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The Frequency of Appearance of the Metaphase Cells from the Primary Tissues of Rat Fetus

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INTRODUCTION

The materials generally used in most chromosomal studies except for a particular purpose are the cultured cells of animal or plant tissues. For the purpose of the studies of the chromosomal aberrations possibly obtainable by the administration of teratogens to the early embryo, the examination of chromosomes from the primary tissues of embryo is the most desirable, since it seems not to be able to rule out completely the possibilities of some disturbing effects by culture conditions (Bloom et al, 1970) and/or culture media per se. Another reason why the primary tissue should be used is to examine the immediate effects of the teratogens upon the embryonic chromosomes within relatively short period of time, 2 to 5 days after administration of teratogens. However, it was impossible, if any, to find out the reports regarding the chromosomal studies, particularly the frequency of appearance of metaphase cells from the primary tissue of rat fetus.

The intention of the present work is to develop a simple procedure of smear preparations from the chemical-free primary tissues of early rat fetus and to standardize the conditions by which an appropriate number of metaphase cells enough to analyze the cromosomal aberrations could be revealed.

MATERIALS AND METHODS

The animals used are 7 fetuses from the 11th day and 3 fetuses from the 13th day of pregnant rats (Sprague-Dawley). The fetuses were treated separately according to their fetal age.

Preparation of tissue suspensions: The rats of 11th day of pregnancy were sacrificed by decapitation. All the fetuses removed from them were pooled in a Petri dish containing 4 to 5 ml of warm Hanks balanced salt solution (BSS) and minced carefully with a sharp ophthalmologic scissors for 5 minutes. The crude tissue suspension was diluted with 20ml of warm Hanks BSS and dispensed evenly in 16 graduated conical centrifuge tubes. Again, the additional warm Hanks BSS was added to make 9.9ml of final volume in each tubes. These suspensions were divided into 4 groups, 4 tubes in each group. The tissue stspensions from the 13th day of fetal age were also prepared as described above.

Throughout the procedure we used the glasswares which had been cleared with oxidizing acids and neutral detergent but no attempts were made to employ an aseptic technique,

since the experiment lasted not more than three hours until the process of fixation, it seems there was no time for the development of any significant bacterial infection. Careful handling was necessary to collect materials because the fetuses at the 11th day of pregnancy were small and extremely fragile.

Colchicine treatment: 0.2mg of colchicine (crystal, pure, E. Merck AG. Darmstadt, Germany) was dissolved in a ml of Hanks BSS. With successive dilution 0.1mg/ml, 0.01mg/ml and 0.001mg/ml solutions were prepared. To each 4 groups of suspensions 0.1ml of a colchicine solution of 4 different concentrations was added to make final volume of every suepension to be 10ml, so that the final con--entration of each suspension would become 10^{-3}mg/ml, 10^{-4}mg/ml, 10^{-5}mg/ml and 2×10^{-3}mg/ml, respectively. The suspensions treated with colchicine were incubated at 37°C with various time intervals ranging from 15 minutes to 120 minutes. Five minutes prior to the end of each exposure the suspensions were brought to spin. After spin for 5 minutes at 600 rpm, the supernatant was discarded and the sediments were trypsinized with warm trypsion solution, 0.25% in Hanks BSS(pH 7.8) for 15 minutes at 37°C. Trypsinization was rapidly stopped by adding 0.5ml of rabbit serum. Spin again and hypotonic treatment with warm 1% sodium citrate solution for 10 minutes at 37°C, and then, successively followed by spinning, fixation with acid-methanol (1:3) for 10 minutes at room temperature and the change of fixative two or more times until the supernatant became clear. Final supernatant was discarded and the smears were made by ignition method over the flame and stained with Giemsa solution.

Observation: Ten smears were made from each suspension. Randomly selected twenty visual fields from each smear were carefully observed under low magnification (×100) of light microscope. Total cells and the metaphase cells from 200 visual fields per group were counted. From the sum of cell counts the ratio of the cells arrested in metaphase per 1,000 cells was calculated.

RESULTS

In Giemsa stained smears of colchicine treated primary tissue, variable number of cells arrested in metaphase could be successfully observed from the fetuses of 11th day and 13 th day of fetal age though there were some differences in their cell counts. The identification of chromosomes from those metaphase

Table 1. Number of metaphase cells/1,000 cells in the rat fetus of 11th day of fetal age after exposure to various duration and to different concentration of colchicine

Time of exposure (min.) / Conc. of colchicine (mg/ml)	15			30			60			120		
	Total cells	Metaph. cells	Metaph. cells/10^3 cells	Total cells	Metaph. cells	Metaph. cells/10^3 cells	Total cells	Metaph. cells	Metaph. cells/10^3 cells	Total cells	Metaph. cells	Metaph. cells/10^3 cells
2×10^{-3}	7,002*	66	9.4	2,528	27	10.7	4,242	100	23.6	8,558	261	30.5
10^{-3}	12,780	143	11.2	10,015	186	18.6	6,857	154	22.5	11,400	423	37.1
10^{-4}	3,137	25	8.0	6,825	114	16.7	8,944	137	15.3	3,416	87	25.5
10^{-5}	10,490	77	7.3	8,311	84	10.1	6,269	126	20.1	8,085	122	15.1
			av. 9.0			av. 14.0			av. 20.4			av. 27.1

* Sum of cells appeared in 200 visual fields (×100)

Table 2. Number of metaphase cells/1,000 cells in the rat fetus of 13th day of fetal age after exposure to various duration and to different concentration of colchicine

Time of exposure (min.) / Conc. of colchicine (mg/ml)	15			30			60			120		
	Total cells	Metaph. cells	Metaph. cells/10^3 cells	Total cells	Metaph. cells	Metaph. cells/10^3 cells	Total cells	Metaph. cells	Metaph. cells/10^3 cells	Total cells	Metaph. cells	Metaph. cells/10^3 cells
2×10^{-3}	21,184*	74	3.5	27,396	53	1.9	15,577	68	4.4	18,215	136	7.5
10^{-3}	39,516	107	2.7	20,593	15	0.7	19,513	24	1.2	20,147	89	4.4
10^{-4}	19,149	60	3.1	17,871	49	2.7	13,326	76	5.7	16,138	134	8.3
10^{-5}	15,753	58	3.7	20,904	46	2.2	19,065	75	3.9	16,641	133	8.0
			av. 3.3			av. 1.9			av. 3.8			av. 7.1

* Sum of cells appeared in 200 visual fields (×100)

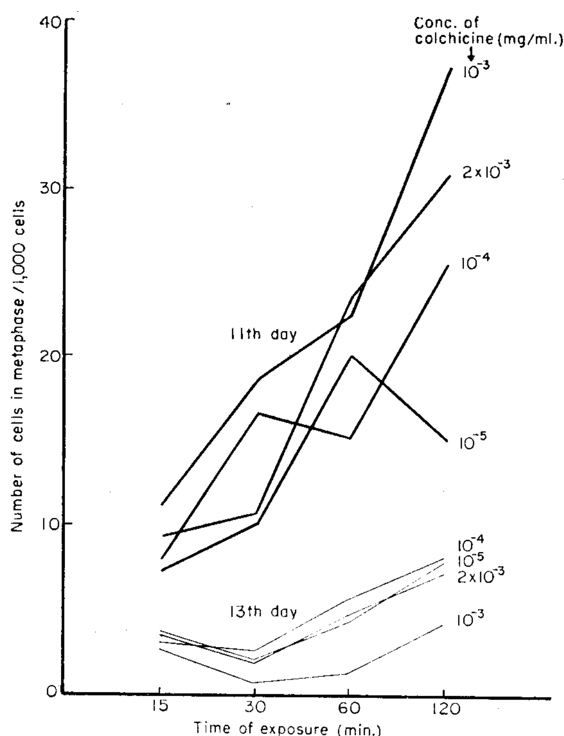

Fig. 1. Number of metaphase cells appeared in 1,000 cells in the rat fetus of 11th and 13th day of fetal age after exposure to various duration and to different concentration of colchicine.

cells was not difficult and clear enough to find even under the low magnification of light microscope. As the results assembled in the Table 1, in the fetus at the 11th day of fetal age, the number of cells arrested in metaphase increased with the prolongation of the duration of exposure to colchicine. The average numbers of cells in metaphase per a thousand cells were 9.0 (7.3-11.2) in 15 minutes, 14.0(10.1-18.6) in 30 minutes, 20.4 (15.3-23.6) in 60 minutes and 27.1 (15.1-37.1) in 120 minutes exposure. As shown in Figure 1, the increasing pattern of the number of metaphase cells was somewhat proportional to the duration of exposure to colchicine with a little exception. There was no marked differences in the number of metaphase cells among the groups treated with different colchicine concentrations when the duration of exposure was same.

In the fetus at the 13th day of fetal age, the increment of the cells in metaphase according to the prolongation of the duration of exposure was also shown (Table 2, Fig. 1), that is, 3.3(2.7-3.7) metaphase cells/1,000 cells in 15 minutes exposure, 1.9(0.7-2.7) in 30 minutes, 3.8(1.2-5.7) in 60 minutes and 7.1 (4.4-8.3) in 120 minutes, respectively. But the increment of the number of mataphase cells was not so marked as compared with that of fetus of 11th day. Lesser cell counts in the groups of 30 minutes exposure than that of 15 minutes exposure were noticed. As in the fetus of 11th day, there

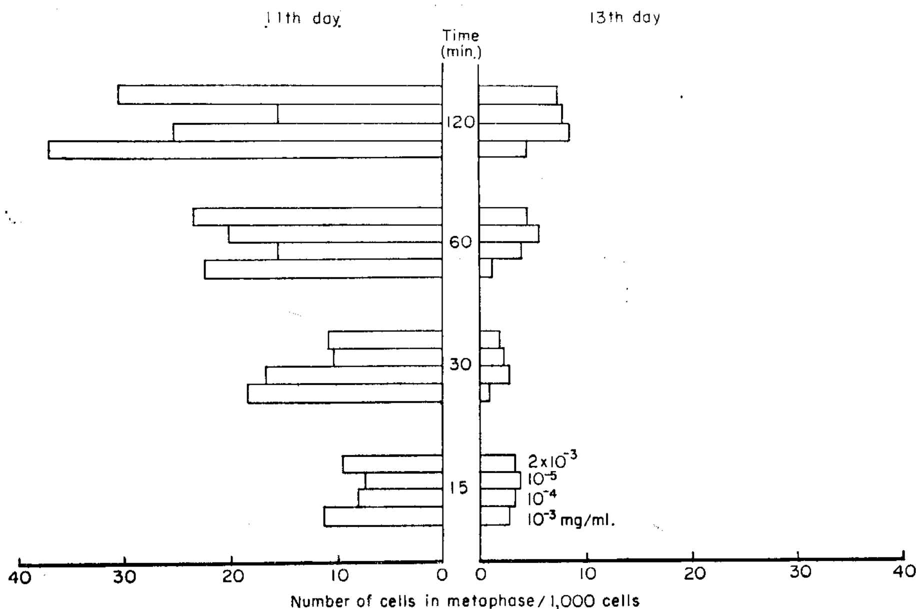

Fig. 2. Comparison of the number of metaphase cells appeared in 1,000 cells in the rat fetus of 11th and 13th day of fetal age after treated with different colchicine concentrations and different time of exposure

were no obvious difference in the appearance of mataphase cells by the treatment with different colchicine concentrations among the groups which have been exposed same duration to colchicine.

DISCUSSION

At the beginning of this experiment we put stress on the duration of exposure to colchicine rather than the concentration of the drug, since the employed colchicine dosages were in the range of minimal effect to block the mitosis at metaphase without any toxicity, that is, from 2×10^{-3}mg/ml to the amount of no significant blocking effect, 10^{-5}mg/ml (Murray et al, 1951). However, there are some differences from us which will be discussed later.

As has been expected, with the prolongation of the duration of exposure to colchicine the number of cells arrested in metaphase increased in both materials of the 11th and 13th day of fetal age. Though it also is expected that the more prolonged the exposure in a certain range the more cells in metaphase will appear, the examination of the appropriate frequency of metaphase cells in a short exposure time was the authors' intention. Hence, the durations of exposure we had planned were no more than two hours.

The discrepancy in the increment of metaphase cells between the fetuses of 11th and 13th day of fetal age was interested. Langman (1969) emphasized that the difference of growth rate in early embryonal development is probably due to the difference of proliferative activity, namely cell division. Similar opinion has been presented by Balinsky(1970) who pointed out that the increasing rate of embryonal body weight decreases with the age of embryo. The ratio of the number of dividing cells to total embryonal cell mass is larger in the embryo at the 11th day than that of 13th day, even though the absolute number of

dividing cells may be much larger in the latter. This agrees with our results of an increase in the number of metaphase cells in the 11th day fetus. In the fetus of 11th day, some irregularities in the increase of number of metaphase cells between the groups of 10^{-4} and 10^{-5}mg/ml of colchicine concentrations might be due to some technical errors in the course of experiment. Regarding the lesser counts of metaphase cells in the 30 minutes exposure of 13th day of fetal age than that of 15 minutes, we are unable to offer an explanation at present.

Colchicine concentration: Many workers, Ludford(1936), Nebel(1937), Levan(1938) and others, had studied that the colchicine deleteriously affects the spindle structure, or more than the spindle, that is, the maturation of kinetochore (Jokelainen 1968) to stop or delay the mitotic progress in the dividing cells. Although the mechanism of action of colchicine has recently been studied in part, (Borisy et al. 1967, Schelanski 1967), the exact mechanism of inactivating the spindle has not been clearly defined yet.

Murray et al. (1951) have reported on the effective colchicine concentration, and found that 10^{-5} molar in concentration could induce a marked blocking of mitosis without general toxicity while 10^{-4} molar appeared to be generally toxic and 10^{-6} molar produced little if any mitotic arrest. Gaulden and Carlson (1951) have also investigated the effects of colchicine, 2.5×10^{-6} molar in concentration, on the grasshopper neuroblast and concluded that despite the lower concentration of colchicine, the effect was depend upon the phase of cell division at the time of application of chemicals: no detectable effects on the spindle at anaphase, no blocking of mitosis but some retardation at metaphase, and a successful blocking of mitosis in metaphase occured if colchicine applied at prometaphase. On the other hand Kato et al. (1967) reported that the induction of chromosomal pulverization in binucleate human cells infected with leukovirus appeared to be dependent upon the concentration of colcemid, a colchicine derivative. They found that the higher the concentration of colchicine used, the more the pulverized chromosomes occured. Since Taylor (1965) showed that the colchicine concentration as low as 5×10^{-8} molar could arrest the mitosis successfully in human cancer cell, we have employed rather lower concentrations for the purpose of elimination of undue or toxic effect that might be induced by the higher colchicine concentration. The colchicine adopted in this experiment were thus 2×10^{-3}mg/ml as the highest dose which corresponds approximately to 5×10^{-6} molar and 10^{-5}mg/ml as the lowest dose which equals to 2×10^{-8} molar.

We could observed a number of cells arrested in metaphase after colchicine treatment even with the lowest dose although there are some differences between the groups of various exposure. There was, however, no marked difference in the number of metaphase cells by the different colchicine doses when the duration of exposure to colchicine was same (Fig. 2). It could be thought that there might be several debatable points: a) the colchicine concentrations are too low to inhibit the mitosis, which means that the consequences are same as if no colchicine has been employed; b) the duration of exposure is too short to be enough for the action of colchicine especially in the exposures for 15 minutes, 30 minutes and 60 minutes; c) both the dosages of colchicine and the durations of exposure are not

sufficient. To rule out such point these should have been compared to the colchicine-untreated subjects, but we did not include it in our experiment because of inadequacy in examining the separate chromosome in the lack of colchicine effect. However, it seems unlikely that these colchicine concentrations are so low as to be of no effects. By using Hela and cultured human amnion cells Kleinfeld and Sisken (1966) observed that 97 to 100% of the cells entering mitosis were blocked at metaphase with the colchicine concentretion of 10^{-5} mg/ml, following a lag period of 60 minutes after colchicine treatment. Taylor(1965), and Borisy and Taylor (1967) also observed that 10^{-7} molar of colchicine concentration was sufficient to block essentially all the cells in the culture as they reached metaphase with an exposure of 6 to 8 hours. These suggest that a little differences in shorter exposed groups in our experiment may not be due to the insufficient concentration of colchicine but due to the shorter exposure.

Another interesting observation was reported by Williams et al. (1966). They investigated the mitotic indices of the embryonic brain and the crypts of Lieberkühn of maternal gut with administration of 1 to 8 mg of colcemid /kg body weight to the female rat at 14th day of pregnancy. They found the optimum dose for the gut of adult rat and the dose arresting the maximum number of mitosis in the embryonic brain was 1 mg/kg body weight, and that the number of mitosis in the maternal gut was essentially constant at all dose levels but in the embryonic brain the mitotic figures decreased in a dose-dependent manner, that is, inversely proportional to the dose employed. As indicated in Fig. 2, cell counts of metaphase cells in the fetus at the 13th day of fetal age show somewhat similar aspect but it is not uniform in mode or rather irregular, and it seems unwise that the comparison is made at present. The method of these workers, also the material was different, was quite differed from us in the route of administration of colchicine, in vivo than in vitro, which indicates that some other factors in vivo affecting the action of colchicine may exist.

Duration of exposure: It is known that the mitosis arresting effects of colchicine is dependent upon the duration of exposure and the degree of development of spindle at the time of exposure as well as the concentrations(Inoue 1952, Gaulden et al. 1951). It seems that, though a certain level of the concentration of colchicine should be reached in the cell for the effective inhibition of mitosis by disruption or inactivation of the spindle structure and the maintenance of its inhibiting action, the action of the colchicine is not an all-or-nothing type of phenomenon but a progressive one proportional to the concentration and the duration of exposure.

In comparison with the initial lag period of 1 hour after colchicine treatment (Kleinfeld et al. 1966) the exposure time to colchicine less than 60 minutes in our experiment seems short. However, though it may not be sufficient, it may not entirely be insignificant. On this regrad there are several feasible reasons: a) colchicine penetrated the cells in all stages of cell cycle rapidly and equilibrated with external colchicine within 15 minutes (Taylor 1965); b) in the colchicine untreated human amnion cell culture the mitotic time was 35 to 43 minutes and in most mammalian cells the entire period of mitosis is about 60 minutes (Kleinfeld et al 1966); c) the colchicine can delay the mitotic progress

even in low concentration although it cannot block the mitosis completely(Gaulden et al. 1951); d) the period of recovery from the arrest after being washed free of colchicine is more than 50 minutes at least (Kleinfeld et al. 1966).

From the end of exposure to colchicine to initial fixation we spent 35 minutes at most. Therefore, the colchicine-treated dividing cells cannot completely recover and complete the mitosis, differing from the untreated dividing cells which can proceed to the completion of mitosis without any delay or blocking.

It seems that the further accumulation of arrested cells in metaphase may definitely be induced by the prolongation of the duration of exposure. But the blocking in metaphase for 5 or more hours may cause sudden violent blebbing and death of cell. Exposure for 2 hours was sufficient duration to observe the metaphase cells in an appropriate number. Thus 2 hour exposure and 10^{-3}mg/ml of colchicine concentration would be the quite adequate conditions to study the metaphase chromosomes in case of using the primary tissues.

SUMMARY AND CONCLUSION

As a preliminary experiment for the study of chromosomal aberrations by administration of teratogens to the early embryo, the present experiment has been attempted to standardize the conditions by which an appropriate number of metaphase cells could be revealed by using the primary tissues from the rat fetuses of the 11th day and 13th day of fetal age. The authors have reached the conclusion as followings.

1) The primary tissue, without culture, from the rat fetus can be adopted in chromosome study by its sufficient supplying of metaphase cells in a short exposure to colchicine of no more than two hours.

2) The frequency of appearance of metaphase cells is related to the age of fetuses and the duration of exposure.

3) No significant difference in the appearance of metaphase cells by diffenet colchicine concentrations in the range from 2×10^{-3} mg/ml to 10^{-5}mg/ml could be found.

4) The condition of an exposure to colchicine for 2 hours at least, a colchicine concentration less than 2×10^{-3}mg/ml in the fetus of 11th day of fetal age is appropriate for the chromosome study on the teratological base.

—國文抄錄—

흰쥐 胎仔의 Primary Tissue를 사용한 細胞 分裂 中期細胞의 發顯頻度에 關한 實驗

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各種 Teratogen이 흰쥐 胎仔의 染色體에 미치는 영향을 研究하기 위한 豫備實驗으로 染色體 分析에 充分한 程度의 中期 細胞 發顯의 條件을 標準化하기 위하여 妊娠 第11日 및 條13日의 흰쥐 胎仔의 培養하지 않은 Primary Tissue를 Colchicine 濃度別, Incubation 時間別로 處理한 뒤 塗沫標本을 만들어 光學顯微鏡 低倍率(10×10)下에서 中期細胞의 發顯頻度를 比較 觀察하여 다음과 같은 結論을 얻었다.

1) 培養하지 않은 흰쥐 胎仔의 Primary Tissue도 2時間 以下의 짧은 Colchicine 處理에 依해 充分한 中期細胞의 發顯을 보이므로 染色體 分析 研究에 使用될 수 있다.

2) 中期細胞의 發顯 頻度는 胎仔의 胎齡및 Colchicine 處理時間과 密接한 關係가 있다.

3) Colchicine 濃度 2×10^{-3}mg/ml에서 10^{-5}mg/ml의 범위 內에서는 Colchicine 濃度差에 依한 中期細胞 發顯頻度에 顯著한 差異가 없었다.

4) 畸形學的인 意味에서의 染色體 研究에는 다음과같

은 條件이 적합하다.

胎齡：第11日,

Colchicine 濃度：2×10^{-3}mg/ml 以下

Colchicine 處理時間：2時間

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