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The Development and Prevalence of the Transfer Technique for Frozen-Thawed Embryos of Japanese Black Beef Cattle in Tochigi Prefecture

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Abstract. The conditions of embryo transfer by the stepwise method, in which frozen-thawed embryos are transferred on day 7 (day 0=onset of estrus) , were investigated with the aim of increasing pregnancy rates in frozen-thawed embryo transfer. The use of a vaginal speculum to prevent bacterial infection when passing an embryo transfer gun through the vagina yielded a pregnancy rate equal to or higher than that with application of a sheath cover to the transfer gun. Administration of a sedative, xylazine, to recipient cattle for preventing movement at the time of embryo transfer improved the pregnancy rate. The influence of the time from thawing of frozen embryos to transfer and of the transportation of the recipient by truck upon pregnancy rate was investigated. Embryo transfer within 60 minutes after aspiration into a straw or transportation of the bovine recipient, 1.5 hours each way before and after transfer, had no influence on pregnancy rate. Relations of the embryonic developmental stage and morphological quality after thawing of frozen embryos to pregnancy rate were investigated in recipients of nulliparous Holstein heifers. The pregnancy rate increased as the embryonic developmental stage advanced from compacted morula, early blastocyst, and blastocyst in that order. The pregnancy rate obtained with blastocyst stage embryos was significantly ($P<0.05$) higher than that with compacted morula stage embryos, and there was no significant difference in pregnancy rates between excellent morphological quality and good morphological quality for compacted morula stage embryos. When correlation of luteal function and pregnancy rate was investigated in bovine recipients, pregnancy rate showed a tendency to increase with increasing blood progesterone (P) concentration on the day before (on day 6 after estrus) and the day of embryo transfer. The pregnancy rate in bovine recipients, which showed a blood P concentration of ≥2.5 ng/ml on the day before embryo transfer, was significantly ($P<0.05$) higher than that in those with a blood P concentration of <2.5 ng/ml. Pregnancy rate showed a tendency to increase with decreasing blood estradiol-17β (E_2) concentration on the day of embryo transfer. Activation of luteal function by administration of human chorionic gonadotropin (hCG) in cycling cattle was investigated for its effect on increasing pregnancy rate in bovine recipients. A follicle coexisting with cyclic CL ovulated and induced CL formed after injection of hCG 1,500 IU 5 days after ovulation. The blood P concentration was significantly ($P<0.05$) higher in the administration group than in the control group, and the blood E_2 concentration rapidly decreased, showing a lower concentration than in the control group. These results suggest the possibility that the pregnancy rate could be improved by administration of hCG. Pregnancy rate following intramuscular injection hCG 1,500 IU was comparatively investigated in parous Japanese Black beef cattle receiving frozen-thawed embryos 7 days after estrus. Pregnancy rate was 67.5% in the group in which hCG was administered on day 6 after estrus, and was significantly ($P<0.05$) higher than that in the control group (45.0%) and the group in which hCG was administered on day 1 after estrus (42.5%), revealing that hCG administration facilitated pregnancy. Transfer of frozen-thawed embryos in the blastocyst stage within 60 minutes after the aspiration into a straw, with a vaginal speculum after administration of

xylazine is suggested as a way of improving pregnancy rate in bovine recipients with favorable luteal function and in those with luteal function activated by administration of hCG on the day before embryo transfer.
Key words: Pregnancy rate, Bovine frozen embryo transfer, Vaginal speculum, Xylazine treatment, Period from thawing to transfer, hCG treatment

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Bovine embryo transfer is now practiced in the livestock industry. The transfer of embryos with excellent hereditary competence aims to improve the hereditary competence of beef cattle, and has come to play a big role in increasing economic productivity in livestock industry management. According to an investigation by the Livestock Industry Bureau of the Ministry of Agriculture, Forestry and Fisheries [1], the number of offspring yielded by bovine embryo transfer has steadily been increasing together with an increasing number of cattle receiving transfer and increasing pregnancy rates; the number of offspring has been at least 10,000 from 1993 onward. Approximately 70% of the calves produced by embryo transfer are Japanese Black beef cattle, and the embryo transfer technique in Japan has been utilized with the aim of increasing production of Japanese beef cattle, since beef trade liberalization.

Frozen-thawed embryo transfer has been on the increase since 1979 when the first production of a calf was reported in Japan [2]. Since 1996, frozen-thawed embryo transfer has occupied about 80% of transfer cases. However, the pregnancy rates of frozen embryo transfer have remained at low levels, 34–47%, since 1987. Likewise, pregnancy rates of fresh embryo transfer have been static at 49–53% since 1987. These low levels of pregnancy rates have prevented the wider use of transfer of fresh and frozen-thawed embryos, and improvements in pregnancy rates are recognized as being indispensable to their wider use. In general, the following are known to be associated with the improvement of pregnancy rate in frozen embryo transfer: 1) methodological factors for embryo transfer; 2) embryonic factors; and 3) endocrinological factors in bovine recipients. With the aim of improving the pregnancy rates of frozen embryo transfer, the relations of various factors, which might influence pregnancy rate, to pregnancy rate were elucidated and a strategy for the improvement of pregnancy rate was assessed in the present study.

Materials and Methods

Collection, freezing and thawing of embryos

Superovulation was induced by intramuscular injections of 20 mg of follicle-stimulating hormone (FSH) (Antrin, Denka Pharmaceuticals, Co., Ltd., Japan) in decreasing doses and $PGF_{2\alpha}$ (Veterinary Pronalgon F Injection; Upjohn Pharmaceuticals, Ltd., Japan) injections given in a total dose of 30 mg of dinoprost (20 mg in the morning and 10 mg in the evening) 3 days after the start of FSH administration [3]. Artificial insemination was performed 12 and 24 hours after the appearance of standing estrus, and the embryos were collected non-surgically on day 7 (the day of appearance of estrus designated as day 0) by flushing the uterus with a Foley catheter [4]. All morula to blastocyst stage embryos, which were morphologically evaluated as having excellent or good quality, were frozen and stored by the glycerol equilibrium method in the two-stepwise method according to Seidel [5]. The embryos were re-evaluated in the post-thawed state for the morphological quality and embryonic developmental stage in Dulbecco's phosphate buffered saline containing 0.4% BSA .

A single thawed embryo, which was re-evaluated as being excellent or good in quality after thawing, was aspirated into a 0.25 ml straw. It took 16–17 minutes after the initiation of thawing for the aspiration of the embryo to be completed. The straw was attached to an embryo transfer gun (Cassou, France), which was wrapped in thermal paper towels, kept at 37 C, and transported to the site of transfer.

Embryo transfer method

On transferring the embryos, the vulva of the recipient was flushed with a disinfectant for external topical use (Propodyne Scrub, Santen Pharmaceuticals, Co., Ltd., Japan). The site of transfer was located in the central portion of the uterine horn ipsilateral to the ovary with corpus luteum (CL). The embryo transfer was conducted with the same equipment by the same person.

Diagnosis of pregnancy

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

Statistical analysis

Pregnancy rate was analyzed by chi-square test and Fisher's exact probability test [6]. Significant differences in changes of plasma concentrations of hormones were analyzed by the general linear models procedure of the Statistical Analysis System (SAS) [7]. Differences at a P value less than 5% ($P<0.05$) were considered to be statistically significant.

Determination of plasma progesterone (P) and estradiol-17β (E_2) concentrations

Blood samples were collected from the jugular vein into heparinized tubes and were centrifuged at 3,000 rpm for 15 min immediately after collection. Blood plasma specimens were stored at –20 C until assayed. Plasma P and E_2 concentrations were determined by radio-immunoassay [8].

Experiment and Results

1. Methodological factors for embryo transfer

1) Pregnancy rate of non-surgical frozen embryo transfer using vaginal speculum: The method for embryo transfer to bovine recipients, which is currently widely practiced involves non-surgical transfer in the early luteal phase, i.e., about 7 days after estrus, when the uterus is susceptible to bacterial infection. When embryos are transferred through the cervix, a number of bacteria are present in the bovine vaginal vestibule [9], indicating the need for prevention of bacterial contamination accompanying passage of the embryo transfer gun through the vagina. Therefore, a sheath cover is applied to the embryo transfer gun to keep the insertion of the transfer gun from the cervix into the uterus sanitary. In this study, the utility of vaginal speculum for embryo transfer instead of the sheath cover, was investigated as a method for preventing bacterial contamination accompanying passage of the embryo transfer gun through the vaginal vestibule.

Embryos were transferred with an embryo transfer gun, to which a sheath cover was not applied in group A (153 head), and to which a sheath cover (Fujihira Kogyo, Tokyo) was applied in group B (control group; 158 head). Group A receiving embryos from the gun without the sheath cover showed a pregnancy rate of 56.9% (87/153), while the rate in Group B using the gun covered with the sheath cover was 54.4% (86/158) (Table 1). The results indicate the usefulness of the vaginal speculum for non-surgical embryo transfer with an embryo transfer gun without the sheath cover [10].

2)Effect of xylazine tranquilization during embryo transfer on pregnancy rate: To improve pregnancy

Table 1. Pregnancy rate of non-surgical frozen-thawed embryo transfer using vaginal speculum in cow

Group	Protective Sheath		Pregnancy rate (%)							
			Japanese Black		Holstein		Japanese Black × Holstein		Total	
A	Absence	Nulliparous	3/3[a)]	(100)	28/39	(71.8)	4/10	(40.0)	35/52	(67.3)
		Parous	44/83	(53.0)	3/10	(30.0)	5/8	(62.5)	52/101	(51.5)
		Total	47/86	(54.7)	31/49	(63.8)	9/18	(50.0)	87/153	(56.9)
B	Presence	Nulliparous	2/5	(40.0)	42/62	(67.7)	5/9	(55.6)	49/76	(64.5)
		Parous	30/69	(43.5)	2/6	(33.3)	5/7	(71.4)	37/82	(45.1)
		Total	32/74	(43.2)	44/68	(64.7)	10/16	(62.5)	86/158	(54.4)

[a)] The number of pregnant recipients/The number of recipients to which embryos were transferred.

Table 2. Xylazine tranquilization of recipient at the time of non-surgical compact morula embryo transfer and pregnancy rate in Holstein heifers

Division [A]	Group[B]					
	Xylazine[a)]			Non- Xylazine		
	Excellent	Good	Total [C]	Excellent	Good	Total [C]
Stanchion	18/34[b)]	4/6	22/40	16/33	2/4	18/37
Stalls	(52.9[c)])	(66.7)	(55.0)	(48.5)	(50.0)	(48.6)
Treatment	13/25	2/5	15/30	9/19	3/7	12/26
Stalls	(52.0)	(40.0)	(50.0)	(47.4)	(42.9)	(46.2)
All together	31/59	6/11	37/70	25/52	5/11	30/63
	(52.5)	(54.5)	(52.9)	(48.1)	(45.5)	(47.6)

There were no significant differences between Xylazine administered and non-administered groups.
No interactions was found between the groups and the divisions [A × B × C].
[a)] Xylazine 20 mg was injected intramuscularly 5–15min before embryo transfer.
[b)] The number of the pregnant recipients/The number of the recipients to which embryos were transferred.
[c)] % .

rate by smoothly conducting embryo transfer without damage to the uterus, it is desirable that the bovine recipient is restrained during transfer by resting in a treatment stall. However, the number of farms having such a treatment stall is very limited. Currentry, most cases of embryo transfer to nulliparous Holstein heifers are conducted while they are hitched to stanchion stalls without resting in treatment stalls. When transfer is conducted with bovine recipients restrained by stanchions, the administration of a sedative with the aim of suppressing restlessness at the time of transfer as much as possible may be effective for the improvement of pregnancy rate. The drug, xylazine, which has been widely used as a sedative in cattle has been reported to have an uterotonic effect [11]. No reports have revealed the pregnancy rate when the drug was administered before embryo transfer with the aim of sedating the recipient. In the present experiment 20 mg of xylazine was intramusculary injected (i.m.) to nulliparous Holstein heifers before embryo transfer to obtain the sedative effect, then one frozen-thawed compacted morula stage embryo of Japanese Black beef cattle was transferred. The results of the assessment of pregnancy rate are shown in Table 2.

The pregnancy rate in the group to which xylazine was administered before transfer (xylazine administration group) tended to be higher than in the control group to which the embryos were transferred without xylazine administration to recipients kept in stanchion stalls (Division 1) and those kept in treatment stalls (Division 2) (55.0% vs 48.6% and 50.0% vs 46.2%, respectively). The xylazine administration group showed a tendency for fewer recipients in which it took at least 5 minutes for the manipulation of embryo transfer in Divisions 1 and 2 (12.5% vs 24.3%, respectively), and for fewer recipients in which the blood adhered to the tip of the embryo transfer gun in Divisions 1 and 2 (13.3% vs 19.2%, respectively)(Table 3). These results suggest that administration of xylazine during embryo transfer is effective for improvement of pregnancy rate when nulliparous Holstein heifer recipients are kept in stanchion stalls during transfer [12].

3) Influence of period from thawing to transfer of embryos and trucking of recipients on pregnancy rate: There are two ways of conducting bovine frozen-thawed embryos transfer: in one , the thawed embryo is transported from the clinical setting to the scene of transfer, and in the other, the bovine recipient is transported to the embryo transfer center by truck, undergoes transfer, and is returned by truck after transfer. Seidel *et al.* [13] have advocated that bovine frozen-thawed embryo transfer should be conducted in as a short a time as possible, within 30 minutes if possible, after thawing for obtaining high pregnancy rates with the transfer. However, this has not yet been adequately investigated, or no guide on the basis of fundamental data has been presented.

In the present experiment, the influence of the

Table 3. Relationship between the time required for manipulation of embryo transfer and pregnancy rate

Division [A]	Xylazine [B]	Number of recipients	Time required for transfer [C]			
			<5 minutes		≥5 minutes	
			Number of recipients	Pregnancy Rate	Number of recipients	Pregnancy Rate
Stanchion Stalls	Administration Group	40	37(92.5[a)])	20/37 [b)](54.0)	3(7.5)	2/3(60.7)
	Control group	37	30(81.1)	14/30(46.7)	7(18.9)	4/7(57.1)
Treatment Stalls	Administration Group	30	26(86.7)	12/26(46.2)	4(13.3)	3/4(75.0)
	Control group	26	21(80.8)	9/21(42.8)	5(19.2)	3/5(60.0)

There were no significant differences in the proportion of recipients and pregnancy rate. No interactions were found between the groups and the divisions [A × B × C].

[a)] %.

[b)] The number of the pregnant recipients/The number of the recipients to which embryos were transferred.

time taken for the period from thawing to transfer of the bovine frozen embryo, and of the transportation of the recipient cattle by truck, on pregnancy rate was investigated.

In Experiment 1, one excellent or good compacted morula embryo was aspirated into a straw for transfer 16–17 minutes after the initiaton of thawing. The embryos were transported to the breeding farm and transferred to the recipients, 96 nulliparous Holstein heifers, 105 Japanese Black cows, and 35 cross breeds. Pregnancy rates were determined 11–20 (Group 1), 21–30 (Group 2), 31–40 (Group 3), 41–50 (Group 4) and 51–60 minutes (Group 5) after aspiration of the embryo into the straw. The rates were 50.0% (2/4) to 73.9% (14/19) in Groups 1 through 5 of the Holstein heifers, 50.0% (3/6) to 62.5% (20/32) in Groups 2 through 5 of the Japanese Black cows, and 43.8% (7/16) to 60.0% (6/10) in Groups 2 to 4 of the cross breeds (Japanese Black strain × Holstein strain). No significant differences were found among the groups (Table 4).

In Experiment 2, Holstein heifer recipients were transported from the breeding farm to our Embryo Transfer Institute by truck for 1.4–1.6 hours (58–62 kilometers one way). The recipients were maintained in stalls for approximately 20 minutes until thawing of the frozen embryos and aspiration of the thawed embryos into the straw were completed. Then procedures similar to those of Experiment 1 were conducted: the embryos were transferred within 10 minutes (Group 6) and 11–20 minutes (Group 7) after aspiration. Thus, pregnancy rates of the recipients receiving embryos within 30 minutes of arrival after transportation by truck were determined. They were 69.7% (23/33) and 66.7% (6/9) in Groups 6 and 7, respectively, with no significant difference. These data indicate that pregnancy rate was not affected by the time period between thawing and transfer, if the transfer was completed within 60 minutes after aspiration which was initiated 16–17 minutes after commencement of thawing of compacted morula embryo, or the 1.5-hour (one way) trucking of recipients (Table5) [14].

2. Embryonic factors

1) The influence of developmental stage and morphological quality of frozen-thawed embryos on pregnancy rate: Relations of embryonic developmental stage and morphological quality on pregnancy rate were investigated. One hundred eighty Japanese Black beef cattle embryos in compacted morula to blastocyst stages, whose morphological quality was excellent, were frozen-thawed and re-examined after thawing. Embryos in compacted morula to blastocyst stages, whose morphological quality was judged to be excellent or good, were transferred to 172 nulliparous Holstein

Table 4. Influence of time from aspiration of a frozen-thawed compact morula embryo into a straw for transfer on pregnancy rate following non-surgical embryo transfer

Group	Embryo Quality	Group 1 11–20[1)]	Group 2 21–30	Group 3 31–40	Group 4 41–50	Group 5 51–60	Total
Holstein (Nullipara)	Excellent	7/13[2)] (53.8)[3)]	4/8 (50.0)	3/7 (42.9)	13/17 (76.5)	2/4 (50.0)	29/49 (59.2)
	Good	–	1/2 (50.0)	28/43 (65.1)	1/2 (50.0)	–	30/47 (63.8)
	All	7/13 (53.8)	5/10 (50.0)	31/50 (62.0)	14/19 (73.7)	2/4 (50.0)	59/96 (61.5)
Japanese Black (Mullipara)	Excellent	0/2 (0)	15/31 (48.4)	18/28 (64.3)	13/20 (65.0)	2/5 (40.0)	48/86 (55.8)
	Good	–	5/8 (62.5)	2/4 (50.0)	3/6 (50.0)	1/1 (100)	11/19 (57.9)
	All	0/2 (0)	20/39 (51.3)	20/32 (64.0)	16/26 (61.5)	3/6 (50.0)	59/105 (56.2)
Cross breed[4)] (Mullipara)	Excellent	–	3/6 (50.0)	7/16 (43.8)	6/9 (66.7)	–	16/31 (51.6)
	Good	–	2/3 (66.7)	–	0/1 (0.0)	–	2/4 (50.0)
	All	–	5/9 (55.6)	7/16 (43.8)	6/10 (60.0)	–	18/35 (51.4)

[1)] Period from aspiration of embryo into straw to completiono f transfer.
[2)] Pregnant cattle/recipient cattle.
[3)] %.
[4)] Japanese Black × Holstein.

Table 5. Influence of recipient's transportation by truck on pregnancy rate in non-surgical transfer of frozen-thawed compact morula embryos

Breed	Embryo Quality	Group 6 0–10[1)]	Group 7 11–20	Total
Holstein (Nullipara)	Excellent	22/31[2)] (71.0)[3)]	5/8 (62.5)	27/39 (69.2)
	Good	1/2 (50.0)	1/1 (100)	2/3 (66.7)
	All	23/33 (69.7)	6/9 (66.7)	29/42 (69.0)

[1)] Period from aspiration of embryo into straw to completion of transfer.
[2)] Pregnant cattle/recipient cattle.
[3)] %.

heifers, one each, on day 7 after estrus.

Pregnancy rates of 62.0% (49/79), 66.7% (26/39), and 81.0% (34/42) were achieved in the transfers of the compacted morula, early blastocyst, and blastocyst stages, respectively, for the transfer of the excellent quality embryos. The pregnancy rate obtained with blastocyst stage embryos was significantly ($P<0.05$) higher than that with compacted morula embryos. As to the morphological quality, the excellent quality embryos of the compact morula stage resulted a pregnancy rate of 62.0% (49/79). The pregnancy rate of the good quality embryos of the morula stage was 58.3% (7/12) revealing no significant difference between the two qualities. These results indicate that the pregnancy rate increases as the embryo developmental stage advances from compacted morula to blastocyst, whereas post-thawed morphological qualities, excellent and good, have no influence on pregnancy rate in frozen-thawed embryos transferred to bovine recipients on day 7 after estrus (Table 6) [15].

Table 6. The effect of embryonic developmental stage and morphological quality on pregnancy rate obtained with non-surgical transfer of frozen-thawed embryos

Stage	Quality	
	Excellent	Good
Compact morula	62.0[a)] (49/79[b)]) ※	58.3 (7/12)
Early blastocyst	66.7 (26/39)	–
Blastocyst	81.0 (34/42) ※	–
Total	68.1 (109/160)	58.3 (7/12)

[a)] Pregnancy rate (%).
[b)] Number of pregnant heifers/Number of transferred recipients.
※ Significant difference ($P<0.05$) was detected between the two.

Table 7. Relationships between the blood P and E_2 levels on the day before embryo transfer and results of pregnancy

P(ng/ml) E_2(pg/ml)	< 0.5	0.5~ <1.0	1.0~< 1.5	≥1.5	Total	
<1.5	0/1[a](0)[b]	0/2 (0)	0/1(0)	3/5 (60.0)	3/9 (33.3)	13/34 (38.2) ※
1.5~< 2.5	1/3(33.3)	2/5 (40.0)	1/5 (20.0)	6/12 (50.0)	10/25 (40.0)	
2.5~< 3.5	3/4 (75.0)	7/11 (63.6)	4/6 (66.7)	3/7 (42.9)	17/28 (60.7)	25/39 (64.1) ※
≥ 3.5		4/4 (100)	4/5 (80.0)	0/2 (0)	8/11 (72.7)	
Total	4/8 (50.0)	13/22 (59.1)	9/17 (52.9)	12/26 (46.2)	38/73(52.1)	

a) Number of pregnant cows/Number of recipients. [b] Pregnancy rate (%).
※ There was a significant (P<0.05) difference between these values.

Table 8. Relationships between blood Pand E_2 concentrations on the day of embryo transfer and results of pregnancy

P(ng/ml) E_2 (pg/ml)	< 0.5	0.5~<1.0	1.0~< 1.5	≥ 1.5	Total	
<1.5		0/1 (0)		0/3 (0)	0/4(0)	7/17 (41.2)
1.5~< 2.5		4/6 (66.7)	0/2 (0)	3/5 (60.0)	7/13(53.8)	
2.5~< 3.5	3/4[a](75.0)[b]	7/15 (46.7)	2/3 (66.7)	1/2 (50.0)	13/24 (54.2)	31/56 (55.4)
≥ 3.5	3/5 (60.0)	7/12 (58.3)	5/9 (55.6)	3/6 (50.0)	18/32 (56.3)	
Total	6/9 (66.7)	18/34 (52.9)	7/14 (50.0)	7/16 (43.8)	38/73 (52.1)	

[a] Number of pregnant cows/Number of recipients. [b] Pregnancy rate (%).

Table 9. Correlations of luteal development, presence of follicles (≥1.0 cm in diameter), the blood E_2 and P levels, and E_2/P[a] ratio on the day before transfer (day 6) with results of pregnancy

Coexistent follicles	Development of CL												Total			
	Good development				Poor development				Cystic CL							
(≥1.0 cm in diameter)	P level (ng/ml)	E_2 level (pg/ml)	E_2/P ratio	Pregnancy rate(%)	P level (ng/ml)	E_2level (pg/ml)	E_2/P ratio	Pregnancy rate(%)	P level (ng/ml)	E_2 level (pg/ml)	E_2/P ratio	Pregnancy rate(%)	P level (ng/ml)	E_2 level (pg/ml)	E_2/P ratio	Pregnancy rate(%)
Presence	2.8±1.1	1.4±0.8※	0.7±0.8	43.8(7/16)	2.0±1.0	2.5±1.3	1.7±2.1	33.3(2/6)	1.7±0.4※	1.3±0.5	0.8±0.3	0(0/3)	2.5±1.1	1.5±1.1	0.9±1.2	41.7(10/24)
Absence	2.9±1.0	0.9±0.4※	0.4±0.3	64.7(22/34)	2.1±0.6	2.0±1.8	1.4±1.0	54.5(6/11)	2.8±0.6※	0.9±0.1	0.4±1.0	33.3(1/3)	2.8±1.1	1.3±1.0	0.6±0.7	57.1(28/49)
Total	2.8±1.0※	1.2±0.8※※	0.6±0.7※※	58.0 (29/50)	2.0±0.8※	2.2±1.6※※	1.6±1.8※※	47.1 (8/17)	2.1±0.7	1.2±0.5	0.7±0.4	16.7 (1/6)	2.6±1.0	1.4±1.1	0.8±1.1	52.1(38/73)

[a] $\times 10^3$.
There was a significant (P<0.05) difference between ※ and ※.
There was a significant (P<0.05) difference between ※※ and ※※.

3. Endocrinological factors for bovine recipients

1) The relationship of plasma P and E_2 concentrations on the day before and the day of frozen-thawed embryo transfer to pregnancy rate: Plasma P and E_2 concentrations on the day before (on day 6 ; day 0 = onset of estrus) and the day of embryo transfer were determined in parous Japanese Black beef cattle, and their relations to pregnancy rate were investigated. The pregnancy rate showed a tendency to increase with increasing plasma P concentration of recipient cattle on both the day before and the day of embryo transfer. The pregnancy rate (60.7%) in cattle with a plasma P concentration of ≥2.5 ng/ml on the day before embryo transfer was significantly (P<0.05) higher than that (38.6%) in cattle with plasma P concentration of <2.5 ng/ml. As for the relationship between plasma E_2 concentration and pregnancy rate, pregnancy rate showed a tendency to increase with decreasing plasma E_2 concentration on the day of transfer (Table 7–8).

Based on the rectal examination of the ovaries on the day before embryo transfer, CL was divided into three categories : favorable development, poor development and cystic. The presence or absence of a coexisting follicle ≥1.0 cm diameter with CL was also investigated and the relation of these clinical parameters to plasma P and E_2 concentration and the pregnancy rate was investigated. In the recipients, plasma P concentration was not different between favorable luteal development and the poor luteal development types of CL. However, the plasma E_2 concentration tended to be higher and the

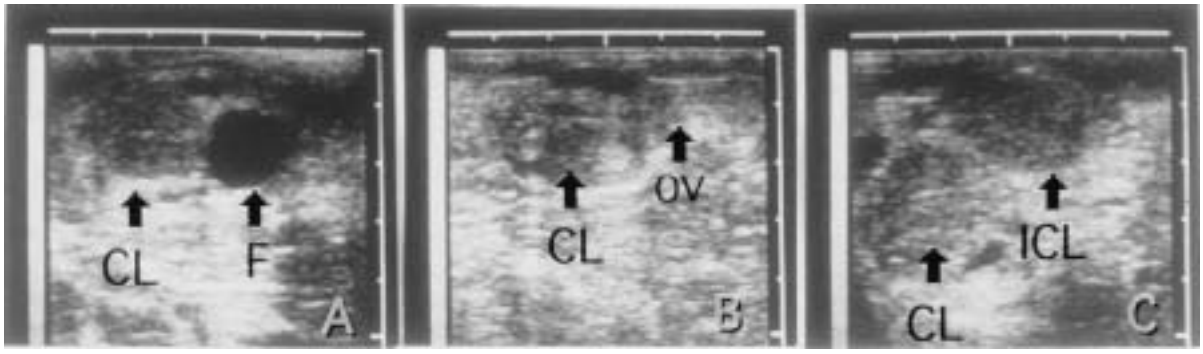

Fig. 1. Representative ultrasonographic findings of hCG-induced ovulation and consequent formation of CL on the first dominant follicles in the cattle treated with hCG on day 5. A: CL periodicum and coexistent follicles (F) on the day of hCG administration (day 5). B: Ovulation (OV) sites of coexistent follicles 2 days after hCG administration. C: CL periodicum (lower left CL) and induced CL (ICL; higher right ICL) 7 days after induced ovulation (9 days after hCG administration).

pregnancy rate tended to be lower in the recipients bearing coexisting follicles with diameter ≥1.0 cm than in the recipients without a follicle (Table 9) [3, 16].

2) The effect of hCG on the development and function of bovine corpus luteum

To fundamentally assess the procedure for improving pregnancy rates in embryo transfer, hCG (1,500 IU) was i.m. once to two groups of 4 Holstein cows: hCG was administered on the day of ovulation (Day0-hCG group); and hCG was administered 5 days after ovulation (Day 5-hCG group). The response of the ovaries to hCG and changes of plasma P and E_2 concentrations after hCG injection were investigated.

In the Day5-hCG group, ovulation was induced in all cattle 1 or 2 days after administration of hCG and sequential formation of induced CL was observed after the induced ovulation (Fig. 1). The plasma P concentration became significantly ($P<0.05$) higher than that in the control group 6, 7, 12, 13, and 14 days after ovulation. Plasma E_2 concentration decreased rapidly after hCG administration 5 days after ovulation, and became lower than that in the Day5-control group (Fig. 2). These results reveal that the luteal activity was increased and plasma E_2 concentration was decreased by administration of hCG 5 days after ovulation, suggesting the hCG treatment is effective at increasing the pregnancy rate in embryo transfer [17].

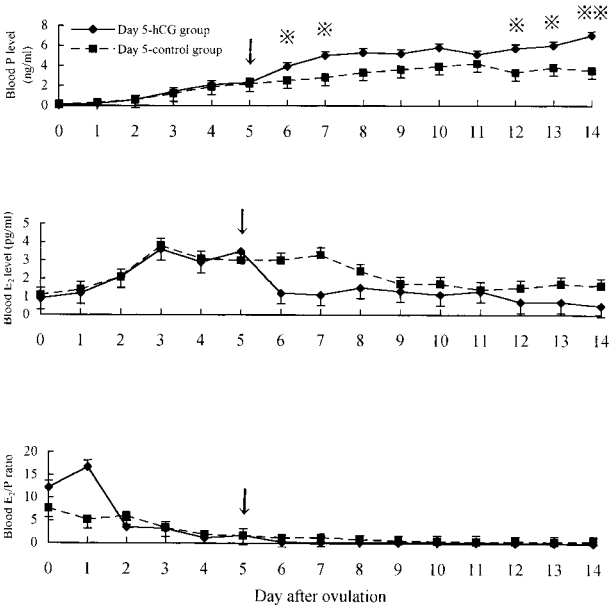

Fig. 2. Changes in blood P and E_2 concentrations and E_2/P ratio in the groups administered hCG on day 5. ↓: 1,500 IU of hCG or 5 ml of saline solution was administered. Values represent means ± SEM. ※ Significant difference ($P<0.05$) was detected between the hCG and the control groups. ※※ Significant difference ($P<0.01$) was detected between the hCG and the control groups.

3) Improvement of pregnancy rate by administration of hCG to bovine recipients of transferred frozen-thawed embryos

To investigate the improvement of pregnancy rate by the method of increasing luteal function with hCG, which was administered during the period between estrus and embryo transfer, 120

Table 10. Effect of hCG administration on day 1 or day 6 on pregnancy rate in bovine recipients

Group	hCG 1,500 IU administration[a)]	Pregnancy rate (%)
A	Day 1[c)]	17/40[b)] (42.5)
B	Day 6	27/40 (67.5)[d)]
C	Saline, Day 6	18/40 (45.0)[e)]
	Total	62/120 (51.7)

a) Intramuscular injection.
b) Number of the pregnant cows / Number of transferred embryo.
c) Day 0 = onset of estrus.
d,e) Different superscripts indicate significant differences within columns (P<0.05).

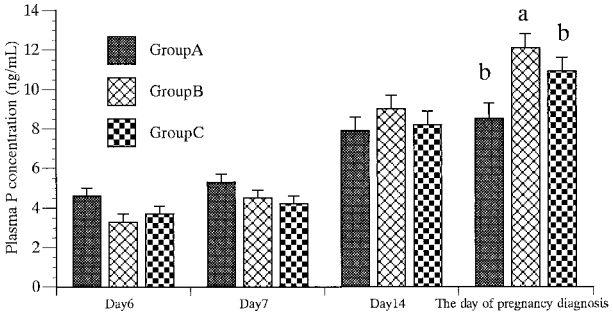

Fig. 3. Plasma P concentration on days 6, 7 and 14, and the day of pregnancy diagnosis. Data are presented as means ± SEM. Day 0=onset of estrus. Different superscripts show a significant difference (P<0.05).

parous Japanese Black beef cattle, which were kept at Japanese beef cattle breeding farms were divided at random into three groups, each containing 40 head. 1,500 IU of hCG was i.m. in Group A one day after estrus; 1,500 IU of hCG was i.m. in Group B 6 days after estrus; and 5 ml of saline was i.m. in Group C as a control 6 days after estrus. Embryo transfer was conducted 7 days after estrus in these groups, and the pregnancy rate was compared among these groups. Blood samples were collected from 12 recipients, which were randomly selected from each group 6, 7, and 14 days after estrus and at the time of pregnancy diagnosis, and plasma P and E_2 concentrations were determined.

The pregnancy rate in Group B was 67.5%, which was significantly (P<0.05) higher than 45.0% in Group C and 42.5% in Group A (Table 10). Plasma P concentration 14 days after estrus tended to be higher in Group B than those in Group C and Group A. At the time of pregnancy diagnosis, the plasma P concentration of pregnant recipients in

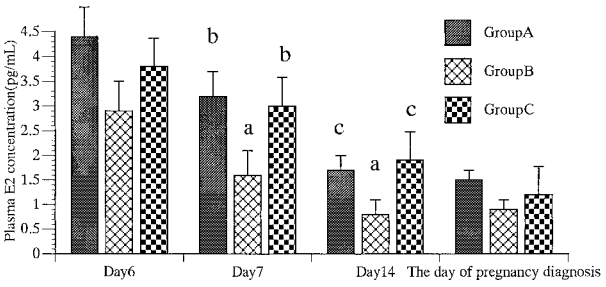

Fig. 4. Plasma E_2 concentrations on days 6, 7 and 14 and day of pregnancy diagnosis. Data are presented as means ± SEM. Day 0=onset of estrus. Different superscripts within days show a significant differene (a, b; P<0.05, a, c; P<0.03).

Group B was significantly (P<0.05) higher than those in Group C and Group A (Fig. 3). Plasma E_2 concentration 7 and 14 days after estrus were significantly (P<0.05) lower in Group B than those in Group C and Group A (Fig. 4) [18].

Discussion

The bovine uterus is known to have a condition which prevents bacterial infections in estrus and a condition which facilitates bacterial infections in the luteal phase [19]. Suzuki *et al.* [9] investigated bacterial flora in the bovine vagina, and found that the number of bacteria is large in the vaginal vestibule regardless of the estrous cycle and is small in sites deep in the vagina and the cervix. These observations indicate that it is important for improvement of pregnancy rate to prevent intravaginal bacterial contamination accompanying the passage of an embryo transfer gun through the vagina in embryo transfer to the cervix. The pregnancy rate in embryo transfer using a vaginal speculum was favorable, approximately 55%, regardless of the presence or absence of a sheath cover applied to the embryo transfer gun. One possible reason for this result was considered to be that bacterial contamination accompanying insertion of the transfer gun through the vaginal vestibule was prevented by the use of the vaginal speculum even in the absence of the sheath cover on the transfer gun.

Tervit *et al.* [20] and Sreenan *et al.* [21] have suggested possible reasons for low pregnancy rates in non-surgical embryo transfer in cattle as damage and hemorrhage occurring on the endometrium

due to the an embryo transfer gun . The number of the farms having treatment stalls for embryo transfer is very limited. Most recipients are hitched to stanchions and restrained for embryo transfer. Accordingly, it is highly probable that the endometrium is damaged by the transfer gun as a result of the recipient's movement during transfer. In this experiment, xylazine was administered to nulliparous recipient Holstein heifers during embryo transfer with the aim of sedating them. The pregnancy rate in the xylazine administration group tended to be higher than that in the control group, though there was no statistically significant difference. The proportion of the cattle in which it took at least 5 minutes for the embryo transfer maneuver, and the proportion of the cattle which showed blood adhering to the tip of the transfer gun in the xylazine administration group were low, though there was no significant difference. The combined use of xylazine administration and epidural anesthesia facilitated the embryo transfer maneuver and allowed embryo transfer without damage to the uterus by the transfer gun.

A thawed embryo was aspirated into a piece of straw 16–17 minutes after the initiation of thawing. The transfer gun was wrapped with a paper towel kept preliminarily at 37 C, and the embryo in the transfer gun was transported to the scene of transfer in a container at room temperature. When the embryo was transferred within 60 minutes after the embryonic aspiration into the straw, there was no decrease in pregnancy rate as the time lapse between embryo aspiration and transfer increased. The result is assumed to indicate that transfer of an embryo with excellent or good morphological quality within 60 minutes after embryonic aspiration into the straw does not impair the embryonic growth.

Some reports have shown that transportation by truck causes stress to bovine recipients and elevates plasma cortisol concentration [22], and that transportation within a short time after delivery particularly induces a marked elevation of blood cortisol concentration [23]. In the present experiment, the recipient cattle were transported from the site of breeding to the embryo transfer center in a journey which took 1.4 to 1.6 hours each way. They were hitched to treatment stalls for approximately 20 minutes for transfer, and returned again to the farms within 30 minutes after the end of transfer. Pregnancy rates in this experiment were comparable to those in the transfer of a frozen-thawed embryos transported to the actual site of breeding of recipient cattle. Because there was no decrease in the pregnancy rates of the recipient cattle transported by truck for 1.4 to 1.6 hours each way , transportation was not considered to cause sufficient stress as would influence pregnancy rate. These results suggest that a method, in which recipient cattle are transported to an embryo transfer center by truck for transfer, would also be practical, when frozen embryo transfer is conducted in nulliparous Holstein heifers that are bred on farms located at least an hour's journey time each way from the embryo transfer center.

Recipient-donor estrous synchrony is needed for the attainment of high pregnancy rates in bovine embryo transfer [24–26]. The pregnancy rate in embryo transfer is known to be influenced by the embryonic developmental stage. In cases of surgical transfer of fresh embryos carried out 6 to 8 days after estrus, in which donor estrous cycle was synchronized with the recipient estrous cycle within a range of ± 12 hours, the pregnancy rate with early morula embryos was significantly lower than each of that achieved with compacted morula, early blastocyst, and blastocyst stage embryos [28]. In the present study, frozen-thawed embryos with morphologically excellent or good quality in compacted morula, early blastocyst, and blastocyst stages were transferred to recipient cattle 7 days after estrus, and the relations of embryonic developmental stage and morphological quality to pregnancy rate were investigated. The pregnancy rate with blastocyst embryos was significantly higher than that with compacted morula embryos, and the pregnancy rate tended to increase as the developmental stage advanced to the blastocyst stage. With regard to this matter, it was reported in rabbits that pregnancy rate was high when frozen embryos were transferred to a rabbit oviduct in an estrous cycle stage relatively earlier than the embryonic developmental stage, because frozen embryos are retarded in their development by injuries due to freezing and thawing [29]. In the present study, the pregnancy rates with blastocyst and early blastocyst embryos were higher than that with compacted morula embryos. This was probably because the embryos at blastocyst and early blastocyst stages were transferred to recipient cattle with uterine estrous cycle stages equal to or

earlier than the embryonic developmental stage, leading to synchronization of the subsequent embryonic developmental stage with the uterine estrous cycle stage.

Some reports have shown that persistent P secretion from the ovaries is needed for the maintenance of bovine pregnancy [30, 31], and early embryonic death has been indicated as occurring when plasma P concentrations are low [32, 33]. It has also been reported that P accelerates intrauterine embryonic development [34]. Low plasma P concentration, therefore, causes embryonic death with a high frequency, interferes with accomplishment and maintenance of pregnancy, thus decreasing pregnancy rate. As for the relationship between plasma P concentration and pregnancy rate in bovine recipients, Stubbings and Walton [35] reported that the pregnancy rate in non-surgical frozen-thawed embryo transfer was low in nulliparous heifers with a plasma P concentration of ≤3 ng/ml. In the present study, pregnancy rate was as high as 56.9% in recipients that showed a plasma P concentration of ≥2.5 ng/ml on the day before or the day of embryo transfer. This result was consistent with the results of a previous report [35]. Therefore, a high pregnancy rate might be obtained by selecting cattle that show a plasma P concentration of ≥2.5 ng/ml on the day before or the day of embryo transfer. In the present study, findings of CL, which were obtained by rectal examination on the day before embryo transfer, were divided into three types, favorably developed CL, poorly developed CL, and cystic CL, according to a previous report [36], and the relationship between plasma P concentration and pregnancy rate was investigated. The results were consistent with those of a previous report [37]. The mean plasma P concentration was high in the cattle with favorably developed CL, and low in the cattle with poorly developed or cystic CL.

It has been reported that not only P secretion from CL but also E_2 secretion from follicles is needed for the maintenance of bovine pregnancy [38]. Elsewhere, it was reported that plasma P concentration was low and plasma E_2 concentration was high in the cattle from which unfertilized oocytes and degenerative embryos were collected 7 days after estrus [39]. In the present study, there were relationships between pregnancy rate and the plasma P and E_2 concentrations on the day before and the day of embryo transfer. The pregnancy rate was 73.3% in the cattle which showed a plasma P concentration of ≥2.5 ng/ml and a plasma E_2 concentration of <1.5 pg/ml on the day before embryo transfer. This rate was significantly ($P<0.05$) higher than 37.2% in the cattle that showed plasma P<2.5 ng/ml and E_2 >1.5 pg/ml concentrations. On the other hand, pregnancy rate was 33.3% in the cattle that showed a plasma P concentration of <2.5 ng/ml and a plasma E_2 concentration of ≥1.0 pg/ml on the day of embryo transfer, while pregnancy rate was as high as 55.6% in the cattle with plasma P and E_2 concentrations of ≥2.5 ng/ml and <1.0 pg/ml, respectively. These results support previously reported data for the day before embryo transfer [39]. On the basis of findings of rectal examination conducted on the day before embryo transfer, CL were divided into three types, favorably developed CL, poorly developed CL, and cystic CL, according to the form of CL, and relations of pregnancy rate to plasma P and E_2 concentrations and E_2/P ratio were also investigated. The plasma P concentration was significantly high and plasma E_2 concentration was significantly low in the recipient cattle with favorably developed CL, as compared to those in the recipient cattle with poorly developed CL. The recipients were divided into groups with and without follicles ≥1.0 cm in diameter, and relations of pregnancy rate to plasma P and E_2 concentrations and E_2/P ratio were investigated. Pregnancy rate was low in the recipient cattle with high blood E_2/P ratio, in which follicles ≥1.0 cm in diameter coexisted with CL. The results suggest that it is necessary to devise a method of increasing the P secretion function while simultaneously decreasing plasma E_2 concentration during the period from estrus to embryo transfer, and of accelerating luteinization by inducing ovulation of follicles coexisting with CL. It is also suggested that the effect of the method to improve pregnancy rate should be investigated.

Some reports have shown that hCG has a stimulant effect on CL [40, 41], and that plasma P concentraion is elevated by hCG administration [42, 43]. To progress research into hCG administration aiming at facilitating pregnancy by embryo transfer, 1,500 IU of hCG was administered on the day of ovulation or 5 days after ovulation, and changes in ovaries after the treatment and fluctuations in plasma P and E_2 concentrations were investigated. The stimulative effect of hCG on

luteinization and P secretion function was investigated. In the functional luteal phase, the tissue area of cyclic CL was significantly larger in the group, to which 1,500 IU of hCG was administered 5 days after ovulation, than in the control group. In all cases of the group treated with hCG 5 days after ovulation, the dominant follicles ovulated on the day or 2 days after hCG administration. This was followed by induced CL formation. Twenty-four and 48 hours after hCG administration, the plasma P concentration in the group, to which hCG was administered 5 days after ovulation, was significantly higher than that in the control group. The same group showed high plasma P concentration thereafter as well. The plasma P concentration was significantly higher than that in the control group 12, 13, and 14 days after ovulation. Some reports have shown that the elevation of plasma P concentration observed during the 5-day period from hCG administration 5 days after estrus in Holstein cattle is attributable to direct stimulation of cyclic CL by the hCG administration [40], and that the plasma P concentration started being elevated 2–3 days after ovulation when ovulation was induced by hCG administration to cattle with ovarian quiescence [44]. From these reports, the elevated plasma P concentration, which was observed 3–12 hours after hCG administration, was considered attributable to the direct stimulating effect of hCG on cyclic CL. The reason for the significantly higher plasma P concentration in the group treated with hCG 5 days after ovulation, which was observed 7 days after hCG administration, is proposed as follows: as a result of the increased tissue area of cyclic CL and induced CL, the total area of CL tissue in the hCG administrated group was significantly larger than that in the control group; P secretion from cyclic CL was elevated and was incremented by P secretion from induced CL, thus the ability to secrete P was elevated in total. Furthermore, the group administered hCG 5 days after ovulation showed a lower plasma E_2 concentration than that in the control group on the day after administration. Mature follicles in estrus are known to be desensitized and to show a rapid decrease in E_2 secretion in response to surge of luteinizing hormone (LH) [45]. It has also been reported that the first dominant follicles develop with an increase in plasma E_2 concentration in cattle [46]. These findings and observations of the present study suggest that administration of 1,500 IU of hCG 5 days after ovulation leads the first dominant follicles to ovulate, thereby decreasing the plasma E_2 concentration after hCG administration.

When hCG was administered to bovine recipients one day and 6 days after estrus, the pregnancy rate was 67.5% in the group administered 6 days after estrus, which was significantly higher than in the control group and the group administered hCG one day after estrus. It is assumed that an endocrine environment, in which high blood P concentration and low plasma E_2 concentration and E_2/P ratio over the period from the day of embryo transfer to about 14 days after estrus, when the maternal body recognizes the embryo, was achieved by administration of 1,500 IU of hCG to the recipients 6 days after estrus. As a result, the intrauterine environment would become suitable for embryo development, thereby significantly improving the pregnancy rate.

On the basis of the observations of the present study, administration of hCG 5 days after ovulation increased P secretion function and simultaneously decreased plasma E_2 concentration, induced of ovulation of coexisting follicles with CL and subsequent new formation induced CL, and stimulated development of cyclic CL, resulting in an improved pregnancy rate in embryo transfer.

The above results suggest that pregnancy rate can be improved in bovine embryo transfer by administration of xylazine to bovine recipients before embryo transfer, transfer of frozen-thawed embryos in the blastocyst stage to bovine recipients with a favorable luteal function, or to those with a luteal function increased by administration of hCG on the day before embryo transfer, and by transfer of frozen-thawed embryos within 60 minutes after embryonic aspiration into a straw with the application of a vaginal speculum.

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References

1. **MAFF Statistics**. The national average conception rate in 1999. *ET News Letter* 2001; 23: 91 –92.
2. **Sugie T, Tunoda Y, Soma T**. Storage and transfer of frozen embryos. *Japan J Anim Reprod* 1979; 25: 203–205.
3. **Nishigai M, Kamomae H, Tanaka T, Kaneda Y**. The relationship of blood progesterone and estrogen concentrations on the day before and the day of frozen-thawed embryo transfer to pregnancy rate in Japanese Black cattle. *J Reprod Dev* 2000; 46: 235–243.
4. **Elsden RP ,Hasler JF, Seidel GE Jr**. Non-surgical recovery of bovine eggs. *Theriogenology* 1976; 5: 23–32.
5. **Seidel GE Jr, Elsden RP,Brink Z**. Cryopreservation of bovine embryos in media with chemically defined macromolecules. *Theriogenology* 1990; 33: 322 (abst).
6. **StatView User's Resource Forum**. StatView 4.5 for Macintosh Official Guidebook. Nankodo Co., Ltd. Tokyo; 1996.
7. **Statistical Analysis System**. SAS User's Guide. Statistics, Version 6.03 Edition, Cary, NC, SAS Inst. Inc; 1988.
8. **Taya K, Watanabe G, Sasamoto S**. Radioimmunoassay for progesterone, testosterone and estradiol-17β using ^{125}I-iodohistamine radioligands. *Japan J Anim Reprod* 1985; 31: 186–197.
9. **Suzuki T, Takahashi Y, Shimohira I**. Bacteriological Investigation of cervical and vaginal mucus in cows by season and estrous stage. *Japan J V M* 1982; 35: 235–241.
10. **Nishigai M, Kamomae H, Tanaka T, Kaneda Y**. Pregnancy rate of non-surgical frozen embryo transfer using vaginal speculum in cow. *J Reprod Dev* 1996; 42: 97–99.
11. **Sagner G, Hoffmeister F, Kroneberg G**. Pharmacological basis of a new drug for analgesia, sedation and relaxation in veterinary medicine,Bayer Va 1470 or "Rompun". *Dtsh tierarztl Wschr* 1968; 75: 565–572.
12. **Nishigai M, Kamomae H, Tanaka T, Kaneda Y**. Effect of Xylazine tranquilization during embryo transfer to bovine recipients on pregnancy rates. *J Reprod Dev* 1997; 43: 41–46.
13. **Seidel GE Jr, Elsden RP**. Protocol in corporating current recomendation for freezing bovine embryos. Techniqes for Freezing Mammalian Embryos: 1988 Short Course Proceedings. Colorado State University 1988: 85–86.
14. **Nishigai M, Kamomae H, Tanaka T, Kaneda Y**. Influence of period from thawing to transfer of embryos and trucking of recipients on pregnancy rate in bovine embryo transfer. *J Reprod Dev* 1998; 44: 1–6.
15. **Nishigai M, Kamomae H, Tanaka T, Kaneda Y**. The influence of developmental stage and morphological quality of frozen-thawed embryo on pregnancy rate in bovine embryo transfer. *J Reprod Dev* 1999; 45: 301–306.
16. **Nishigai M, Kamomae H, Tanaka T, Kaneda Y**. Pregnancy rate and blood progesterone concentrations on the previous day and the day of frozen embryo transfer in parous recipient cows of Japanese Black. *J Reprod Dev* 1998; 44: 413–419.
17. **Nishigai M, Kamomae H, Tanaka T, Kaneda Y**. The effect of human chorionic gonadotropin on the development and function of bovine corpus luteum. *J Reprod Dev* 2001; 47: 283–294.
18. **Nishigai M, Kamomae H, Tanaka T, Kaneda Y**. Improvement of pregnancy rate in Japanese Black cows by administration of hCG to recipients of transferred frozen-thawed embryos. *Theriogenology* 2002; 58: 1597–1606.
19. **Hawk HW, Brinsfield TH, Turner CD, Whitmore GW, Norcross MA**. Effect of ovarian status on induced acute inflammatory responses in cattle uteri. *Am J Vet Res* 1964; 25: 362–366.
20. **Tervit HR, Cooper MW, Goold PG, Haszard GM**. Non-surgical embryo transfer in cattle. *Theriogenology* 1980; 13: 63–71.
21. **Sreenan JM, Diskin MG**. Factors affecting pregnancy rate following embryo transfer in the cow. *Theriogenology* 1987; 27: 99–113.
22. **Murata H, Hirose H**. Impairment of lymphocyte blastogenesis in road- transported calves observed with a whole blood culture technique. *Jpn J Vet Sci* 1990; 52: 183–185.
23. N**anda AS, Dobson H, Ward WR**. Relationship between an increase in plasma cortisol during transport-induced stress and failure of oestradiol to induce a luteining hormone surge in dairy cows. *Research in Veterinary Science* 1990; 49: 25–28.
24. **Albihn A, Gustafsson H, Rodriguez-Martinez H**. Maternal influence on the early development of asynchronously transferred bovine embryos. *Anim Reprod Sci* 1991; 24: 25–35.
25. **Hasler JF, MaCauley AD, Lathrop WF, Foote RH**. Effect of donor-embryo-recipient interactions on pregnancy rate in large-scale bovine embryo transfer program. *Theriogenology* 1987; 27: 139–168.
26. **Rowson LEA,Moor RM, Lawson RAS**. Fertility following egg transfer in the cow: effect of method, medium and synchronization of oestrus. *J Reprod Fertil* 1969; 18: 517–523.
27. **Rowson LEA, Lawson RAS, Moor RM, Baker AA**. Egg transfer in the cow: synchronization requirements. *J Reprod Fertil* 1972; 28: 427–446.
28. **Shneider HJ Jr, Castleberry RS, Griffin JL**. Commercial aspects of bovine embryo transfer. *Theriogenology* 1980; 13: 73–85.

29. **Tsunoda Y, Soma T, Sugie T**. Effect of post-ovulatory age of recipient on survival of frozen-thawed rabbit morulae. *J Reprod Fertil* 1982; 65: 483–487.
30. **Bulman DC, Lamming GE**. Milk progesterone levels in relation to conception, repeat breeding and factors influencing acyclicity in dairy cows. *J Reprod Fertil* 1978; 54: 447–458.
31. **Leslie E, McDonald S, Mcnutt SH, Nichols RE**. On the essentiality of the bovine corpus luteum of pregnancy. *Am J Vet Res* 1953: 539–541.
32. **Lamming GE, Darwash AO, Back HL**. Corpus luteum function in dairy cows and embryo mortality. *J Reprod Fertil Suppl* 1989; 37: 245–252.
33. **Lukaszewska J, Hansel W**. Corpus luteum maintenance during early pregnancy in the cow. *J Reprod Fertil* 1980; 59: 485–493.
34. **Gisert RD, Morgan GL, Short EC, Zavy MT**. Endocrine events associated with endometrial function and conceputus development in cattle. *Reprod Fertil Dev* 1992; 4: 301–305.
35. **Stubbings RB, Walton JS**. Relationship between plasma progesterone concentrations and pregnancy rates in cattle receiving either fresh or previously frozen embryos. *Theriogenology* 1986; 26: 145–155.
36. **Hasler JF, MaCauley AD, Lathrop WF, Foote RH**. Effect of donor-embryo-recipient interactions on pregnancy rate in large-scale bovine embryo transfer program. *Theriogenology* 1987; 27: 139–168.
37. **Sunagawa M, Kasahara T, Tsunoda R, Ohtsu S**. Morphological investigation of corpus luteum and plasma progesterone levels in bovine recipients. *Jpn J Anim Reprod* 1987; 33: 206–208 (In Japanese).
38. **Mann GE, Lamming GE, Fray MD**. Plasma oestradiol and progesterone during early pregnancy in the cow and the effects of treatment with buserelin. *Anim Reprod Sci* 1995; 37: 121–131.
39. **Maurer RR, Echternkamp SE**. Hormonal asynchrony and embryonic development. *Theriogenology* 1982; 17: 11–22.
40. **Kerbler TL, Buhr MM, Jordan LT, Leslie KE, Walton JS**. Relationship between maternal plasma progesterone concentration and interferon-tau synthesis by the conceptus in cattle. *Theriogenology* 1997; 47: 703–714.
41. **Veenhuizen EL, Wagner JF, Tonkinson LV**. Corpus luteum response to 6-chlro-17-aceto oxyproges-terone and hCG in cow. *Biol Reprod* 1972; 6: 270–276.
42. **Fricke PM, Reynolds LP, Redmer DA**. Effect of human chorionic gonadotrophin administered early in the estrous cycle on ovulation and subsequent luteal function in cows. *J Amin Sci* 1993; 71: 1242–1246.
43. **Walton JS, Holbert GW, Robinson NA, Leslie KE**. Effects of progesterone and human chorionic gonadotropin administration five days post insemination on plasma and milk concentrations of progesterone and pregnancy rates of normal and repeat breeder dairy cows. *Can J Vet Res* 1990; 54: 305–308.
44. **Kamomae H, Kaneda Y, Domeki I, Nakahara T**. Ovarian changes and plasma progesterone and estradiol-17β levels in ovarian quiescent heifers after treatment with hCG. *Japan J Amin Reprod* 1986; 32: 13–23 (In Japanese).
45. **Suzuki Y**. Hormone and ReproductionáU. Gakkai Publishing Center, Tokyo. 1979: 65–105.
46. **Kaneko H, Kishi H, Watanabe G, Taya K, Sasamoto S, Hasegawa Y**. Changes in plasma concentrations of immunoreactive inhibin, estradiol and FSH associated with follicular waves during the estrous cycle of the cow. *J Reprod Dev* 1995; 41: 311–320.