CYTOGENIC STUDIES OF CYCLOHEXYLAMINE, A METABOLITE OF CYCLAMATE[1]

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ABSTRACT

Nine wistar M W-3 pathogen-free rats, selected for this study, were observed for the effects of cyclohexylamine (CHA) on chromosomes. The rats were divided into three groups, each consisting of three animals. Group I served as control, group II received doses of 20 mg/kg body weight, and group III received 50 mg/kg body weight of CHA. The experiment ran for seven consecutive weeks, with CHA being injected intraperitoneally for five consecutive days. One day after the last injection, blood was taken from the tail vein and prepared *in vitro* for chromosome analysis. The prepared chromosomes were first observed under light microscope and oil immersion, and chromosome spreads which appeared to be abnormal were photographed for detailed examination.

The results indicate that CHA did not produce any significant chromosme abnormalities during the course of this study. It is suggested that longer term experimentation utilizing a larger number of animals and employing *in vitro* technique should be considered in an effort to resolve conflicting results concerning the effects of cyclamates and CHA on chromosomes.

INTRODUCTION

With the Federal Drug Administration's (FDA) ban on cyclamate in September of 1969, a great deal of interest has arisen in this field. Prior to and prompting the ban, a number of researchers studied the biological effects of cyclamate. Fits-

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baugh, *et al.* (1951) and Nees and Derse (1965) showed reduced growth rate. Other authors showed bladder cancer (Bryon and Extuch, 1970) and thyroid adenoma (U.S. Food and Drug Administration 1968). Further complications occurred when Kojuwa and Ichaibagese (1966) and later Chedd (1968) found that cyclamate is converted to cyclohexylamine (CHA) within the intact organism. This compound appears to be especially dangerous, and additional reports utilizing CHA showed chromosome abnormalities in red cells (Stoltz, *et al.*, 1970), leukocyted and monolayer cells (Stone, *et al.*, 1969), and bone marrow and germ cells (Legator, *et al.*, 1969). CHA has also been shown to affect the cardiovascular system (Rosenblum and Rosenblum, 1968, and Yamamura, *et al.*, 1968) and the nervous system (Legator, *et al.*, 1969). Contrary to these results, a special report prepared for the FDA clearly defined that neither subcutaneous or topical application of cyclamate had any effect in initiating carcinogenic activity (U.S. Food and Drug. Admin., 1968).

The abundance of evidence concerning the specific effects of cyclamate and CHA remains contradictory, and considerably more information is needed to assess accurately the physiological and pathological effects of cyclamate and its metabolite CHA. The purpose of this study was to determine the effects of different-sized doses of CHA injected intraperitoneally on the chromosomes of circulating blood of rats.

METHODS AND MATERIALS

Nine Wistar M W–3 pathogen-free rats were used for this study. All of the animals were less than one year old and were maintained three in a cage at a room temperature of 23.5°C ± 1°C. Food and water were provided *ad libitum*. The rats were divided arbitrarily into three groups of three rats each. Group I served as control, group II received doses of 20 mg/Kg body weight of CHA, and group III received doses of 50 mg/Kg; all injected doses were adjusted to a pH of 7.4 with HCl. Table 1 shows the vital data and doses for the animals used during the experiment.

The experiment ran for seven weeks, during which time the following procedure was followed. Each week of the experiment, each experimental animal was injected, intraperitoneally, with cyclohexylamine for five consecutive days. Twenty-four hours after the fifth injection, blood was taken from each rat's tail vein. Blood from each animal was transferred by using a heparinized sterile capillary pipette to a commercial chromosome preparation medium (chromosome medium 1A, Grand Island Biological Co.).

The preparation was incubated at 37°C ± 1°C for 72 to 96 hours. At the end of the incubation period, 0.1 ml of colchicine was added to each tube, and the total sample was maintained at the incubation temperature for 1½ to 2 hours. The preparation was then centrifuged at 1000 RPM for 5 minutes, at which time 5 ml of buffered saline solution was added slowly to each sample, after which the sample was again incubated at 37°C for 15 minutes. Following this, one drop of freshly mixed fixitive (acetic acid-alcohol 3:1) was added and the mixture was again centrifuged at 1000 RPM for five minutes. An additional 2 ml of fixitive was added and maintained at 0°C for twenty minutes. Finally an additional 2 ml of fixitive was added to maximize chromosome stability. After the last centrifugation, approximately one third of the supernatant was retained and mixed with the remaining cells, which were then placed on a cold, wet slide. The excess fluid was drained on a paper towel and the slide passed over a small flame to partially dry it. When the slides were completely air dry, they were treated with Geimsa stain for fifteen minutes and then with Jenner stain for another fifteen minutes. They were then rinsed three times in distilled water, again air dried, and a cover glass was mounted. Four to five slides were prepared from each animal each week in this manner.

The mounted slides were first scanned, using the lower power of a standard light microscope, to locate well-separated chromosome spreads. These identified spreads were then examined again, using an oil-immersion lens. Fifty spreads of the metaphase stage of the chromosomes were examined for each of the three groups. Spreads which appeared on vision to show possible abnormalities were photographed and enlarged for detailed re-examination. These were then karyotyped, using the classification of Hungerford and Nowell (1963).

At the conclusion of the experiment, all of the animals were sacrificed by administering a sharp blow on the head. The internal organs and glands were examined visually, with the aid of a dissecting microscope, for gross abnormalities.

TABLE 1

Vital data of experimental animals and daily doses of CHA

Rat No.	Group No.	Daily Dose of CHA	Sex	Body Weight (grams)
1.	I	0	M	458
2.			M	502
3.			F	260
4.	II	20 mg/kg	M	477
5.			F	276
6.			F	276
7.	III	50 mg/kg	F	308
8.			F	273
9.			M	480

RESULTS

The percent of cells with abnormal chromosomes and the changes in these values during the course of the experiment are presented graphically in Figure 1. All of the animals were generally healthy and in good condition at a start of the experiment. During the latter part of the first week of the treatment, the CHA-

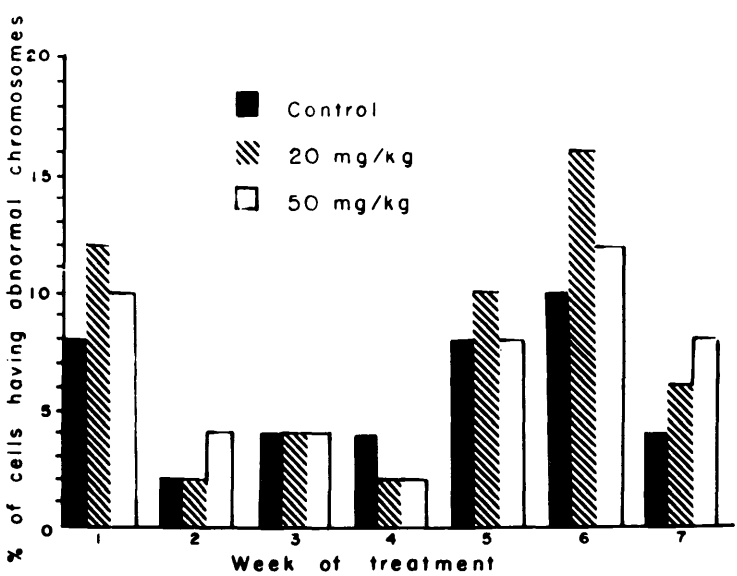

FIGURE 1. Histogram of percentates of cells having abnormal chromosomes. On the abscissa are the seven experimental weeks.

treated rats appeared to be in poor condition, their eyes being pale and watery. As the experiment progressed, these animals seemed to regain their health gradually, and at the termination were in good condition. At the termination of this experiment, none of the animals showed urinary bladder tumors or any other pathological signs in other internal organs.

Although this study employed a small number of experimental animals, it was believed that the difference in the CHA doses and the duration of the experiment would produce discernible differences as far as abnormal chromosomes are concerned. Observation of these data suggest that there were no discernible differences. This is emphasized by the fact that the number of abnormal spreads and percent of cells with abnormal chromosomes are nearly identical for both the control and the experimental groups. Observation of these raw data (Table 2)

TABLE 2

Accumulative dose, number of chromosome spreads, abnormal observation, and percent of abnormal spreads for seven weeks of experimentation

Expt. No.	Group No.	Accumulative dose mg/kg/week	Spreads used for analysis	No. of abnormal spreads	% of cell with Abnormal chromosomes
1.	I	0	50	4	8
	II	100	50	6	12
	III	250	50	5	10
2.	I	0	50	1	2
	II	200	50	1	2
	III	500	50	2	4
3.	I	0	50	2	4
	II	300	50	2	4
	III	750	50	2	4
4.	I	0	50	2	4
	II	400	50	1	2
	III	1000	50	1	2
5.	I	0	50	4	8
	II	500	50	5	10
	III	1250	50	4	8
6.	I	0	50	5	10
	II	600	50	8	16
	III	1500	50	6	12
7.	I	0	50	2	4
	II	700	50	3	6
	III	1750	50	4	8

indicate that, from week to week, the percent of cells with abnormal chromosomes was variable. However, in all experimental weeks, the values for the experimental groups were similar to those of the control groups. This would suggest that there were no differences between the experimental and control groups and that the week-to-week differences that were observed were due to variations in preparation techniques.

DISCUSSION AND CONCLUSIONS

A major difficulty of *in vitro* studies of chromosomes using micro-culture techniques is the control and regulation of cellular growth. The factors which are known to affect such growth are: temperature of incubation, incubation time of the cells, non-sterile technique, and the number of viable leucocytes which are present in the blood at the time when the blood sample was taken. We found that 38°C, as compared to 37°C, gave better cellular growth. Incubation times of 90 to 94 hours also seemed to yield better cell growth then did the usually recomended 72 hours. The number of viable leucocytes is almost impossible to

regulate, as it depends on the physiological conditions of the individual animal. During each week of the experiment, all of the samples were treated uniformly; however some of the sample cells exhibited very good growth and others very poor growth, which leads one to suspect that there may well be other unknown factors which affect yield.

Another problem in the present study was the variation, from each experimental animal, in the number of usable chromosome spreads. On occasion there were yields of two to three chromsome spreads per field. In other instances, there were either no usable spreads or only four or five on an entire slide.

The results of this study were compared with those of other available published data. Legator, *et al.* (1969) first reported on *in vivo* studies and stated with confidence that a dose of 10 mg/kg/day for five days IP injection in rats will increase chromosome breaks in bone-marrow cells. Stoltz, *et al.* (1970) also reported on *in vitro* studies of human leucocytes, where treatment with cyclamate, CHA, and other related chemicals did induce chromosome breaks, breaks which did not develop in untreated controls. At the present time, the above studies have not yet been duplicated by other laboratory investigators.

There are no published reports available at the present time that indicate negative effects of CHA on chromosome abnormalities. In an article appearing in the *New Scientist* in 1968, and another which appeared in *Family Health* in 1969, experiments are described which were performed at the Institute of Experimental Pathology and Toxicology, Albany Medical College, in New York. Studies of chromosome breaks of 17 prisoners, who ingested from 3 to 16 grams of cyclamate and who excreted CHA in their urine, demonstrated no significant increases in chromosome breaks. The detailed procedure of the above experiment was not released by the original researchers, so no real comparison with this study can be made. Also, in a letter to the editor of *Science* discussing the then recently proposed FDA ban on the use of cyclamates, Inhorn and Meisner state (1970) that: "Work in our laboratory and elsewhere has shown no mutagenic effect when very high concentrations of cyclamate were put into cultures of normal human cells." But again the detailed procedure used in this study is presently not available. Although we utilized only a limited number of animals, the results of this study also suggest that there is no increase in numbers of chromosome breaks in rats which were injected with 50 mg/Kg/day for periods of up to seven weeks.

The fact that we found no urinary bladder tumors agrees with the two-year study of Price (1970), in which fifty rats were fed CHA and only one animal developed a bladder tumor. It also agrees with Shabad's (1963) work, in which CHA was administered both orally and subcutaneously without producing any bladder tumors. We, in fact, were unable to find tumors of any kind associated with body organs on our animals.

It is recognized that this combination *in vivo* and *in vitro* study has limitations. It is possible that gross damage occurred *in vivo* study because damaged cell lines could not have repoduced and would not be present for *in vitro* analysis. It did not appear that fewer usable spreads were obtained from the experimental animals than were available from the control animals. However, this is an observation not supported by any other reported data.

In conclusion, our data support the premise that CHA has little if any effect on the chromosomes of rats; however, for the above results, it would appear that a more extensive study using larger numbers of animals, longer treatment, and expanded *in vitro* techniques would be desirable.

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