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**CHROMOSOME STUDIES  
ON HETEROSEXUAL TWINS IN CATTLE**

**II SIGNIFICANCE OF SEX-CHROMOSOME CHIMERISM (XX/XY)  
IN EARLY DIAGNOSIS OF FREEMARTIN**

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In the previous paper<sup>5)</sup>, the present authors reported that there existed sex-chromosome chimerism (XX/XY) in both freemartins and their male co-twins, and that this phenomenon may be due to a cell interchange between them as a result of vascular anastomosis in the chorionic blood vessels.

The main purpose of the present work is to elucidate further the incidental rate of the chimerism between freemartins and their co-twins, and thus give a fundamental basis for employing the blood culture technique for early diagnosis of the freemartin.

**MATERIALS**

Six pairs of heterosexual twins were used in the present study, together with 5 non-freemartin heterosexual twin females having one or more calvings, as controls. Some of the animals were those used in the previous study<sup>5)</sup>. The source of samples for chromosomal study was blood, but in one case blood and bone marrow. An account of materials, together with chromosomal data, is given in table 1.

**METHODS**

The procedures for leukocyte culture and short-term culture of bone marrow cells were principally similar to those used in the previous study<sup>5)</sup>. But, partially, the procedure for leukocyte culture was revised, as shown schematically in figure 1. For chromosome preparations, the air-drying method by ROTHFELS and SIMINOVITCH was employed.

The culture medium of the present study was based on the formula of MAKINO et al. which was partly modified from that of MCCOY et al. The medium contains 14 different kinds of amino acids and 8 kinds of vitamins dissolved in EARL's balanced salt solution. The chemical components of the medium are shown in table 2.

TABLE 1 *Materials and results of chromosome counts in blood of heterosexual twins (Holstein)*

NO.	ANIMAL NUMBER	AGE	MALE			FREEMARTIN		
			No. of cells counted	Sex-chromosome		No. of cells counted	Sex-chromosome	
				XX	XY		XX	XY
		days						
1	F 21	Fetus	51	20	31	53	19	34
2	F 18	3	51	16	35	53	19	34
3	F 19	3	50	35	15	50	32	18
4* <sup>1</sup>	F 8	16	65	33	32	74	45	29
			55* <sup>2</sup>	34	21	57* <sup>2</sup>	32	25
5* <sup>1</sup>	F 10	25	58	12	46	56	13	43
6* <sup>1</sup>	F 1	169	146	133	13	111	98	13
NO.	ANIMAL NUMBER	AGE	MALE			NON-FREEMARTIN		
		yr. mo.						
7	F 12	3. 1	—			55	55	0
8	F 11	3. 8	—			214	214	0
9	F 17	6.10	—			50	50	0
10* <sup>1</sup>	F 9	7. 2	—			75	75	0
11	F 15	10. 2	—			65	65	0

Notes : \*<sup>2</sup> Previously reported case

\*<sup>2</sup> Bone marrow cells

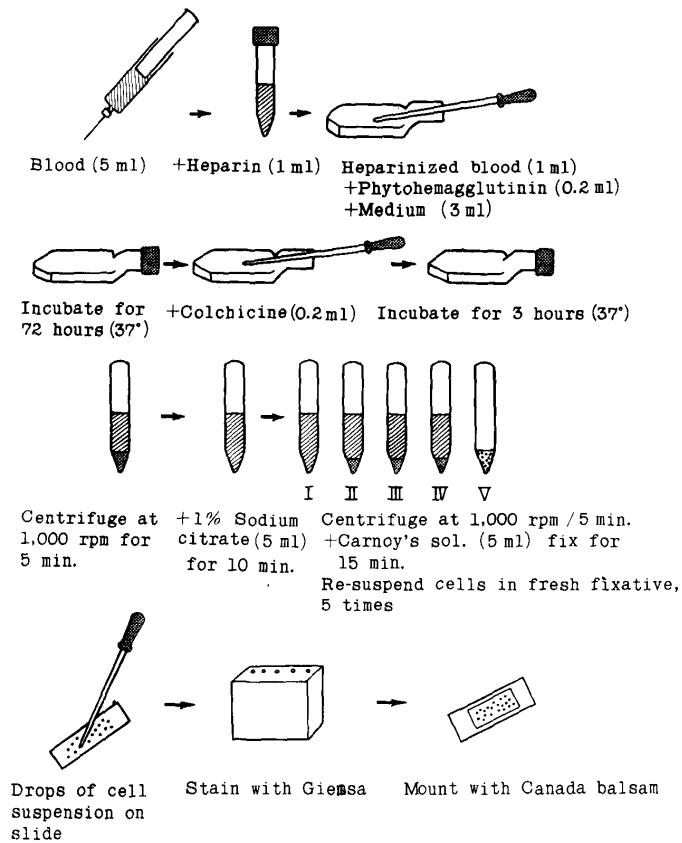
— Not examined

TABLE 2 Chemical components of culture medium

I Balanced salt solution (B. S. S.)		
1	Sodium chloride (NaCl)	68.0 g
	Potassium chloride (KCl)	4.0
	Sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ )	1.52
	Sodium bicarbonate ( $\text{NaHCO}_3$ )	22.0
	Dextrose	10.0
	Triple distilled water (T. D. W.)	1 000.0 ml
2	Calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ )	2.6 g
	Magnesium sulfate ( $\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$ )	2.0
	T. D. W.	1 000.0 ml
II Amino acids		
	l-arginic HCl	0.420 g
	l-histidine HCl·H <sub>2</sub> O	0.209
	l-lysine HCl	0.364
	dl-threonine	0.357
	dl-valine	0.351
	l-leucine	0.393
	l-isoleucine	0.393
	dl-phenylalanine	0.330
	l-tyrosine	0.181
	dl-tryptophane	0.061
	dl-methionine	0.298
	l-cysteine HCl	0.242
	l-glutamine	2.193
	l-asparagine H <sub>2</sub> O	0.504
	T. D. W.	1 000.0 ml
III Vitamins		
	Thiamine hydrochloride	0.001 g
	Choline chloride	0.001
	Calcium pantotenate	0.001
	Folic acid	0.001
	Pyridoxamine dihydrochloride	0.001
	Nicotinamide	0.001
	Biotin	0.001
	Riboflavin	0.0001
	T. D. W.	100.0 ml
IV Culture medium		
	B. S. S. 1	250.0 ml
	T. D. W.	1,450.0
	Amino acids	250.0
	Vitamins	25.0
	B. S. S. 2	250.0
	Penicillin	200 000.0 units
	Phenol red	0.5 ml

Notes: Adjust pH with 1N NaOH or 1N HCl (pH 7.3).

Filter the combined solution through a SEITZ filter of No. 85.

FIGURE 1 *Blood culture technique*

## RESULTS

In table 1 are presented chromosomal findings involving the ratio of XX-cells to XY-cells obtained from blood cultures and bone marrow cells of heterosexual twins. Both freemartins and their co-twins (Nos. 1~6) showed 2A-XX/2A-XY chimerism without exception. The chimera ratio varied considerably with each individual, but it tended to parallel among co-twins. In order to ascertain this tendency statistically, chi-square tests were used. In each pair of co-twins, the chi-square value showed no significant difference ( $P < 0.05$ ) either in leukocytes or bone marrow cells. Further statistical analysis, by means of chi-square tests, between the chimera ratios in leukocytes and bone marrow cells in No. 4 failed to show any evidence of significant difference ( $P < 0.05$ ).

On the other hand, observations of 5 normal cows (Nos. 7~11) from the non-freemartin heterosexual twins as controls revealed a normal female complement with an XX-mechanism in cultured leukocytes without exception.

## DISCUSSION

The pioneer works by KELLER & TANDLER, and also LILLIE<sup>7,8)</sup> showed that

in heterosexual twins in cattle with fused chorionic membranes and vascular anastomosis between dizygotic twins early in embryonic life, the male individual exerts a very characteristic influence on the differentiation and the development of the gonads of its female co-twin. Thus, the male is normal at birth but the female has usually been modified into a sterile freemartin. It is generally accepted that intersexuality of the freemartin in cattle twins may be interpreted as a result of the influence of sex hormones produced by the gonad in the male, so that the female acquires male characteristics although its genetic sex remains unchanged. It must be admitted that this theory is an attractive one, but so far it is entirely lacking in experimental support. So, ASDELL stated that LILLIE's hormone theory has been criticized on several points as follows: Firstly, no one has shown that the male fetus produces hormones at the early stage of gestation which could modify the sexual differentiation of the female fetus. Secondly, vascular anastomoses of the chorionic blood vessels have been found in other species in which the freemartin condition does not occur. Thirdly, injection of sex hormones into pregnant animals of other species has modified the development of the accessory sex organs but never that of the gonads themselves.

It is interesting to note that in the present investigation sex-chromosome chimerism was detected in cultured leukocytes and bone marrow cells in all the examined cases of freemartins and their co-twins. This makes it impossible to interpret thoroughly the etiology of the freemartin only by the hormone theory postulated by LILLIE<sup>7,8)</sup>, because it is commonly believed that in Mammalia some hormones may be able to interfere with the sex differentiation and change the phenotype sexuality, but may be unable to exert an effect on changing genotype conditions. Therefore, a new interpretation should be postulated for explaining the etiology of the freemartin condition.

It should be reasonable to think that some ancestral cells with the ability to produce leukocytes may be transported by the blood stream from the male fetus to the female or inversely, through anastomosed chorionic blood vessels, and that the ability will be maintained through postnatal life. This assumption is strongly supported by the fact of the high incidence of chromosome chimerism in bone marrow cells. In addition, blood typing studies by OWEN, HOSODA et al. and SCHINDLER have shown that establishment of a vascular anastomosis results in a common circulation and an interchange of cells which are ancestral to the hemopoietic cells of postnatal life. Thus, these authors considered co-twin calves as so-called erythrocyte mosaic individuals.

One of characteristic findings in the present study is the fact that the chimera ratios tend to parallel with each other among co-twin individuals. The question arises whether the degree of vascular anastomosis of the fetal membrane or function

of the heart in twin fetuses might have an effect on the extent of XX/XY ratio. If the freemartin condition is mainly due to the effect of the Y chromosome which was transferred from a male partner, the degree of deviation in the development of the sex organs of a freemartin might probably be quantitatively controlled by the number of Y chromosomes that migrated, in other words, by the extent of cell chimerism. In order to clarify these problems, however, much more work will be required.

From the viewpoint of diagnosis of freemartins, many investigators and practitioners have paid attention to the clinical signs, such as abnormal development of the clitoris, the small vulva, the plentiful vulvar hair, short length of the vagina, lack of estrus or arrested development of the udder and teats<sup>1,3,11,15</sup>). These clinical diagnoses are sometimes uncertain because occasionally there are freemartins with almost normal external sex organs. In such cases, a decisive diagnosis cannot be made until 6 months or more, when a rectal examination of internal sex organs can be allowed or the first estrus can be checked. This may cause the owner considerable economic loss. Recently, HOSODA et al. and also SCHINDLER postulated that detection of erythrocyte mosaic by means of blood type analysis can be employed for an accurate diagnosis of freemartins. Blood typing in cattle, however, requires considerably complicated techniques.

It is hoped to be able to use chromosome analysis with leukocyte culture for a complete early diagnosis of the freemartin, because all of the freemartins studied were of sex-chromosome chimerism without exception, while no chimerism was observed in non-freemartin heterosexual twins which had calved once or more.

Another interesting aspect concerns the male co-twin with a freemartin. It seems reasonable to distinguish cytologically such males from single born males or homosexual twins, because the former was of sex-chromosome chimerism and the latter was not. Further study is needed to ascertain whether the chimerism produces any effect on the fertility of the male twin or of his offspring.

#### SUMMARY

Chromosome analysis was carried out on 6 pairs of heterosexual twin cattle and also 5 normal cows from non-freemartin heterosexual twins. Culture of leukocytes from blood and colchicine treatment of bone marrow cells were used.

The results may be summarized as follows:

- 1) Sex-chromosome chimerism (XX/XY) was detected in all cases of 6 pairs of the freemartin and its male co-twin. The statistical analyses between XX/XY ratios in freemartins and their co-twins, and also the ratios in leukocytes and bone marrow cells showed no significant differences ( $P < 0.05$ ).

- 2) In all cases of 5 normal cows from the non-freemartin heterosexual twins

as controls, no sex-chromosome chimerism was observed. Thus, it is hoped to be able to use chromosome analysis with leukocyte culture as an accurate early diagnostic method for freemartins.

3) It seems reasonable to think that the male calf co-twin with a freemartin may be cytologically distinguished from single born or homosexual twin males, because the former was of sex-chromosome chimerism.

4) Basic mechanisms of bovine freemartinism were discussed from the viewpoint of chromosomal cytology.

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## EXPLANATION OF PLATES

### PLATE I

- Fig. 2 Completely fused chorio-allantois with anastomosed blood vessels of No. 1  
(scale: cm)
- Fig. 3 Heterosexual co-twin fetuses  
Male (upper) and freemartin (bottom) of No. 1  
(scale: cm)
- Fig. 4 Dorsal view of sex organs of freemartin of No. 1  
BG: Bilateral gonads  
UT: Uterus  
SV: Seminal vesicle-like structures  
VA: Vagina  
CL: Clitoris
- Fig. 5 Sex-chromosome chimerism in blood culture from the above freemartin  
Arrows show XX (upper) and XY (bottom).  
× 1,000

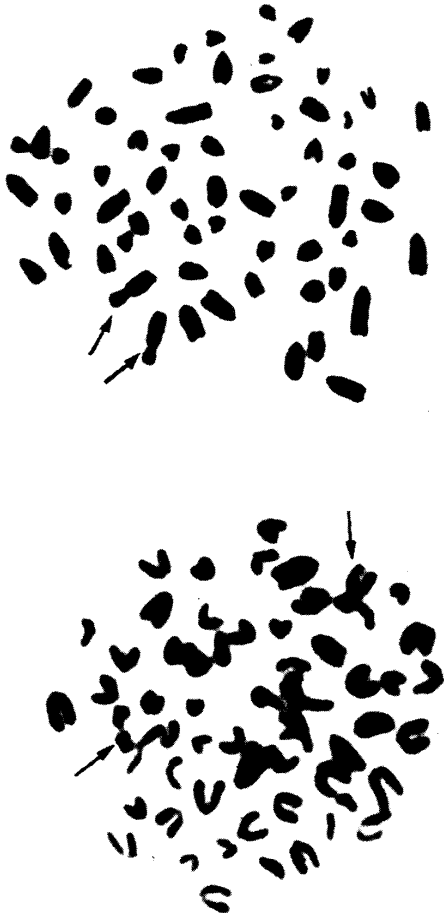
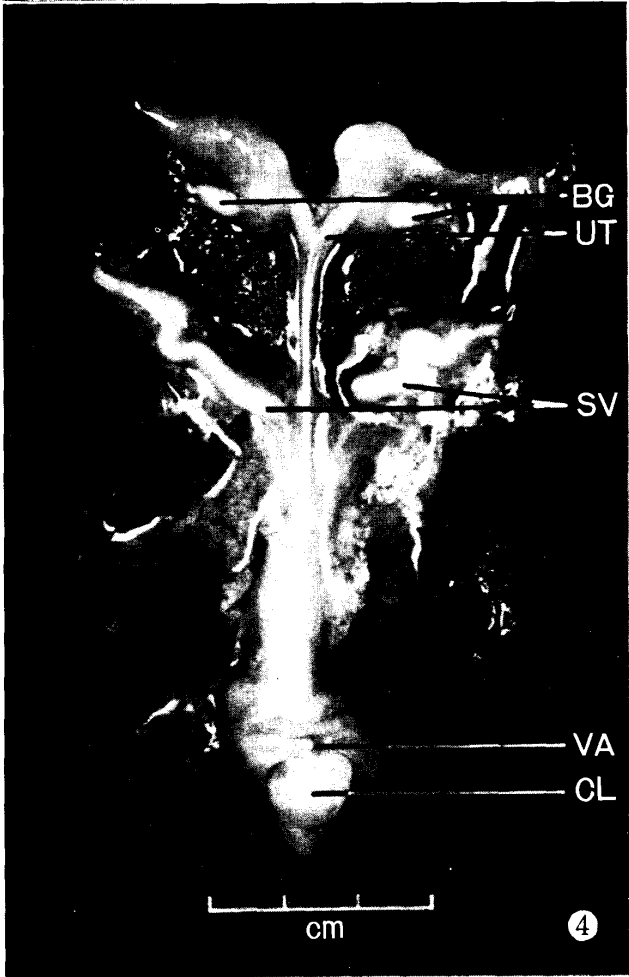


PLATE II

Figs. 6 & 7 Sex-chromosome chimerism in blood cultures from the heterosexual co-twins 169 days postnatal (No. 6)

Fig. 6 indicates 2A-XY (left) and 2A-XX (right) in male calf.  
× 1,000

Fig. 7 shows 2A-XX (left) and 2A-XY (right) in freemartin.  
× 1,000

