Pregnancy Rate and Blood Progesterone Concentrations on the Previous Day and the Day of Frozen Embryo Transfer in Parous Recipient Cows of Japanese Black

Masahiko NISHIGAI1,2), Hideo KAMOMAE1), Tomomi TANAKA1) and Yoshihiro KANEDA1)

1)Laboratory of Veterinary Reproduction, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509,
2)Nasu ET Institute, 7-10, Shimakata, Kuroiso, Tochigi 329-3152, Japan

Abstract. Blood progesterone (P) concentrations were determined on the previous day (6 days after onset of estrus; day 6) and the day of embryo transfer (7 days after onset of estrus; day 7) in recipients of Japanese Black beef cow to clarify the correlation between luteal function and pregnancy rate in the stepwise transfer method of frozen Japanese Black embryos. When the blood P concentrations on both day 6 and day 7 in the bovine recipients increased, the pregnancy rate also tended to increase. Pregnancy rates in the recipients with blood P concentration <2.5 ng/ml and ≥2.5 ng/ml on day 6 were 38.6% (17/44) and 60.7% (34/56), respectively, showing a significant (P <0.05) difference. The pregnancy rate in the recipients with blood P concentration <2.5 ng/ml on day 7 was 35.7% (10/28), while the corresponding rate was 56.9% (41/72) in the recipients with blood P concentration of ≥2.5 ng/ml, showing no significant difference. The pregnancy rate in the recipients in which blood P concentration was <2.5 ng/ml on day 6, and increased to ≥2.5 ng/ml on day 7 was low at 45.0% (9/20), while the rate in the recipients which showed blood P concentration of ≥2.5 ng/ml on both day 6 and day 7 was as high as 61.5% (32/52). The relationship between the blood P concentration and luteal development on day 6 corresponding to pregnancy rate was investigated by dividing corpus luteum (CL) into the following three groups by rectal palpation findings: favorably developing, poorly developing and cystic groups. The mean blood P concentration was 2.8 ng/ml in the favorably developing CL, whereas the levels were as low as 2.0 ng/ml and 2.1 ng/ml in the poorly developing CL and cystic CL, respectively. The pregnancy rate for the favorably developing CL was 55.3% (42/76) which tended to be higher than for the poorly developing CL (44.4% (8/18)), and cystic CL, (16.7% (1/6)), respectively. These observations showed that a high pregnancy rate is achieved by selecting cows with blood P concentration of ≥2.5 ng/ml on day 6 and day 7 of embryo transfer.

Key words: Bovine frozen embryo transfer, Plasma progesterone, Corpus luteum quality, Pregnancy rate.

In recent years, bovine frozen embryo transfer has become popular among Japanese Black cow breeding farmers owing to the improvement of adequate freezing techniques and the sequential rise of pregnancy rates [1].

The frozen embryo is transferred by the following three methods: (1) the direct transfer method [2] in which a thawed embryo is transferred without removal of the cryoprotectant; (2) the one-step
straw method [3] in which an embryo, frozen with the use of glycerol as cryoprotectant, and glycerol are first diluted in the sucrose solution layers that are formed in the same straw before the embryo is directly transferred; (3) the stepwise method [4] in which a thawed embryo is transferred following the gradual removal of cryoprotectant outside the straw and confirmation of the survivability and morphological quality of the embryo.

In one of the popular techniques for frozen embryo transfer, the stepwise method, the bovine candidate for recipients is subjected to rectal palpation on the day prior to embryo transfer (6 days after onset of estrus; day 6) to assess the aptitude for transfer by examining the growth of the corpus luteum and the state of accessory reproductive organs, after which the transfer of the frozen-thawed embryo is performed on the next day (7 days after onset of estrus; day 7). However, it is often difficult to assess the luteal growth, i.e. judge the luteal function precisely by rectal palpation.

Some reports have shown the blood progesterone (P) concentration to be significantly involved in the completion and maintenance of bovine pregnancy [5, 6]. In embryo transfer, if blood and milk concentrations are determined in candidate recipient cows before transfer and the optimal recipient cows can be selected, the embryo transfer performed under such conditions should lead to high pregnancy rates. The correlation between the P concentrations and the rate of pregnancy have been investigated by determining blood P concentrations on day 6 [7] and day 7 [8–11] in bovine recipients, but no positive relationship between them has as yet been revealed. With the direct transfer and the one-step straw methods, there is the possibility that degenerated embryos will be transferred, because these methods allow no confirmation of the survivability or quality of post-thawing embryo [4]. For this reason, frozen-thawed embryo transfer was performed by the stepwise method in this experiment.

This experiment was conducted to clarify the correlations between blood P concentrations on day 6 and/or day 7 and rates of successful pregnancy with attention to the state of luteal development on day 6 in Japanese Black parous cows.

Materials and Methods

Bovine recipients

One hundred Japanese Black parous cows were used as recipients. They were assessed for corpus luteum (CL) by palpation per rectum by one veterinarian 6 days after the second and/or third postpartum estrus, which occurred ≥60 days after calving. All the cows were found to have no abnormalities in reproductive organs.

Determination of blood progesterone concentration

Blood samples were collected from the jugular vein of each recipient on day 6 and day 7. Immediately after blood collection, the samples were centrifuged, and the heparinized plasma was collected. The plasma samples were stored, frozen at -20°C, until assay. Plasma P concentrations were determined by radioimmunoassay [12]. The sensitivity of the assay was 0.5 pg/tube, and intra- and inter-assay coefficients of variation were 5.6% and 9.7%, respectively.

Examination and classification of corpus luteum

The CL was examined by palpation per rectum on day 6. According to previous reports [8, 13, 14], they were classified as favorably developing CL, poorly developing CL, and cystic CL. A favorably developing CL has a major axis of 1.5 cm or more and a clear crown, a poorly developing CL has a major axis of less than 1.5 cm and a clear crown, and a cystic CL is associated with palpable retention of luminal fluid and an obscure crown.

Collection, freezing and thawing of embryos

The embryos used for study were collected, frozen and thawed according to previously reported methods [15], as follows. Intramuscular administration of follicle-stimulating hormone (FSH) (Antrin, Denka Pharmaceuticals, Co., Ltd., Kanagawa) to the Japanese Black cow donors was started on day 10 (the day of estrus onset was designated day 0), with decreasing doses for 3 consecutive days, and PGF2α (Veterinary Pronalgon F injection, UpJohn Pharmaceuticals, Ltd., Tokyo) of 30 mg dinoprostone (20 mg in the morning and 10 mg in the evening) was injected intramuscularly to induce superovulation. The donors were inseminated at 12 and 24 h after the onset of estrus, and the embryos were recovered by non-surgical uterine
flushing on day 7. The recovered embryos were classified according to Lindner and Wright [16], and the embryos at late morula to blastocyst stage, which were evaluated as excellent or good, were subjected to two-step glycerol equilibration according to the method of Seidel and Elsden [17], followed by cryopreservation.

The embryos were thawed and glycerol was removed thereafter according to the method of Seidel and Seidel [18]. Subsequently, a thawed embryo was evaluated in the post-thawed condition for morphological quality in phosphate buffer solution containing 0.4% bovine serum albumin according to the Elsden’s classification [19]. The embryos evaluated as being excellent or good were divided into three developmental stages, the late morula, early blastocyst and blastocyst.

A thawed embryo was aspirated into 0.25 ml straw and the straw was attached to an embryo transfer gun (Cassou embryo transfer gun for heifers, Cassou Corp., France). Then, the transfer gun was wrapped in a thermal paper towel and transported to the place of transfer in a container.

**Embryo transfer method**

As reported previously [15], the vulva was washed with a disinfect solution for external use (Prepodyne Scrub, Santen Pharmaceuticals, Co., Ltd., Osaka) and a vaginal speculum that had been immersed in a disinfectant solution (Osvan solution, Takeda Pharmaceutical Industries, Co., Ltd., Osaka) was inserted into the vagina to dilate it for insertion of the transfer gun deep into the vagina to the external os of the uterus without touching the vaginal wall. The vaginal speculum was pulled out immediately after insertion of the transfer gun and the frozen-thawed embryo was transferred.

The site of transfer was at the central portion of the uterine horn ipsilateral to the ovary with CL and a single embryo was transferred by means of the same equipment by the person in the same procedure. In order to securely immobilize recipients and to smoothly conduct the transfer, each recipient underwent intramuscular injection of 20 mg of the Xylazine (2% Celactal Injection, Bayer, Tokyo) and caudal epidural anesthesia with 3 ml of 2% lidocaine hydrochloride (2% Xylocaine Injection, Fujisawa Pharmaceuticals, Co., Ltd., Osaka) 5~15 min before embryo transfer.

**Diagnosis of pregnancy**

The recipients, in which estrus did not recur, were examined for pregnancy by rectal palpation 40~50 days after the embryo transfer.

**Statistical analysis**

Correlations between blood P concentrations on day 6 and day 7 of transfer and pregnancy rate (Table 1~Table 3) were calculated by chi-square test and Fisher’s exact probability test [20]. With regard to the luteal development according to the blood P levels on day 6 corresponding to the attainment of pregnancy and testing the difference in the mean P level (Table 4), significant difference was tested by GLM procedure of the general linear models procedure of the Statistical Analysis System (SAS) [21]. Statistical significance was considered to be present when the probability value was below 5% (P<0.05).

**Results**

Blood P concentrations on day 6 ranged from 0.2 to 6.0 ng/ml with a mean of 2.6 ± 1.0 (SD) ng/ml and the corresponding levels on day 7 ranged from 0.1 to 7.1 ng/ml with a mean of 3.2 ± 1.4 ng/ml in the recipients. A significant difference was found in the mean levels between day 6 and day 7 (P<0.05).

The blood P levels of bovine recipients were divided into the following 4 groups to assess relationships between the blood P concentration and pregnancy rate: <1.5, 1.5 ~<2.5, 2.5 ~<3.5 and ≥3.5 ng/ml. The results that are closely related to the blood P concentration and pregnancy rate were obtained on day 6 and day 7. The pregnancy rate was comparatively assessed according to these 4 groups of blood P concentration in the present study. The pregnancy rate tended to increase with the blood P concentration on day 6, the day prior to transfer increased. The pregnancy rate was as high as 60.7% (34/56) in the 2 groups with blood P concentrations of ≥2.5 ng/ml, while the rate was 38.6% (17/44) in the other 2 groups with blood P concentrations <2.5 ng/ml. There was a significant difference in pregnancy rate between ≥2.5 ng/ml and <2.5 ng/ml groups (P<0.05).

On day 7, the pregnancy rate also tended to increase with the change in the blood P concentration from <1.5 ng/ml to 2.5~<3.5 ng/ml. The pregnan-
Frequency rate tended as high as 56.9\% (41/72) in the 2
groups with blood P concentrations of ⟩= 2.5 ng/ml, while the rate was 35.7\% (10/28) in the other 2
groups with blood P concentrations <2.5 ng/ml. However, there was no significant difference be-
tween ʾ= 2.5 ng/ml and <2.5 ng/ml groups (Table 2).

Table 3 shows correlations of blood P concentra-
tions on day 6 and day 7 with the pregnancy rate.
Although the pregnancy rate was only 45.0\% (9/20)
in the recipients with blood P concentrations <2.5 ng/ml on day 6 and ʾ= 2.5 ng/ml on day 7, pregnancy rate was high at 61.5\% (32/52) in the recipients with blood P concentrations of ʾ= 2.5 ng/ml on both day 6 and day 7.

Table 4 shows correlations of the blood P concentra-
tion in relation to luteal development on day 6 and the results of pregnancy in the recipients. The mean blood P concentration was 2.7 ± 1.0 ng/ml in the cows with favorably developing CL, 2.2 ± 1.2 ng/ml in those with poorly developing CL and 2.1 ± 0.7 ng/ml in those with cystic CL. Pregnancy rates were 55.3\% (42/76), 44.4\% (8/18) and 16.7\% (1/6) in the cows with favorably developing CL, poorly developing CL and cystic CL, respectively. Thus, the pregnancy rate tended to be low in the cows with poorly developing CL and those

Discussion

Continuous secretion of P from the ovary is known to be necessary for maintenance of bovine pregnancy [5, 6]. In the present study, the pregnancy rate tended to increase as the blood P concentration on both the previous day and the day of embryo transfer increased, suggesting that the blood P concentrations on the previous day and the day of embryo transfer influence the pregnancy rate. With regard to the relationship between the blood P concentration on the day of embryo transfer and pregnancy rate in bovine recipients, Niemann et al. [8] investigated frozen embryo trans-
fer, and Remsen and Roussel [9] did the same for fresh embryo transfer. These groups reported the pregnancy rate to be highest in recipients with P concentrations ranging from 2 to 5 or from 2 to 6 ng/ml. In addition, Niemann et al. [8] and North-
ey et al. [11] showed that pregnancy rates declined when blood P concentrations were <2.0 ng/ml in bovine recipients. Stubbings and Walton [10] have reported pregnancy rates to be low, when the blood P concentrations on the day of embryo transfer were ʾ= 1.0 ng/ml in fresh embryo transfer and
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3.0 ng/ml in frozen embryo transfer in heifers, in which embryos were non-surgically transferred. In the present study, the pregnancy rate was 60.7% and 56.9% in recipients with blood P concentrations of 2.5 ng/ml on day 6 and day 7, respectively. These results are essentially consistent with the results of previous reports [8–11]. The pregnancy rate was 61.5% in the recipients with blood P concentrations of 2.5 ng/ml on both day 6 and day 7, while the pregnancy rate was 45% in the recipients which showed blood P concentrations of <2.5 ng/ml on day 6 and 2.5 ng/ml on day 7. From these results, it was estimated that the blood P concentration must attain 2.5 ng/ml in the early period within 6 days after onset of estrus for the pregnancy rate to be improved.

It is considered possible in individual cows to estimate the stage of the estrous cycle depending on the morphology of CL [22], and to evaluate luteal function to some extent from the condition of luteal tissue formation (growth of CL or luteinization). Based on these estimations, bovine recipients are classified according to the CL morphology palpated per rectum, and a transfer is carried out giving priority to optimal recipients. In the present study, CL were classified into 3 groups, i.e. the favorably developing, poorly developing and cystic groups, according to methods described in previous reports [8, 13, 14] by palpation per rectum on the day prior to embryo transfer, and correlations of the 3 CL groups with blood P concentrations and pregnancy rate were investigated. The mean blood P concentration was 2.7 ± 1.0 ng/ml in the cows with favorably developing CL, while those in the cows with poorly developing CL and cystic CL were 2.2 ng/ml and 2.1 ng/ml,

<table>
<thead>
<tr>
<th>Blood P concentration (ng/ml)</th>
<th>Favorable</th>
<th>Poor</th>
<th>Cystic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1.5</td>
<td>1/7 (14.3)</td>
<td>2/4 (50.0)</td>
<td>0/1 (0)</td>
<td>3/12 (25.0)</td>
</tr>
<tr>
<td>1.5~&lt;2.5</td>
<td>10/20 (50.0)</td>
<td>4/8 (50.0)</td>
<td>0/4 (0)</td>
<td>14/32 (43.8)</td>
</tr>
<tr>
<td>2.5~&lt;3.5</td>
<td>20/35 (57.1)</td>
<td>2/6 (33.3)</td>
<td>1/1 (100)</td>
<td>23/42 (54.8)</td>
</tr>
<tr>
<td>≥3.5</td>
<td>11/14 (78.6)</td>
<td>–</td>
<td>–</td>
<td>11/14 (78.6)</td>
</tr>
<tr>
<td>Total</td>
<td>42/76 (55.3)</td>
<td>8/18 (44.4)</td>
<td>1/6 (16.7)</td>
<td>51/100 (51.0)</td>
</tr>
</tbody>
</table>

Mean P = 2.7 ± 1.0 ng/ml. 

a) Major axis of ≥1.5 cm with a clear crown.
b) Major axis of <1.5 cm with a clear crown.
c) Associated with palpable retention of luminal fluid inside the cavity.
d) Number of pregnant cows / Number of recipients.
e) Pregnancy rate (%) in parenthesis.
f) Mean ± SD.
respectively. The pregnancy rate was as high as 55.3% in the favorably developing CL group, but 44.4 and 16.7% in the poorly developing CL and cystic CL groups, respectively.

With regard to the relationship between luteal development and pregnancy rate, some reports have indicated no direct relationship [8–10, 13, 14, 23], and another report has shown that the pregnancy rate was high when CL with a major axis of 1.5–2.0 cm were present in recipients without concomitant follicle development [24]. Humbolt et al. [25] have also shown the pregnancy rate to be only 27.3% in cows with 1.0–2.0 cm CL in size as compared to 47.6% in those with \( \geq 2.0 \) cm CL in size. The present results are comparable to those of Humbolt et al. [25]. The blood P concentration may correlate closely with the attainment of pregnancy, which depends on the development of CL.

The CL with cavity 0.7–1.0 cm or more in size are called cystic CL in cattle [26–28]. The pregnancy rate in the cows with cystic CL was only 16.7% (1/6) in the present study. It is noteworthy that the mean blood P concentration in non-pregnant cows was also low at 1.8 ± 0.5 ng/ml, while the blood P concentration was 3.2 ng/ml in one impregnated cow with cystic CL on the day prior to transfer. Hasler et al. [13] reported that the pregnancy rate in cows with cystic CL was comparable to that in cows with favorably developing CL in surgical transfer of fresh embryos. Some reports have also shown the blood P concentration in cows with cystic CL to be lower than that in cows with normal luteinization [28, 29], while others have shown the blood P concentration in cows with cystic CL to be the same as that in cows with normal luteinization [30, 31]. Taking these observations into consideration, P-secreting function is considered to depend on the quantity of luteal tissue, and cystic CL does not impair pregnancy if the quantity of luteal tissue is good enough and the P-secreting function is normal, while the pregnancy rate is impaired if the quantity of luteal tissue is small and P secretion is subnormal.

As discussed above, the rectal examination of luteal development before embryo transfer provided information for luteal function in most bovine recipients. So, the necessity of the rectal examination for selecting recipients was confirmed. However, Ott et al. [32] reported the blood P concentration to be low in 18% of the cows that were evaluated as having normally developing CL by rectal examination. In the present study, the percentage of the cows in which the blood P concentrations were <2.5 ng/ml was 35.5% of cows having favorably developing CL, supporting the results of Ott et al. [32]. Based on these observations, the maneuver of determining the blood P concentration is effective for precisely evaluating luteal function. From this viewpoint, improvement of a simple method for determining the P concentration to select optimal bovine recipients is eagerly awaited. Blood P concentrations and pregnancy rates were low in the cows with poorly developing CL and those with cystic CL. From these observations, improvement in the pregnancy rate is anticipated with the application of treatments designed to raise blood P concentrations in these cows designated for use as recipients.

The following conclusions are drawn from the results of this study: (1) the pregnancy rate increased as the blood P concentration increased in bovine recipients in non-surgical transfer of frozen-thawed embryos and (2) the pregnancy rate is considered to be improved by selecting recipient cows whose blood P concentration are \( \geq 2.5 \) ng/ml on both the previous day and the day of transfer. These results suggest that elevation of luteal function at a relatively early period, i.e. within 6 days after onset of estrus, may improve the pregnancy rate.

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