EVALUATION OF SEX CHROMATIN OF CALF'S NEUTROPHILS AS A DIAGNOSTIC TOOL FOR BOVINE FREEMARTINISM AT THEIR EARLY LIFE

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Summary

Bovine drumstick of neutrophil leucocytes was studied on the quantitative and morphological characteristics and was evaluated as a diagnostic measure for bovine freemartin in newborn calves. Nuclear area of neutrophil (A, μm^2) and drumstick area (B, μm^2) were significantly correlated with average diameter of drumstick (ADD, μm) and following regression equations were obtained:
$A = 45(\pm 3)\ ADD - 8$, $r = 0.74$, s.e. ± 0.6, $p < 0.01$,
$B = 1.72(\pm 0.05)\ ADD - 0.98$, $r = 0.93$, s.e. ± 0.1, $p < 0.01$.
Eight female siblings of heterosexual multiplets were diagnosed as freemartin from the results of chromosome analysis. Heterosexual multiplets had a very low frequency of drumstick in the nucleus of neutrophils irrespective of genetic sex. Diameters of drumstick found in freemartin and male cotwin did not differ from those of normal cows. Examinations of drumstick in 800 neutrophils for both female and male siblings are concluded to be the best way to aid the detection of freemartinism of heterosexual twins at early life.
(**Key Words**: Sex Chromatin, Heterosexual Twin, Freemartin, Drumstick, Calf)

Introduction

The causes of freemartinism have not been completely understood. More than 90% of twining females are infertile because of chorionic vascular anastomosis with a resultant common circulation in the twin fetuses. Thus, both male and female fetuses show male and female populations of leucocytes and erythrocytes in their blood cells. A genetical female with a karyotype of a mixture of XX and XY chromosomes has been reported in cattle, horses, sheep, goats and swine to exhibit freemartinism (Bruere and Macnab, 1968, Harvey, 1976, Macrum, 1974). Blood grouping to detect the presence of two red cell populations and analysis of chromosomes may be the most accurate diagnostic procedure for the detection of freemartinism. These methods, however, require sophisticated skills and equipments. Bhatia and Shanker (1983) investigated the sex chromatin of the peripheral blood neutrophils of six bovine freemartins and suggested the drumstick being a useful tool for the detection of freemartin. Studies on the drumstick in calves are scarce, there are, however, few reports on quantitative and morphological characteristics of bovine drumsticks (Bhatia et al., 1982, Bhatia and Shanker, 1985, Onuma, 1964).

The present study was carried out to estimate the quantitative and morphological characteristics of bovine drumstick and to evaluate the drumstick as a diagnostic tool for the identification of bovine freemartinism in newborn calves.

Materials and Methods

Experiment 1

Sixty seven Holstein cows proved to be fertile were used in the investigation of sex chromatin. The mean age of the cows was 4.3 ± 2.4 years. Blood samples were collected from the caudal auricular vein and blood smears prepared were dried and fixed in methanol and subsequently stained by Leishman's solution for 10 min. The stained smears were washed with phosphate buffer solution (pH 7.2) until a pink color appeared. The smears were then stained with Giemsa solution for 20 min and washed thoroughly by tap water. The smears were examined under a light microscope having oil immersion. Microscopic examinations were carried out for 200 neutrophils. The

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widest and smallest diameters of both the drumstick and nucleus of the neutrophils were determined as follows. A standard line measured with the help of an ocular micrometer was set for each nucleus and drumstick. The drumstick and nucleus were photographed and enlarged to an appropriate size. The enlarged photograph was then set on a digitizing equipment and measured for the standard line, diameters and circumference of drumstick and nucleus. The measured values were fed into a microcomputor to calculate the area of the nucleus (A), the drumstick (B), the widest, smallest and mean diameters of drumstick and the ratio of A to B.

Experiment 2

A total of 8 heterosexual bovine multiplets born in farms in the vicinity of Tottori city located in north-western part of Japanese mainland, were used in this experiment of which one case triplets and the others were twins. Samples of 2 male cotwins in 2 twins were not obtained because in one case the male cotwin died and the other was sold before sampling. Thus, number of animals examined totaled 15 calves at the age less than 5 months except for female of No. 1 multiplet. In this trial, chromosome analysis was carried out together with sex chromatin analysis. The procedure of sex chromatin analysis was the same as that of Experiment 1 except for the number of neutrophils examined. As the frequency of drumstick was very low in 200 neutrophils, the examination was extended to include 800 neutrophils but the frequency of drumstick was calculated on the basis of 200 neutrophils. The chromosome analysis was carried out for 50 metaphase neutrophils in each animal.

Results

Experiment 1

Figure 1 shows the distribution of the drumstick frequency in normal cows. More than one drumstick was found in each specimen. The frequency of 2 drumsticks per 200 neutrophils was observed in 24 cows. Frequencies of 1 to 4 drumsticks per 200 neutrophils were observed in 62 cows (94%). The frequency of drumstick averaged 2.7 (S.D., 1.3) per 200 neutrophils of each cow. Table 1 shows mean dimensions of drumstick and neutrophil nucleus in normal cows.

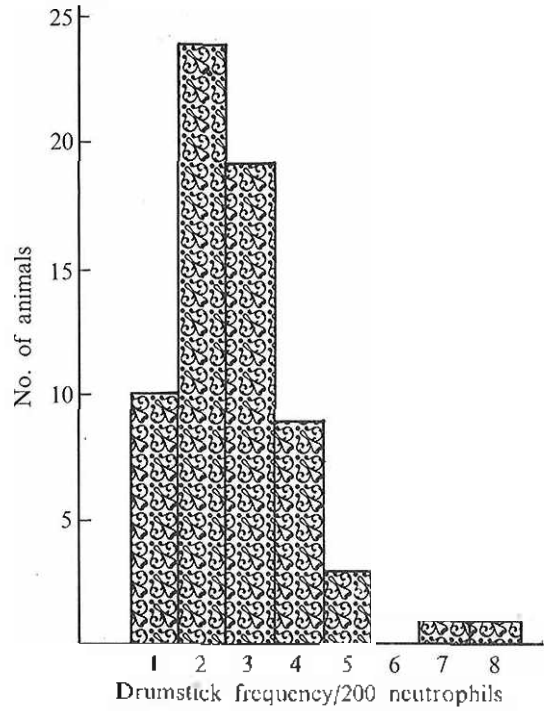

Figure 1. Distribution of drumsticks in normal female cattle.

The nuclear area (A) averaged 50 μm^2 with range from 31 to 71 μm^2. Drumstick area (B) ranged from 0.68 to 1.98 μm^2 with the mean value of 1.24 ± 0.33 μm^2. Mean ratio of A/B and average diameter of drumstick were 41 ± 7 and 1.29 ± 0.18 μm, respectively. Drumstick area (B, μm^2) was significantly correlated with average diameter of drumstick (ADD, μm) ($r = 0.93$, $p < 0.01$). Regression analysis gave the following equation:

$$B = 1.72\ (+0.05)\ ADD - 0.97,\ r^2 = 0.86,\ s.e. \pm 0.1 \quad \cdots\cdots (1)$$

Also, ADD (μm) was significantly and positively correlated with nuclear area (A, μm^2) ($r = 0.74$, $p < 0.01$). The following regression equation was obtained:

$$A = 45\ (\pm 3)\ ADD - 8,\ r^2 = 0.55,\ s.e. \pm 0.6 \quad \cdots\cdots (2)$$

Experiment 2

Results of chromosome analyses are shown in table 2. Each animal showed a chimerism of karyotype with diverse extents. The degree of chimerism ranged from 24% of XX cells to 74 % for female calves. No consistent degree of chimerism was found among multiplets or breeds.

TABLE 1. MEAN DIMENSIONS OF DRUMSTICKS AND NEUTROPHIL NUCLEI IN NORMAL FEMALE CATTLE

	Nuclear area (A) (μm^2)	Drumstick area (B) (μm^2)	Ratio of A/B	Diameter of drumstick[1] (μm)
mean ± SD	50 ± 11	1.24 ± 0.33	41 ± 7	1.29 ± 0.18
Maximum	71	1.98	55	1.69
Minimum	31	0.68	26	0.91

[1] Average diameter calculated by dividing the sum of the widest and smallest diameters by 2.

TABLE 2. RESULTS OF CHROMOSOME ANALYSES OF 50 CELLS FOR INDIVIDUALS OF MULTIPLETS

Multiplet	Breed	Sex	Age	XX cells (%)	XY cells (%)
No. 1	H[1]	Female	1.5 yr	24	76
No. 2	JB	Female	5 mo	42	58
No. 3	H	Female	2 wk	74	26
	H	Male	2 wk	66	34
No. 4	JB	Female	3 mo	56	44
	JB	Male	3 mo	54	46
No. 5	H	Female	2 mo	62	38
	H	Male	2 mo	58	42
No. 6	H	Female	2 wk	44	56
	H	Male	2 wk	46	54
No. 7	H	Female	2 wk	40	60
	H	Male	2 wk	38	62
No. 8	JB	Female 1	2 mo	30	70
	JB	Female 2	2 mo	26	74
	JB	Male	2 mo	32	68

[1] Abbreviated notations are as follows: H, Holstein and JB, Japanese Black cattle.

The percentage of XX cells or XY cells was similar between female and male cotwins in the same multiplets irrespective of twins or triplet. Female cotwins in the present study were diagnosed as freemartin based on the results of the karyotype analysis.

Table 3 shows the mean dimension of the drumsticks and neutrophil nuclei in freemartins and male cotwins together with normal cows. There were no significant differences in mean diameters of drumsticks and neutrophil nuclei among animal groups. The drumstick area in

TABLE 3. MEAN DIMENSIONS WITH STANDARD DEVIATION OF DRUMSTICKS AND NEUTROPHIL NUCLEI IN NORMAL COWS, FREEMARTINS AND MALE COTWINS

	No. of drumstick	Nuclear area (A) (μm^2)	Drumstick area (B) (μm^2)	Ratio of A/B	Diameter of drumstick (μm)
Normal cow	163(67)[1]	50 ± 11	1.24 ± 0.33	41 ± 7	1.29 ± 0.18
Freemartin	8(9)	50 ± 9	1.17 ± 0.16	43 ± 5	1.29 ± 0.08
Male cotwin	7(6)	50 ± 16	1.25 ± 0.03	40 ± 7	1.30 ± 0.17

[1] Figures in the parentheses denote the number of animals examined.

freemartin tended to be smaller than male cotwin and normal cows, although statistical significance was not proved. When 200 neutrophils were examined in each animal, only 5 drumsticks were found among 15 animals of the multiplets. No drumstick was found in 7 freemartins out of 9 and 3 male cotwins out of 6. Thus, at least 800 neutrophils should be examined. Heterosexual multiplets showed very low frequency of drumstick irrespective of genetical sex. In extreme cases, no drumstick was found in 800 neutrophils examined as in female No. 6 multiplet and female 2 of No. 8. All male cotwins had a drumstick which is not found in normal male calves because it has been considered as a product of inactivated female sex chromosome. Individuals of multiplets with lower drumstick frequency showed lower percentage of XX cells. A highly significant correlation was observed between drumstick frequency and percentage of XX cells ($r = 0.64$, $p < 0.01$). The mean frequencies of drumstick are presented in table 4. Drumstick frequency was not significantly different between freemartin and male cotwin, but was highly significant between normal cows and freemartin or male cotwin.

TABLE 4. MEAN DRUMSTICK FREQUENCY OF FREEMARTINS AND MALE COTWINS TOGETHER WITH NORMAL COWS

	No. of animal	Drumsticks/200 neutrophils	
		Mean	SD[1]
Normal cow	67	2.70[b]	1.35
Freemartin	9	0.43[a2]	0.40
Male cotwin	6	0.44[a]	0.19

[1] Standard deviation.

[2] Means with different superscripts are significantly different ($p < 0.01$).

Discussion

The mean area of neutrophil nucleus ($50 \pm 11\ \mu m^2$) observed in the present study was similar to that reported by Bhatia and Shanker (1985). Mean drumstick area appeared to be smaller in the present study than the reported values for Brown Swiss, Jersey and Holstein (Bhatia and Shanker, 1985). Individual values varied from 0.68 to 1.98 μm^2 in the present study. Reported values for drumstick ranged from 0.68 to 1.54 μm^2 (Bhatia and Shanker, 1985). Onuma (1964) reported that drumstick area was consistent in the most of mammals. There was a slight variation in drumstick size because of the difference in X chromosome size (Bhatia et al., 1982). From the result of the present study and those reported, it can be inferred that there is no difference in drumstick area among breeds of animals.

Average diameter of drumstick positively correlated with the drumstick area or the nuclear area and the nuclear area significantly regressed on average diameter of drumstick as shown in the equation 2. A larger drumstick appeared to be formed in a larger nucleus of neutrophils. Therefore, the relationship between the average diameter of drumstick and nuclear area of the neutrophil or the ratio of A/B may indicate whether a drumstick is normal. The result obtained in the present study (41 ± 7) is similar to that reported by Bhatia and Shanker (1985, 40.72 ± 2.32). Results obtained in other animals were $61.70 + 3.56$ for goats, 67.31 ± 4.54 for zebu cattle and 78.49 ± 5.97 for buffaloes (Bhatia and Shanker, 1985). These results were higher than the results obtained in European cattle (*Bos taurus*) by the present study and Bhatia and Shanker (1985). The drumstick area is inferred to be constant. Thus, the nucleus of neutrophils may differ in size among animals, which may have reflected to the difference in the ratio of A/B. Mukherjee and Singh (1974) studied quantitative characteristics of sex chromatin and stated that the A/B ratio showed a specific value with a variation range indigenous to a breed of cattle. Average diameter of drumstick was 1.29 ± 0.18

μm in the present study, which agreed with that obtained for European breed cattle by Bhatia and Shanker (1985). Thus, the diameter of drumstick appears to be about the same in European breed cattle.

The result of chromosome analysis showed that all individuals had both XX cells and XY cells of neutrophils. The degree of chimerism was the same between genetical female and male cotwins in the same multiplet. This evidence proves that chorionic anastomosis between heterosexual fetuses which share a common blood circulation to a diverse extent. Freemartinism has been widely recognized to occur only in the female fetus. Male cotwin of heterosexual twins, however, may exhibit reproductive disturbances (Shanker and Bhatia, 1983a). Shanker and Bhatia (1983b) found that a male sibling of heterosexual twins showed poor libido at its maturity and had an exceptionally high proportion of dead sperms in the ejaculated semen. In the present study, reproductive disorders for male siblings were not examined. Thus, the reproductive function of the male cotwins used in the present study was not clarified. The result of chromosome analysis, however, suggests a poor functioning of gonads of male with a high percentage of XX cells. The study on relationship between the chimerism of neutrophils and reproductive disorders may be required for male siblings of heterosexual twins. In this case, sex chromatin analysis may be utilized as a screening tool because the degree of chimerism of neutrophils significantly correlated with the frequency of drumstick.

Dimensions of drumsticks and neutrophil nuclei did not differ significantly among freemartins, male siblings and normal cows. Thus, it is concluded that these characteristics are not influenced by the chimerism of neutrophils. The chimerism, however, resulted in an incidence of drumstick in male siblings. This result shows that the chimerism of neutrophils in female siblings is proven by the appearance of drumstick in the neutrophils of male siblings. Thus, the examination of drumstick in a male cotwin of heterosexual twins may be able to predict freemartin in a female sibling. For the prediction of freemartinism, neutrophils should be examined up to 800 cells to find a drumstick in a male sibling. Normal cows, however, possessed 2.7 drumsticks in 200 neutrophils on average or at least one drumstick in 200 cells. Thus, examinations of drumstick in neutrophils for both female and male siblings may be the best way to aid the early detection of the reproductive malfunction of heterosexual twins. The frequency of drumstick was significantly lower in freemartin than the normal cow. Large numbers of neutrophils have to be analyzed for drumstick when the male sibling is not available for sampling because of its death *in utero* or after birth. In this case, up to 800 neutrophils have to be examined as in case of male sibling. If the frequency of drumstick are proven to be 0.4/200 neutrophils or less, the female offspring of heterosexual multiplets may be diagnosed as freemartin. When the drumstick examination is combined with the chromosome analysis, the accuracy of the diagnosis is increased to a higher level.

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