ovulation was confirmed as the basis of the disappearance of previously existing follicles on MRI [10, 11].

For MRI, microminipigs were sedated using an intramuscular injection of 0.015 mg/kg medetomidine (Dorbene vet; Kyoritsu Seiyaku Corporation, Tokyo, Japan), 0.15 mg/kg midazolam (Dormicum injection 10 mg; Astellas, Tokyo, Japan), and 0.12 mg/kg butorphanol (Vetorphale; Meiji Seika Pharma, Tokyo, Japan) [6]. A 0.4 Tesla MRI system (Hitachi Medical Corporation, Chiba, Japan) equipped with a QD knee coil for signal reception was used for ovarian imaging. T₂-weighted fast spin-echo (FSE) acquisitions were obtained in the transaxial and sagittal planes (repetition time/echo time, 12,000 ms/104 ms; flip angle, 90°; field of view, 180 mm; slice thickness, 3 mm). MRI was performed over the area within 180 mm of the hindmost teat. The acquired images were analyzed using OsiriX software v. 4.1.2 (Pixmeo Sàrl, Bernex, Switzerland).

For insemination, semen was collected manually from a trained male microminipig. The collected semen was diluted using a Modena extender, such that the sperm concentration was 1 × 10⁸/ml, and 15 ml of semen was inseminated into the cervix with a spiral tip catheter.

Approximately 21 days after insemination, pregnancy was provisionally determined for sows that did not exhibit estrus behavior. After a few days, pregnancy was diagnosed provisionally using portable ultrasonography (Tringa LinearVET, Esaote) and then diagnosed definitively at day –1 by another ultrasonography (Noblus, Hitachi).

Estrus induction treatment

Estrus induction was scheduled according to a report by Kurume et al. [3]. First, the pregnancy of microminipigs was confirmed, and then 0.3 mg of cloprostenol (Resipron-C, ASKA Animal Health, Tokyo, Japan) was injected intramuscularly as an abortion treatment. The day that cloprostenol was administered was defined as day 0. Twenty-four hours after the first treatment, 0.15 mg of cloprostenol and 250 IU of eCG (Gonatropin 3000, ASKA Animal Health, Tokyo, Japan) were administered intramuscularly and simultaneously (day 1). Ninety-six hours after the treatment (day 4), 120 IU of hCG (Serotropin, ASKA Animal Health, Tokyo, Japan) was also injected intramuscularly.

Early and late treatments

The abortion treatment was randomly repeated on the same pigs for different durations during their pregnancies. In the early treatment, cloprostenol was administered at 26.5 ± 0.7 days of pregnancy, while in the late treatment, it was administered at 38.3 ± 0.8 days of pregnancy. More than one estrus cycle was confirmed before another trial was performed.

Observation of induced estrus

After the abortion treatment, backpressure testing and vulva observation were performed from day 1 to day 10. MRI scans were acquired from the next day after hCG administration (day 5) to the day of ovulation when follicular images disappeared, and the number and diameter of ovarian follicles were monitored. However, in the late treatment, pig D did not exhibit clear signs of estrus and, although ovarian images were acquired until day 10, no follicle was observed.

Progesterone and estradiol assays

Changes in plasma progesterone and estradiol concentrations were monitored. Blood samples, 2 ml in volume, were collected daily from the jugular vein from day –1 to day 10. Blood samples were placed in tubes containing EDTA-2N, and the plasma was immediately separated using refrigerated centrifugation (4°C, 1,000 × g, 15 min) and stored at –80°C until the determination of hormone
concentrations.

Plasma progesterone and estradiol concentrations were determined using enzyme immunoassay (EIA) (Cayman Chemical Company, Ann Arbor, MI, USA) [10]. An EIA for progesterone was performed three times, and the intra- and inter-coefficients of variation (CVs) were 5.9% and 6.7%, respectively. An EIA for estradiol was performed once, and the intra-CV was 7.3%.

**Statistical analysis**

Differences in the numbers and diameters of follicles before ovulation, as well as plasma progesterone and estradiol concentrations on each day, from day −1 to day 10 between the early and late treatment, were statistically compared using Welch’s t test. The analysis was performed using a spreadsheet (Excel 2010, Microsoft, Redmond, WA, USA) with the ad-in software Statcel 3 (OMS Publishing, Saitama, Japan). Differences with P < 0.05 were considered statistically significant. All data are expressed as mean ± standard error of the mean (SEM).

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