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G-Band Staining of Mouse Embryo Chromosomes by Urea Treatment

Midori Yoshizawa, Wei Zhang and Susumu Muramatsu*

Department of Animal Breeding and Reproduction, Faculty of Agriculture, Utsunomiya University, Utsunomiya 321, Japan

Abstract: *Chromosome preparations of mouse morulae and blastocysts derived from in-vitro fertilization were made by a new air-drying method, and then subjected to various methods of G-band staining. Excellent G-band figures of embryo chromosomes were obtained by a modified method of urea treatment. The G-band method is shown in the present paper.*

Key words: *Chromosome, G-band, In-vitro fertilization, Mouse embryo.*

The necessity for cytogenetical research of mammalian embryos produced by new developmental technology has been increased recently. As such, a new method of chromosome preparation for micromanipulated mouse embryos has been developed [1], and the adequate concentration and duration of mitotic inhibitor treatment for chromosome analysis in mouse embryos have been determined [2, 3]. However, it is difficult to identify each individual chromosome without banding because every chromosome in the mouse is telocentric, and forms a continuous gradient in size. Sexing of mouse embryos at the first cleavage division could be exactly performed using the C-band staining [4]. However, the identification of each individual chromosome, and the pairing of homologous chromosomes for karyotyping were difficult. The chromosomes treated successfully by Q- or G-band staining are possible to identify individually for karyotyping. For an exact analysis of the chromosomes of embryos, a method which is easy to master and reliable in showing bands, is needed.

We undertook the present study in order to determine an adequate method of G-band staining for chromosome preparations of preimplantation embryos in the mouse. We used embryos derived from *in vitro* fertilization as materials for chromosome preparations. These embryos could develop synchronously, so they could show many mitotic metaphases during the defined time. A new air-drying method of chromosome preparation for mouse embryos at the morula or blastocyst stage is also shown in the present study.

Mice used in the present study were kept in an air-conditioned (20 to 24°C), and artificially lit room (14-hr light/day). Egg donors were 2- to 4-month old (BALB/c × C57BL/6) F_1 virgin females, and sperm donors were 2- to 4-month old ICR males. The procedure of *in vitro* fertilization and addition of caffeine (Sigma Chemical Co.) were done according to the previous report [5]. The sperm-suspension was preincubated for 2 hrs, and then diluted with the same medium to give a sperm concentration of approximately 2×10^5 /ml. Egg donors were induced to superovulate by an i.p. injection of 5 i.u. PMSG followed approximately 48 hrs later by 5 i.u. hCG. Eggs surrounded by the cumulus cells were recovered from oviducts excised at 16 hrs after the hCG injection, and then put into the diluted sperm- suspension. The zygotes fertilized were transferred into Whitten's medium [6] at 6 hrs after insemination. After incubation of the zygotes for about 70 hrs in the medium, all but degenerating eggs were incubated in the medium containing vinblastine sulfate (30 ng/ml) for 8 or 10 hrs for the preparation of embryos at the morula or blastocyst stage [3].

Chromosome preparation for morulae and blastocysts was done according to the new method modified from the method of Yoshizawa *et al.* [7]. Embryos rinsed briefly in 1% hypotonic sodium citrate solution were transferred into 0.4 ml of the same hypotonic solution for several minutes. A very small volume (0.01 to 0.02 ml) of fixative of methanol 3: acetic acid 1 was poured into the solution for mild fixation. An embryo was placed on a glass slide, and then a droplet of acetic acid was put on the embryo for the dispersion of blastomeres of the embryo. Immediately, several drops of the fixative were put

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*To whom correspondence should be addressed.

on the embryo to remove acetic acid from it and to fix the cells.

The staining of air-dried chromosome preparations was attempted by various G-banding methods: Trypsin [8], ASG [8], or urea [9]. In conclusion, the method using urea by Kato and Yoshida [10], which was modified by us, was adequate for mouse embryo chromosomes.

The details of the method are as follows: Chromosome preparations of mouse embryos were immersed in a 3:1 mixture of 7M urea solution and phosphate buffer (pH 6.8), and incubated at 37°C for 10 min. After rinse in tap water, the preparations were stained in 2% Giemsa solution diluted with pH 6.8 phosphate buffer for 10 min. When G-band staining was unsuccessful, the same procedure was repeated again after destaining.

The results of chromosome analysis in G-banded preparations, which were treated with urea solution for 10 min, are shown in Table 1. The proportion of the embryos G-banded successfully to the embryos treated was 83.9% (94/112) in single treatment and 73.4% (69/94) in double. Furthermore, the combined proportion was 79.1% (163/206). Chromosomal analysis of embryos treated was possible in 73.2% (82/112) of embryos treated once, and 61.7% (58/94) of embryos treated twice; resulting in 68.0% (140/206) of total embryos. A successful sexing rate was 72.3% (81/112) in embryos treated once and 61.7% (58/94) in embryos treated twice; for 67.5% (139/206) in total embryos. The sex ratio of diploid embryos, male percentage, was 49.6 % with no significant deviation.

Karyotyping the embryos with 2n (Fig. 1) was done according to "the standard karyotype of the mouse" reported by the Committee on Standardized Genetic Nomenclature for Mice [10]. In this metaphase, an X chromosome could be distinguished from no. 1 chromosomes; which were similar in size to it, and a Y chromosome could be distinguished from no. 19 chromosomes. If these chromosomes were stained by conventional Giemsa staining without G-band, they might not be identified exactly to be X and Y chromosomes.

In chromosome preparations from peripheral leucocytes, it is easy to identify fine bands on those chromosomes, because many samples with many metaphase figures varied in size can be obtained. In preimplantation embryos, however, only one sample per embryo can be obtained and the number of metaphases is also defined. Many kinds of mitotic inhibitors ; colchicine, colcemid, vinblastine sulfate, are useful to accumulate metaphases in mammalian embryos. Yoshizawa *et al.* [3] reported an adequate concentration and duration of vinblastine treatment for the production of high incidence of metaphases in mouse embryos. To maximize metaphases in preparation of embryos, cells are treated for longer time than in the case of peripheral leucocytes; 4–17 hrs vs. 1–1.5 hrs, respectively. However, the long treatment with a mitotic inhibitor causes over-contraction of chromosomes, and then makes identification of clear bands on the chromosomes difficult. As is well known, it is difficult to identify clear bands on the over-contracted chromosomes.

Q-, R- and G-banding are applied for karyotyping widely, and Q- and G-banding show the same banding pattern. Using Q-banding, Martin *et al.*, [12] decided the frequency of chromosome abnormalities in female pronuclei in golden hamster eggs penetrated by human sperm. However, a fluorescence microscope is need for observation and analysis of Q-bands, while a conventional microscope provides enough optic equipment for G-bands. Furthermore, G-bands can be kept for a long time in marked contrast with Q-bands. For these reasons we employed G-band staining for karyotyping embryo chromosomes. Although there were several methods of G-band staining of chromosomes, the method

Table 1. Results of chromosome analysis in mouse embryo preparations treated by G-banding with urea

	Times of treatment		
	1	2	Total
No. of embryos			
treated	112	94	206
G-banded	94 (83.9%)	69 (73.4%)	163 (79.1%)
analyzable	82 (73.2%)	58 (61.7%)	140 (68.0%)
sexed*	81 (72.3%)	58 (61.7%)	139 (67.5%)

* sex ratio of diploid embryos : 49.6% (male %).

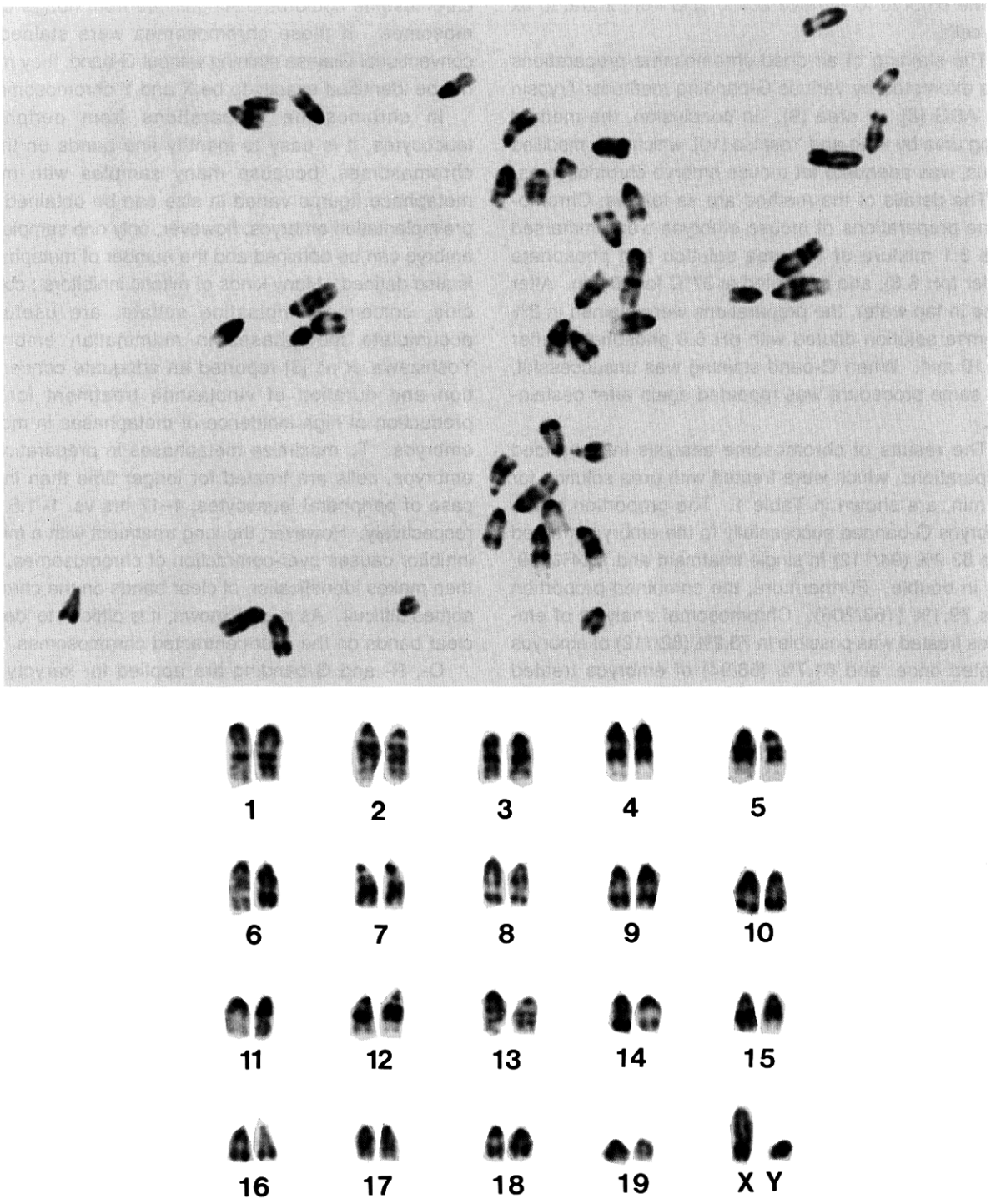

Fig. 1. A G-banded metaphase plate and its karyotype obtained from a mouse embryo. This is sexed as male because of the presence of X- and Y-chromosomes.

with urea was adopted most for repeatability.

Benet *et al.* [13] reported a G-banding technique with trypsin for human sperm chromosomes after penetration of zona-free hamster eggs. Ma *et al.* [14] analyzed chromosomes of human oocytes failing to fertilize *in vitro* using conventional Giemsa-staining, and G-banding after destaining Giemsa. Neither study described the analysis rate by the G-band technique. Benet *et al.* [13], however, viewed the technical points obtaining good G-bands as choosing high quality spreads with long chromosomes, and adapting trypsin treatment to the amount of remaining cytoplasm.

Santaló *et al.* [15] C-banded a part of chromosome preparations, and G-banded a few preparations, when they reported the chromosome compliment of first-cleavage mouse embryos. Why did they not band all of them? It was considered to be very difficult to get clear G-bands in embryo chromosomes constantly. Yoshizawa *et al.* [7] judged sex of first-cleavage mouse embryos derived from *in vitro* fertilization by C-band staining, showing high sexing rate of 93.8%. Luthardt (8) evaluated the relative frequencies of XO and YO embryos by cytogenetical analysis in embryos at first and second cleavage divisions derived from XO mice using G-banding with trypsin. They reported that the proportion of embryos karyotyped in those with countable chromosomes and sexing rates were from 65 to 87% and 70 to 87%, respectively, by G-banding. Although the proportion of embryos with G-banded chromosomes to the treated embryos was 79.1%, analyzed embryos were 68.0% of G-banded embryos in the present study, and a successful sexing rate 67.5% of total embryos.

Our results were close to those of Luthardt [8]. As Benet *et al.* [13] suggest, choosing high quality spreads with long chromosomes as technical points to get good G-bands, we consider also that the reason of unsuccessful banding is over-contraction of chromosomes for long treatment with vinblastine sulfate. However, in the embryos with defined numbers of blastomeres, long treatment with a mitotic inhibitor is needed to accumulate metaphases. We do not have any idea to satisfy the reciprocal objects now. In conclusion, it is desired to find a new mitotic inhibitor which does not contract chromosomes.

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