Effect of Mitogens, Supplement of β-mercaptoethanol and Fetal Bovine Serum Supplementation in Whole Blood Culture Medium for Bovine Chromosome Analysis

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ABSTRACT : We investigated the effects of the mitogen supplements of 3 types, pokeweed mitogen (PWM), phytohemagglutinin (PHA) and concanavalin A (ConA), to a whole blood culture system on the number of metaphase spreads obtained in perinatal bovine chromosome analysis. In addition, the supplementation of β-mercaptoethanol (β-ME) and FBS was examined in such system. Significant differences ($p<0.05$) were seen in the number of metaphase spreads with PHA stimulation compared to both PWM and ConA stimulation. When examined the effects of β-ME supplementation, the number of metaphase spreads was significantly ($p<0.05$) increased at 30 μM β-ME compared to control. When evaluated FBS supplementation during PWM stimulation, no significant effect of the supplementation was found. Finally, the effects of the cortisol concentration (10-20, 20-30 and >30 ng/ml) of the blood samples were examined. There was no significant effect of cortisol concentration ($p>0.05$) among these 3 cortisol concentration groups. The mean percentages of normal metaphase plates (2n=60) from each calf 1) with β-ME, 2) without β-ME and 3) with FBS stimulated with PWM were not significantly different ($p>0.05$). In conclusion, these findings may be useful in cytogenetic screening programs for not only perinatal calves but also for mature cattle. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 5 : 615-621)***

Key Words : Bovine, Chromosome Analysis, β-Mercaptoethanol, Mitogens, Metaphase Plates

INTRODUCTION

It is now widely accepted that cytogenetic investigation of domestic animals, especially of cattle, plays a significant role in the diagnosis of genetic disorders causing reproductive failure, such as Freemartinism, Robertsonian translocations and gonadal hypoplasia (Halnan, 1975; Miyake et al., 1987; Kondoh et al., 1992; Zhang et al., 1992; Llambi and Postiglioni, 1994; Hanada et al., 1995). Generally in the veterinary clinical field, the peripheral whole blood culture method has been widely used for screening of chromosome abnormalities in these cases because of its greater convenience compared with other methods using skin or bone marrow samples (Basrur and Gilman, 1964; Halnan, 1975). Recently, molecular biological techniques utilizing PCR or hybridization have become popular tools for cytogenetic diagnosis even in the veterinary clinical field (Fujishiro et al., 1995; Kawakura et al., 1996, 1997). As a result, the analysis of complex genomes at the molecular level has produced a wealth of data that is gradually revealing a tight relation between function and structure as reflected in cytogenetic banding patterns (Gardiner, 1995). Thus, progress in both molecular biological techniques and chromosome analysis is important for the purpose of the surveillance of and accumulation of basic knowledge about congenital chromosome abnormalities. However, there are cases in which it is impossible to observe metaphase spreads for chromosome analysis, especially in weak calves with asphyxia due to malformations, and the difficulty in obtaining metaphase spreads might be due to insufficient numbers of lymphocytes in the initial cultures or to a poor response of lymphocytes to mitogenic stimulation (Lin et al., 1976; Sakai et al., 1991; Takagi et al., 1992). Additionally, although applications of band staining methods for investigating and identifying chromosomes were reported as early as the 1970's in domestic animals (Halnan, 1976; Ford et al., 1980), they have not yet been carried out routinely in the veterinary clinical field mainly due to the lack of rapid and reliable techniques for chromosome analysis. The banding techniques are not always successful, but a minimal requirement of these techniques is the production of metaphase spreads of sufficient quality and quantity for the chromosome banding techniques (Lin et al., 1976). Therefore, it is very important for cytogenetic investigation of domestic animals, especially of cattle, to establish more effective whole blood culture systems that will increase the number of metaphase spreads obtained for chromosome analysis. Regarding this point, it was reported that the culture conditions of lymphocytes might affect the mitotic cells obtained for cytological preparations (Ray and Burine, 1967). Additionally, it was also reported that several factors might affect the mitogenic response or

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viability of cultured lymphocytes, including the type of supplemented mitogens (Renshaw et al., 1977; Ishikawa, 1987), low-molecular-weight thiol compounds such as β-mercaptoethanol (β-ME) (Fanger et al., 1970; Ishii et al., 1981a,b), lots of FBS (Renshaw et al., 1977) and corticosteroids (Claman, 1972; Guidry et al., 1976). Therefore, it seems very important to investigate in more detail the effects of the above-mentioned factors on the number of the metaphase spreads obtained from cultured lymphocytes and to establish a more effective whole blood culture system for chromosome analysis.

We therefore carried out a preliminary study of clinically normal perinatal calves to investigate the effects of 1) various types of mitogen, 2) supplementation of β-ME or FBS, and 3) the plasma cortisol concentration of the samples, on the number of metaphase spreads obtained for chromosome analysis. Additionally, the effect of supplementation of β-ME on the normality of the chromosome number (2n=60) of metaphase plates was examined.

MATERIALS AND METHODS

General experimental procedure

Blood sampling and chromosome preparation : Fifteen clinically normal perinatal Holstein calves, which were born between November 2000 and February 2001 at the University Farm, were used in this study. Additionally, 3 extra heifers aged from 3 to 8 months were used as controls. Heparinized blood samples (10 ml×2 tubes) were collected under sterile conditions within 12 h after birth. The blood samples were placed on ice and processed for lymphocyte culturing within 30 minutes after collection. For the purpose of chromosome analysis, the whole blood was cultured according to the procedure described by Basrur and Gilman (1964) with some modifications. Briefly, 1 ml whole blood samples were cultured under closed cap conditions at 38.5°C for 72 h in 4 ml of RPMI 1640 containing HEPES buffer and antibiotics (penicillin, 100 IU/ml; streptomycin, 100 μg/ml) in 15 ml conical test tubes (Falcon 2096). In a preliminary experiment, the effect of CO_2 concentration (open cap under 5% CO_2 in air or closed cap) during whole blood culturing was studied, and the number of metaphase spreads was not significantly different in the 2 groups (data not shown). Therefore, in the present study, the closed cap culture method was used. For each blood sample, the number of white blood cells (WBC) and the hematocrit were counted with a blood cell counter, and the lymphocyte counts were estimated from the WBC and hemogram values. Additionally, the plasma from each calf was separated by centrifugation at 1,500×g for 20 minutes and stored at -30°C for measuring the cortisol concentration. At 70 h of culture, colcemid (0.06 μg/ml; Demecolcine Solution, Sigma) was added to the culture medium. Cells were treated with hypotonic 0.75 M KCl solution for 10 to 15 minutes at 37°C, centrifuged at 450×g for 10 minutes and fixed in methanol: acetic acid (3:1 volume:volume) 2 times at room temperature for 30 minutes. After fixation, the pellet was resuspended in 1 ml of fixation medium, and 10 μl of the solution from each calf was dropped onto a cooled slide glass, and the whole metaphase plates on the slides were counted after Giemsa staining.

Cortisol extraction from plasma and hormone assay : The steorids contents were extracted from plasma samples (300 μl) by mixing with 3 volumes of diethyl ether. After evaporation of the diethylether, the residue was dissolved in 300 μl of assay buffer for steroid enzyme immunoassays (EIAs) (40 mM PBS, 0.1% BSA, pH 7.2). Then the samples were diluted to obtain a cortisol concentration within the optimal range of the standard curve. All samples from each calf were analyzed in the same assay in duplicate by a second antibody enzyme immunoassay (EIA) using 96 well ELISA plates (Corning Glass Works, Corning, NY). The procedure for the cortisol EIA was identical to that for the progesterone EIA described earlier (Miyamoto et al., 1992). Basically, standards or samples were incubated with 100 μl of polyclonal antibody (raised in rabbits against cortisol-21-hemisuccinate and donated by Dr. H.H.D. Meyer, Institut fur Physiologie, Freising-Weihenstephan, Germany) (1:50,000 and 100 μl of A-3-CMO-horseradish peroxidase (1:4,000; cortisol-HRP; 4-pregnen-11β, 17, 21-triol-3,20-dione 21-glucosiduronate; Steraloids, Inc, Newport, Rhode Island, USA) for 16 h at 4°C. A standard curve for concentrations ranging from 10 to 10,000 pg/ml was prepared, and the ED50 of the assay was 230 pg/ml. The intra-and interassay CVs were 6.8% and 8.1%, respectively. The cross-reactivities of the antibody were 100% for cortisol, 6.9% for corticosterone, 7.5% for 17a-dihydroxyprogesterone, 6.7% for 20 a-dihydroxy-progesterone, 4.7% for cortisone, 2.6% for deoxy-corticosterone, 0.03% for 5a-pregnanedione, 0.41% for androstenetrione, 1.51% for progesterone, 0.01% for pregnenolone and 0.0007% for estradiol. The average recovery rate of cortisol (1 ng/ml) that was added to plasma samples was 94.23% (n=10).

Experiments

Effect of type of mitogen on the number of metaphase spreads : Whole blood samples were cultured in RPMI 1640 medium supplemented with PWM, PHA or ConA as mitogen without FBS. The mitogen were used at concentrations determined to be suitable in a preliminary experiment as follows. Briefly, based on the concentrations of each mitogen recommended by the supplier, three different concentrations (PWM, Sigma, L-8777; 1, 2, 4 μg/ml; PHA-M, Sigma, L-2646; 5, 10, 20 μg/ml; ConA,

Sigma. C-0412: 10. 20. 40 μg/ml) were tested for chromosome analysis. and PWM (2 μg/ml). PHA (10 μg/ml) and ConA (20 μg/ml) were selected as suitable concentrations for chromosome analysis in this study. The number of metaphase spreads obtained from each mitogen group was determined.

Effects of β-ME and FBS on the number of metaphase spreads : In this experiment. we used PWM as the mitogen based on Renshaw et al. (1977) and on the results of our preliminary experiment showing a good mitogenic response of lymphocytes from newborn calves to PWM. Whole-blood samples were cultured in RPMI 1640 supplemented with 0 (control). 10. 20. 30. 50. 100 or 200 μM β-ME (Sigma. USA) without FBS. Additionally. whole-blood samples were cultured in RPMI 1640 supplemented with FBS (Bio whittaker. Maryland. USA) without β-ME. Preliminary results showed that 200 μM β-ME in the culture medium had a detrimental effect on the mitogenic activity. Therefore, we excluded this group and added one experimental group with 20 μM β-ME and 10% FBS in the culture medium. The number of metaphase spreads obtained from each β-ME concentration group was determined.

Effect of plasma cortisol concentration on the number of metaphase spreads : According to the cortisol concentration in their plasma. the calves were divided into 3 groups: 1) 10-20 ng/ml. 2) 20-30 ng/ml and 3) more than 30 ng/ml. The number of metaphase spreads obtained from each group was determined.

Evaluation of mitotic cell division : Preliminary experiments performed in duplicate cultures clearly showed that there were no significant differences between the number of metaphase spreads on the slides from the duplicate cultures (data not shown). Therefore. in this experiment. a single culture of each experimental group was used. A culture with PWM without β-ME was used as the control. Because of the large variation in the number of metaphase spreads obtained from different calves. the number of metaphase spreads obtained after treatment with different concentrations of β-ME was expressed relative to that obtained from the control (with PWM only). That is, the number of metaphase spreads obtained from the various experimental groups (supplementation with 10. 20. 30, 50, 100 and 200 μM β-ME, and PHA and ConA as mitogens) was divided by obtained from the control.

Evaluation of chromosome number of each metaphase plate : Twenty metaphase spreads (or in cases where that number was not available, the maximum number obtained on 1 slide glass) were photographed (×1,000) with a digital camera (Cool Pix 950. Nikon. Japan). We excluded metaphase plates in which we observed parts of other chromosome spreads around the metaphase spreads. The number of chromosomes in each photographed metaphase plate was counted by 2 people.

Statistical analysis

Pearson's correlation coefficient with p-value was determined to examine the correlation between the number of cultured lymphocytes and the number of metaphase plates (figure 1). Statistical analysis was carried out by repeated measures one-way analysis of variance (ANOVA) and post-hoc testing using STATVIEW (Abacus Concepts. Inc., Berkeley. CA. USA). All percentage data were subjected to arc sine transformation before statistical analysis. Data were considered to be significantly different at $p<0.05$.

RESULTS

Effect of the type of mitogen on the number of metaphase spreads

As shown in figure 1, there was a significant correlation between the number of lymphocytes cultured and the number of metaphase spreads obtained in the case of PWM stimulation (r=0.6, n=15) but not in the cases of PHA and

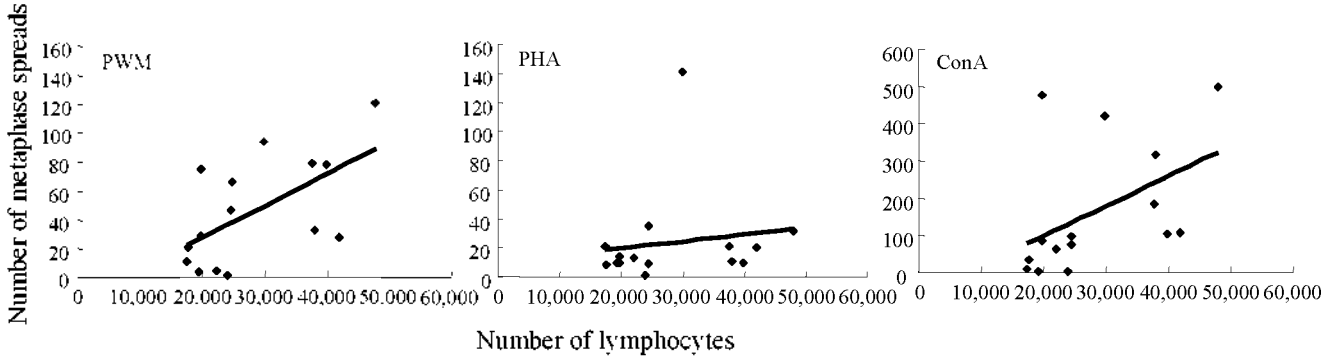

Figure 1. Correlation between the number of lymphocytes and the number of metaphase spreads on the slides prepared using 3 types of mitogen. The equations of the lines are PWM (n=15): y=0.0025x-20.051. r=0.6: PHA (n=15): y=0.0005x+9.0665. r=0.1; ConA (n=15): y=0.008x-67.337. r=0.5.

ConA mitogenic stimulation. The mean ratios between the number of metaphase spreads obtained from each mitogen group and the number obtained from the PWM group are shown in figure 2. Different stimulation patterns were seen in the calf and heifer groups depending on the type of mitogens. In calves, the stimulation ratio with ConA was significantly higher than that with PWM and PHA. On the other hand, in the heifers, the stimulation ratios with both PHA and ConA were significantly higher than that with PWM.

Effects of β-ME and FBS on the number of metaphase spreads

The mean numbers of metaphase spreads obtained with various concentrations of β-ME with PWM as mitogen are shown in figure 3. The mean number gradually increased up to 30 μM β-ME and gradually decreased thereafter as β-ME was increased to 200 μM. There was a significant difference between the number of spreads obtained without β-ME and with 30 μM β-ME. Supplementation of FBS or a mixture of FBS plus 20 μM β-ME in the culture medium had no significant effect compared with the control.

Effect of cortisol concentration on the number of the metaphase spreads

The calves were divided into 3 groups according to the plasma cortisol concentration. The mean cortisol concentrations of the 3 groups were 13.9±0.7 ng/ml, 23.9±1.4 ng/ml and 38.8±1.3 ng/ml. The cortisol concentrations of heifers and FBS were 3.1±1.3 ng/ml and 1.0 ng/ml, respectively. The mean mitotic index (no. of metaphase apreads/total no. of cultured lymphocytes) is

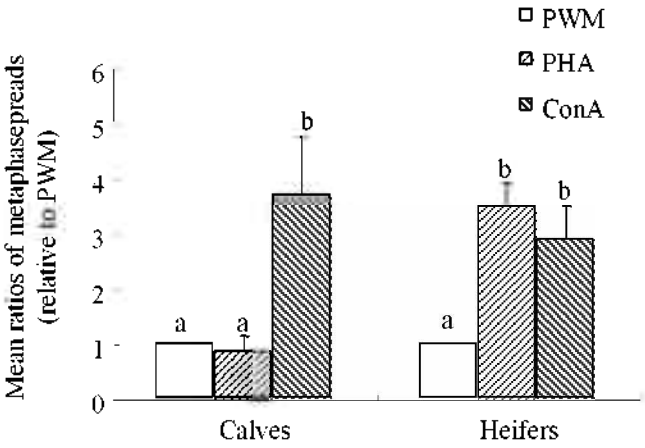

Figure 2. The mean ratios between the number of metaphase spreads (±SEM) obtained using various mitogens and that obtained using PWM with lymphocytes from newborn calves (n=14) and heifers (n=3).
a-b: Significantly different.

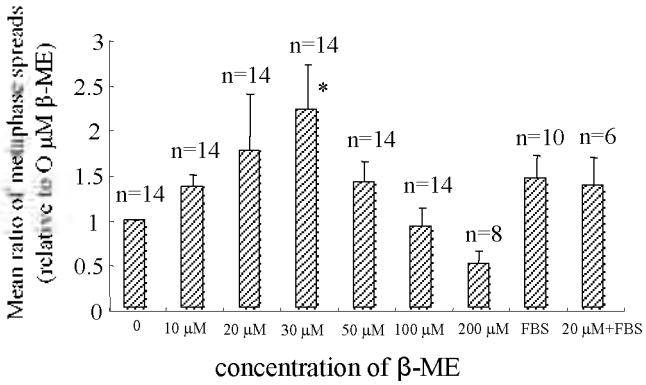

Figure 3. The mean ratios between the number of metaphase spreads (±SEM) obtained with various concentrations of β-ME and that obtained without β-ME (using PWM as mitogen).
* Significantly different compared with control.

shown in table 1. The mean mitotic index of heifers in the PHA stimulation group was significantly higher than that of all of the groups of calves. However, there were no significant differences depending on the cortisol concentration in the PWM and ConA stimulation groups. Figure 4 shows the effect of β-ME supplementation on the number of metaphase spreads with PWM stimulation in each group of calves categorized according to the plasma cortisol concentration. The number of metaphase spreads was not significantly different among the 3 cortisol-concentration groups at any concentration of β-ME. However, addition of up to 30 μM β-ME caused an increase of the number of metaphase spreads and higher concentrations (up to 200 μM β-ME) caused decreases of the number of metaphase spreads within all 3 groups.

Effects of β-ME and FBS on the chromosome number in the metaphase spreads

Five hundred and eighty-two pictures of the fine metaphase plates from 14 perinatal calves were taken (Due to the lack of metaphase spreads, it was not possible to

Table 1. The mean mitotic index (no. of metaphase spreads /total no. of lymphocytes) of calves divided into groups according to plasma cortisol concentration

Cortisol concentration (ng/ml)	Mitogen		
	PWM	PHA	ConA
10-20 (n=4)	0.0020	0.0007[a]	0.0080
20-30 (n=5)	0.0020	0.0014[a]	0.0055
30< (n=5)	0.0014	0.0004[a]	0.0044
Heifers (n=3) (3.1 ng/ml)	0.0023	0.0079[b]	0.0066

[a-b] Significantly different.

obtain data from 1 calf). The rate (%) of normal chromosome plates (2n=60) obtained from lymphocytes of each calf cultured with β-ME, without β-ME or with FBS are shown in table 2. The percentages of normal metaphase plates were not significantly different among the 3 groups. The average rates of normal metaphase spreads of the ME (+), ME (-) and FBS groups were 69.6% (156/224), 64.1% (139/217) and 70.2% (99/141), respectively.

DISCUSSION

These studies clearly demonstrated that the type of mitogen and supplementation of β-ME in the culture medium affected the number of metaphase spreads but did not affect the proportion of metaphase plates which had the normal chromosome number (2n=60) in chromosome analysis of perinatal calves.

PHA and ConA may have specific mitogenic effects on T-lymphocytes, while PWM is mitogenic for both T-and B-lymphocytes (Muscoplat et al., 1974; Renshaw et al., 1977). Although these 3 types of mitogens are commonly used for chromosome analysis, the choice of mitogen seems to depend on the laboratory rather than on more specific criteria. On the other hand, it has been reported that the mitogenic responses of the lymphocytes from perinatal calves varied depending on the mitogen which was used in the culture (Renshaw et al., 1977; Ishikawa, 1987). As shown in figure 1, our results clearly showed significantly different effects of each mitogen on the number of metaphase spreads. Although the mean number of metaphase spreads obtained with PWM stimulation was less than that with ConA stimulation, our results suggested that PWM as well as ConA might be suitable mitogens for chromosome analysis, especially for perinatal calves. In this study, the degree of correlation between the number of metaphase spreads and the total number of lymphocytes with PHA stimulation was lower than that with PWM or ConA stimulation. Also, as shown in figure 2, the patterns of the number of metaphase spreads obtained with PHA compared to PWM were different between calves and heifers. Previously, it was suggested that the 3 types of mitogens may stimulate different populations of lymphocytes (Renshaw et al., 1977). Therefore, although we did not directly observe the lymphocyte blastogenic responses of individual calves in this study, our findings of the different numbers of metaphase spreads may reflect the different lymphocyte blastogenic patterns of these 3 mitogens during the perinatal period. Additionally, the type of mitogen might affect the results of chromosome analysis depending on the age of the calf.

Table 2. The rate (%) of metaphase plates with normal numbers of chromosomes (2n=60) from calves whose whole blood was cultured with or without β-ME or with FBS

Calf No.	ME (-)	ME (+)	FBS
1	5/10 (50)	3/9 (33)	NT
2	4/6 (67)	2/6 (33)	NT
3	7/10 (70)	5/8 (63)	NT
4	11/14 (79)	4/10 (40)	NT
5	6/15 (40)	10/14 (71)	10/18 (56)
6	13/16 (81)	10/12 (83)	11/14 (79)
7	9/13 (69)	13/16 (81)	1/3 (33)
8	12/16 (75)	12/19 (63)	13/15 (87)
9	18/20 (90)	15/20 (75)	14/16 (88)
10	15/19 (79)	15/21 (71)	14/20 (70)
11	10/19 (53)	13/23 (57)	8/10 (80)
12	16/20 (80)	14/21 (67)	14/20 (70)
13	11/21 (52)	14/21 (67)	3/6 (50)
14	19/25 (76)	9/17 (53)	11/19 (58)
Mean	156/224 (70)	139/217 (64)	99/141 (70)

ME (-), without β-ME or FBS; ME (+), with β-ME without FBS; FBS, with FBS without β-ME; NT, Not tested.

β-ME is commonly used in *in vitro* lymphocyte cultures, mainly for promoting cell viability (Fanger et al., 1970; Ishii et al., 1981). In this study, with PWM as mitogen, we clearly found that the supplementation of β-ME in the culture medium increased the number of metaphase spreads in a dose-dependent manner, peaking at 30 μM. It has been reported that low molecular weight thiol compounds such as β-ME reduce cystine to cysteine and also promote the uptake of cysteine, resulting in enhancement of glutathione synthesis (Ishii et al., 1981a,b; Bannai, 1984; Issels et al., 1988). Glutathione is known to have important roles in maintaining the redox state of cells and in protecting cells against the harmful effects of oxidative stress (Meister, 1983). Therefore, although we did not examine the viability of the lymphocytes after 72 h of culture, it is possible that β-ME increased the number of metaphase spreads by increasing the survival of lymphocytes, or by enhancing mitogenic cell reactions in lymphocytes in our study. On the other hand, it has been reported that artifactual errors can be introduced in the conventional lymphocyte culture technique by the use of different lots of FBS because of variations in their ability to support lymphocyte transformation (Johnson, 1965; Muscoplat et al., 1974). It was suggested that calves might be divided into high and low mitogen responder groups with respect to lymphocyte blastogenesis, perhaps due to genetic differences in the immunological response (Whitbread, 1986). In the present study, all lymphocytes were incubated in an autologous serum source without FBS supplementation. Although it is clear that we need to further examine the effects of various lots of FBS, our results suggested that supplementation of a suitable concentration of β-ME in the culture medium of the whole blood culture system might be more effective than

supplementation of FBS.

Corticosteroids are known to inhibit lymphocyte activation (Claman, 1972; Guidry et al., 1976) and it was suggested that the cellular immune capacity of the newborn calf is compromised due to increased plasma glucocorticoid levels in the perinatal period (Osburn, 1973; Osburn et al., 1974). In the present study, the cortisol concentrations of 15 calves ranged from 14 ng/ml to 45.5 ng/ml, which were much higher than those of the control heifers. As shown in table 1, the numbers of metaphase spreads were not significantly different between calves and heifers regardless of the cortisol concentration levels, except for in the case of heifers compared to calves with PHA stimulation. These results may suggest different mechanisms of PHA stimulation of lymphocytes derived from perinatal calves and adult cattles, as we mentioned above. As shown in figure 4, although the number of metaphase spreads did not differ significantly among the cortisol-concentration groups at any, a similar tendency to that seen in figure 3 was observed within each cortisol-concentration group. These findings clearly indicate that not the cortisol concentration but some other unknown factor(s) must cause the low mitogenic activity or low incidence of chromosome plate appearance in certain calves.

The 15 calves whose chromosome numbers were examined in the present study were clinically normal. In this study, no chromosome abnormalities such as chimerism, mosaicism, gaps, or translocations were observed. However, as shown in table 2, there was a tendency for the number of normal chromosomal plates (2n=60) to decrease in cases in which the numbers of countable metaphase plates were low (less than 10). This strongly confirms the conclusion that the greater the number of countable metaphase plates, the more accurate the determination of the chromosome number. The results of this study indicate that there was no significant difference of the rate of normal chromosome number plates (about 70%) regardless of supplementation with β-ME or FBS. Takahashi et al. (1993) reported that normal pregnancy was observed after the transfer to a recipient of blastocysts obtained by *in vitro* culturing with β-ME. Therefore, addition of β-ME to the culture medium appears not to affect the chromosome structure or karyotype.

In conclusion, the results of the present study with clinically normal perinatal calves clearly indicated the usefulness of the whole blood culture system without FBS when supplemented with a suitable concentration of β-ME to increase the number of the metaphase spreads for chromosome analysis. The type of mitogen used in the culture may affect the number of metaphase spreads obtained, but the plasma cortisol concentration of the sample does not affect this number. However, some clinically normal calves produce few metaphase spreads for chromosome analysis. Therefore, further research is needed to identify factors which may affect the results of chromosome analysis.

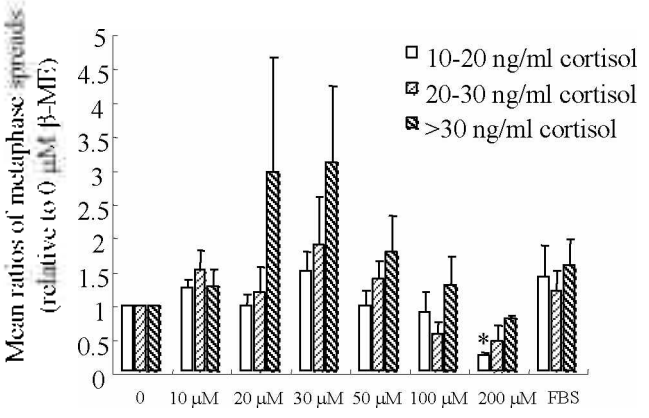

Figure 4. The mean ratios between the number of metaphase spreads (±SEM) obtained from the 3 cortisol-concentration groups with and without β-ME. 10-20 ng/ml, n=4; 20-30 ng/ml, n=5; >30 ng/ml, n=5.

The mean numbers of metaphase spreads (±SEM) of each group without β-ME were 46.3±13.4 (10-20 ng/ml), 64.8±25.6 (20-30 ng/ml) and 48.6±16.4 (>30 ng/ml).

* Significantly different compared with control.

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