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Cytogenetic effects in children treated with methylphenidate

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Abstract

In recent years there has been a surge in methylphenidate (Ritalin) use for treatment of attention deficit/hyperactivity disorder (ADHD) in children. However, there is a paucity of information on whether this drug poses any potential health risks, such as mutagenicity or carcinogenicity, for humans. To address this issue, we investigated whether this central nervous system stimulant produces cytogenetic abnormalities in pediatric patients at therapeutic levels. In a population composed of twelve children treated with therapeutic doses of methylphenidate, we analyzed three cytogenetic endpoints in peripheral blood lymphocytes obtained before and three months after initiation of treatment with this drug. In all participants, treatment induced a significant 3, 4.3 and 2.4-fold increase in chromosome aberrations, sister chromatid exchanges and micronuclei frequencies, respectively ($P=0.000$ in all cases). These findings warrant further investigations of the possible health effects of methylphenidate in humans, especially in view of the well-documented relationship between elevated frequencies of chromosome aberrations and increased cancer risk.

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1. Introduction

Although methylphenidate hydrochloride (Ritalin) has been in use since the 1950s, the use of the drug for the treatment of attention deficit/hyperactivity disorder (ADHD) has increased dramatically in recent years. Between 1990 and 1993, ADHD-related visits

of children to primary care practitioners in the United States (US) increased from 1.7 to 4.2 million per year [1]. Ninety percent of those children were treated with medications, 71% with the central nervous system stimulant methylphenidate. More than 10 million prescriptions were written for methylphenidate in 1996 [2]. Domestic sales in the US showed an increase of almost 500% between 1991 and 1999 with the US consuming approximately 85% of the world's production of methylphenidate.

Considering that methylphenidate has been approved for human use for over 50 years, there are

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surprisingly few studies on the potential for serious side effects, such as mutagenicity and carcinogenicity, in animals or in humans. In a comprehensive 2-year carcinogenicity study in F344 rats and B6C3F1 mice [3], there was a significant increase in hepatocellular tumors in both male and female mice, but not in rats, at the highest dose tested. In another study, methylphenidate caused a decrease in spontaneous mammary gland tumors in female rats [4]. In mutagenicity tests, methylphenidate did not cause mutations *in vitro* in bacteria or in cultured mammalian cells [5–8]. In cell transformation assays, methylphenidate did not transform rat or mouse cells in culture [9–10]. Increases in chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) were reported in a study of Chinese hamster ovary cells in culture [6,11] and an increase in SCEs was observed in an *in vitro* study with human lymphocytes [12].

The lack of chronic studies in humans that address the long term effects of methylphenidate intake, the report of a positive 2-year cancer study in mice, and results from genotoxicity assays prompted the present investigation. Since children can be more vulnerable to genotoxic agents than adults, the study was conducted on a pediatric population to determine if this central nervous system stimulant produces cytogenetic abnormalities in children at therapeutic levels.

2. Materials and methods

2.1. Study design and subjects

A total of 35 children volunteered for the study. They had all been referred to a pediatric clinic at the University of Texas Medical Branch (UTMB) for evaluation of ADHD and consideration of treatment. The study participants were either children that were already patients at the clinic or children that had been referred specifically for behavioral evaluation. Eighteen children out of the thirty-five volunteers were eligible to be enrolled in this pilot study. Details of the clinical evaluation and criteria for enrollment are given in the following section. After being informed of the study, a parent or guardian signed an informed consent that was approved by the Institutional Review Board of UTMB, the child also assented, and the study participant was then enrolled.

The study was designed so that each participant served as his or her own control. None of the children was known to be taking methylphenidate before entering into the study. A 10 ml blood sample was taken before starting methylphenidate, and a second blood sample was collected after three months of treatment with this drug. During the course of the treatment, six subjects were switched to other medications; thus, a total of 12 children completed the study.

2.2. Clinical evaluation and treatment

Each potential study subject had an appointment with, and was seen by a pediatrician experienced in diagnosing ADHD and other behavioral problems for a thorough behavioral evaluation. This evaluation included obtaining behavioral checklists from parents and teachers, interviewing the parents, and talking with and examining each child. The diagnosis of ADHD was made using the DSM IV Criteria in accordance with the current recommendations of the American Academy of Pediatrics [13]. Each child that received the diagnosis of ADHD had to have had attention problems, hyperactive/impulsive problems, or both. These problems had to be ongoing for more than six months, had to have occurred prior to the child's seventh birthday, and had to be present in more than one setting and be more severe than in other children. Most importantly, these problems had to make it difficult for the child to function at home, in school and/or in social settings. The majority of the clinic appointments were 45–60 min in length. Of the children who were diagnosed, 18 were treated with methylphenidate; these were the children who were originally enrolled in the study. The selection of the type of medication to be used was solely a medical decision made by the child's pediatrician. If the diagnosis of ADHD was not made, no medication was prescribed, or if methylphenidate was not prescribed, the child was not eligible for the study. Therapeutic doses of methylphenidate ranged from 20 to 54 mg/day for the children enrolled in this study.

2.3. Cytogenetic analyses

Cytogenetic evaluations using multiple techniques were conducted on peripheral blood lymphocyte (PBL)

samples collected from all study subjects. The first evaluation was done before the start of the methylphenidate treatment. The second evaluation was done three months after the initiation of methylphenidate treatment. Cytogenetic evaluations of all participants were conducted using three different standard protocols: (1) a conventional cytogenetic assay that evaluates CA frequencies [14], (2) a SCE assay, which detects crossing-over events between the sister chromatids of a chromosome [15] and (3) a micronucleus (MN) assay that detects damage to chromosomes or to the mitotic apparatus [16]. The MN assay also allows for scoring of nucleoplasmic bridges (NPB), which are markers of chromosome rearrangements such as dicentric or ring chromosomes [17]. The use of multiple cytogenetic endpoints ensured the detection of different types of genetic damage and allowed for a better understanding of the underlying mechanisms of methylphenidate-induced genetic damage.

Blood cultures from all the samples (initial and three months after treatment) were harvested and stored at -20°C until the slides were prepared and coded for blind scoring by two experienced slide readers who were not aware of their origin. Fifty metaphase cells from each individual's blood sample were scored for CA. For SCEs, 25 metaphases were scored, and for the MN assay, 1000 binucleated cells were scored.

2.4. Statistical analyses

The data was computed using the means of the pre- and post-treatment measurements and the standard error of the change in the means. The Minitab computer program (Minitab Inc, version 14) was used to perform a paired t-test on each of the variables (pre- and post-treatment) yielding *P*-values, all of which were zero to 3 decimal places, and 95% level confidence intervals for the change in means (corresponding to a 0.05 level of significance for testing purposes).

3. Results

3.1. Study subjects

Out of 18 enrolled individuals, 12 study subjects successfully completed the study with three months of treatment with methylphenidate. The mean age of

the children was 8.5 ± 3.5 years. There were 10 males and 2 females in the study. There were 6 Caucasians, 4 African Americans and 2 Hispanic children. The mean \pm SD of the height of the children was 50.8 ± 7.25 inches (range 39–60 inches) and their mean \pm SD weight was 69.09 ± 32.16 lbs. Results from only 11 study subjects were generated before and 3 months after methylphenidate treatment for the SCE and MN assays due to failure of the cultures for these assays in one study participant.

3.2. Chromosome aberration results: individual data

For all the study participants ($n=12$), 50 metaphase cells were analyzed for CA at two different time points: at baseline (before the start of the medication) and 3 months after the beginning of methylphenidate treatment. Chromatid-type aberrations, chromosome-type aberrations and total breaks were recorded. All of the chromatid-type aberrations that were recorded were frank chromatid breaks. Chromosome-type aberrations were mainly deletions and each deletion was counted as two breaks. Gaps were also recorded, but not included in the final aberration frequencies computation. Detailed information on the chromosome aberration findings for each individual studied is presented in Table 1.

3.3. Sister chromatid exchange results: individual data

For all study participants, 25 metaphase cells were analyzed at baseline and at 3 months after methylphenidate treatment. The number of SCEs in each cell was recorded, and the mean number per 25 metaphases for each individual was calculated. Fig. 1 shows the results from 11 individuals enrolled in the study. A significantly higher mean number of SCEs ($P=0.000$) was observed in the PBLs of every volunteer after the 3 months of methylphenidate treatment. It is noteworthy that while participant #1 had an unusually high background level of SCEs, there was still an increase in the SCEs after treatment with methylphenidate.

Table 1

The number and type of chromosome and chromatid aberrations before and 3 months after methylphenidate treatment in each study participant

Participant ^a	#Cells scored	#Chromosome aberrations	#Chromatid aberrations	#Gaps ^b	#Total breaks ^c
1	50	1	1	1	3
1R	50	1	2	2	4
2	50	0	1	1	1
2R	50	1	1	1	3
3	50	0	1	1	1
3R	50	2	1	3	5
4	50	0	0	1	0
4R	50	1	1	2	3
5	50	0	2	1	2
5R	50	0	5	2	5
6	50	1	0	0	2
6R	50	2	1	4	5
7	50	0	2	1	2
7R	50	1	7	5	9
8	50	0	1	0	1
8R	50	0	6	3	6
9	50	1	0	1	2
9R	50	1	3	5	5
10	50	0	2	0	2
10R	50	1	3	4	5
11	50	0	3	1	3
11R	50	1	6	3	8
12	50	0	1	2	1
12R	50	1	1	6	3

^a R indicates samples collected from the same individual 3 months after the start of methylphenidate treatment.

^b Gaps were reported but not included in the total number of breaks.

^c Each chromosome-type aberration was counted as two breaks.

3.4. Micronucleus assay results: individual data

For all the 11 study participants from whom MN data was obtained, 1000 binucleated cells were analyzed at two different time points: at baseline and 3 months after the initiation of methylphenidate treatment. The number of micronuclei (a marker of chromosome breakage and loss) per 1000 binucleated cells was recorded. Fig. 2 shows the MN results for each study subject. These results were similar to the results of the conventional CA and the SCE assays. A significantly higher frequency of MN ($P=0.000$) was observed in PBLs from each study participant after the 3 months of methylphenidate treatment. The number of nucleoplasmic bridges (NPB, a marker of

chromosome rearrangements) per 1000 binucleated cells was also recorded. Fig. 3, shows the results for each study subject. A significantly higher frequency of bridges ($P=0.000$) was observed in PBLs after 3 months of methylphenidate treatment in all the study subjects.

3.5. Cytogenetic summary data

Table 2 summarizes all of the cytogenetic findings of the current study. A significantly higher frequency of aberrant cells, expressed as total breaks per 50 cells, was observed in PBLs of all study subjects after treatment with methylphenidate for 3 months. The mean CA frequency after 3 months of treatment was 5.08, which is approximately a three-fold increase over the observed baseline mean CA frequency of 1.67 per 50 cells ($P=0.000$). The 95% confidence interval for the change in means before and after treatment was 2.44–4.62. A significantly higher mean number of SCEs ($P=0.000$) was observed in PBLs of all study subjects after the 3 months of methylphenidate treatment. The mean number of SCE after 3 months of treatment was 26.27, which was about a 4.3-fold increase over the observed baseline SCE level of 6.09. The 95% confidence interval for the change in means before and after treatment was 12.59–27.76. The mean MN frequency per 1000 cells after 3 months of treatment in all study subjects was 8.46 which is approximately a 2.4-fold increase over the observed baseline MN frequency of 3.55 per 1000 cells ($P=0.000$). The 95% confidence interval for the change in means before and after treatment was 3.04–6.77. The mean NPB per 1000 cells after 3 months of treatment was 6.18, which is approximately 2.8-fold higher ($P=0.000$) than the observed baseline NPB frequency of 2.18 per 1000 cells. The 95% confidence interval for the change in means before and after treatment was 2.59–5.40.

4. Discussion

To our knowledge, this is the first study that addresses the potential clastogenic effects associated with methylphenidate treatment in children. The study was designed so that each individual served as his or her own control. Thus, any increase in cytogenetic

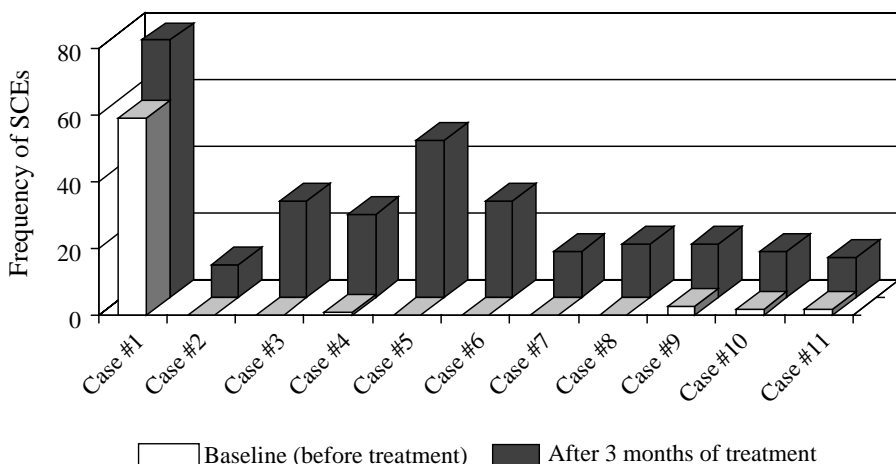


Fig. 1. Mean number of sister chromatid exchanges per 25 cells in each study subject before and 3 months after methylphenidate treatment.

endpoints observed after treatment could be directly attributed to the treatment. A baseline level for each biomarker of genetic damage was established prior to treatment with methylphenidate, and the possible effect of the treatment on that biomarker was determined after three months on the drug. In addition, several markers of genotoxicity were utilized for each sample to give a more comprehensive measure of any potential genotoxic effect that this drug may have. This design optimized the acquisition of relevant data even with a relatively small number of participants, and allowed the achievement of meaningful conclusions while controlling for the effect of

any potential confounders, such as dietary factors or other exposures.

In every individual examined, there was a statistically significant increase in every genotoxic endpoint analyzed with *P*-values equal to zero (up to the fourth decimal) for each of the parameters tested, and 95% confidence intervals for the difference in means quite far from zero. Despite previous evidence that methylphenidate did not cause an increase in MN in mice [7], this study clearly indicates that the use of methylphenidate is associated with an increase in MN in children treated with this drug. In the mouse MN assay, the animals were only treated once with

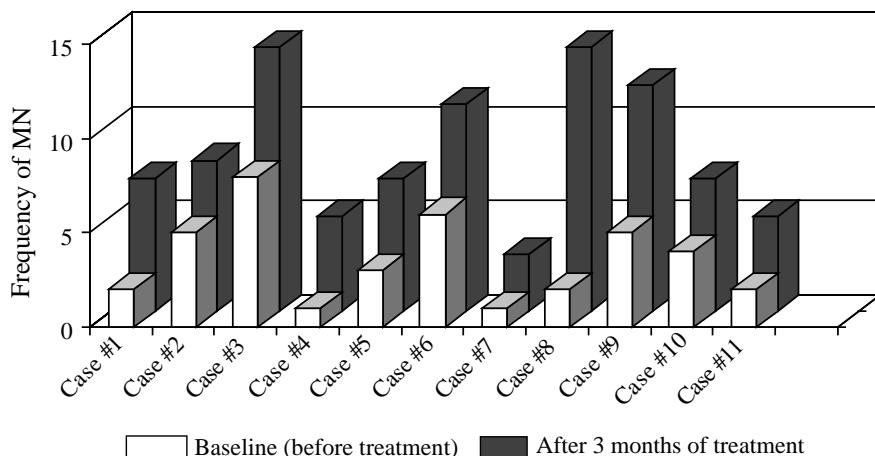


Fig. 2. Mean number of micronuclei in 1000 binucleated cells in each study subject before and 3 months after methylphenidate treatment.

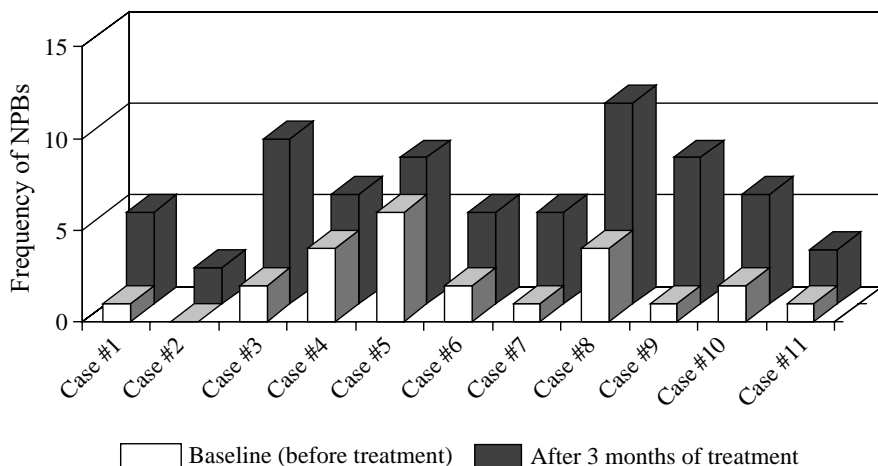


Fig. 3. Mean number of nucleoplasmic bridges in 1000 binucleated cells in each study subject before and 3 months after methylphenidate treatment.

the drug. In our study, the children received therapeutic doses (20–54 mg/day) of methylphenidate daily for 3 months. Thus, extended exposure may be responsible for the difference in the results of the animal studies. It is important to point out that in the animal bioassay studies, where prolonged administration of methylphenidate occurred, liver tumors developed in mice [3]. Differences in metabolism of the drug between species may also have affected the results of these studies. The metabolism of methylphenidate is reported to involve de-esterification, with the primary metabolite in humans being ritalinic acid (80% in urine) [18]. We could not find quantitative data for ritalinic acid production in mice, but the levels of ritalinic acid seen in rats and dogs are significantly lower (35–40% and 23%, respectively, after oral administration) than the levels reported in humans [18]. At present, we do not know the exact mechanism responsible for the genotoxic response that we observed in children following administration of methylphenidate.

We used cytogenetic endpoints to identify the possible genotoxic effects of methylphenidate treatment because of the relevance of these endpoints as biomarkers for genetic damage related to cancer [16]. It is well documented that the frequency of CA in circulating lymphocytes is a relevant biomarker for cancer risk in humans, reflecting both early biological effects of exposure to genotoxic carcinogens and individual susceptibility. This correlation between CA

and cancer risk was found regardless of known exposure to carcinogens, which indicates that an increase in CA frequency is, in itself, a strong biomarker for elevated risk of cancer (reviewed by [19]). Only one study addressing the carcinogenesis of methylphenidate in humans was located in the literature [20]. Its usefulness in addressing the carcinogenesis of methylphenidate in humans is limited because of its design as a hypothesis generating study. Our results, coupled with the positive cancer bioassay data in laboratory animals, provide a strong argument that methylphenidate should be further investigated.

Table 2
Summary of the cytogenetic data

Endpoint	Mean ± SEM before treatment	Mean ± SEM after 3 months of treatment	P-value ^a
CA ^b	1.67 ± 0.27	5.08 ± 0.54	0.000
SCEs ^c	6.09 ± 5.30	26.27 ± 6.03	0.000
MN ^d	3.55 ± 0.68	8.46 ± 1.13	0.000
NPB ^e	2.18 ± 0.54	6.18 ± 0.80	0.000

^a The difference between the levels of each end point before treatment and after treatment was significantly different ($P=0.000$).

^b Mean number of breaks in all study subject before and 3 months after methylphenidate treatment.

^c Mean number of sister chromatid exchanges in all study subject before and 3 months after methylphenidate treatment.

^d Mean number of micronuclei in all study subject before and 3 months after methylphenidate treatment.

^e Mean number of nucleoplasmic bridges in all study subject before and 3 months after methylphenidate treatment.

While twelve subjects may represent a relatively small sample size, our study is remarkable in the consistency of the increase in every type of cytogenetic endpoint monitored, in every child receiving the drug, when the results from the pretreatment period were compared with the results after three months of treatment. While it should be noted that the data should be replicated and expanded by further studies in a larger population before a definitive conclusion about the genotoxicity of methylphenidate can be attained, the results presented herein raise many important questions. For example, what is the reversibility of the cytogenetic effects observed when the use of the drug is terminated? Could the same clastogenic effects observed in children be observed in adults? Would the effects observed be exacerbated with an increased length of drug intake? One alternative treatment available for ADHD is Adderall, which is also an amphetamine-based drug like methylphenidate. However, in a recent study, investigators reported an increase in genetic damage in adult methamphetamine users [21]. Therefore, it is imperative that treatment with amphetamine-based, and other structurally related drugs, be monitored for genotoxic effects in children as well. Another major issue not addressed is the question of the role that genetic susceptibility may play in the observed effects. Although every child showed increases in the measures of genotoxicity monitored, the magnitude of the response varied considerably between individuals. It is now well documented that genetic polymorphisms in certain genes, such as metabolic and DNA repair genes, affect the level of genetic damage in individuals exposed to genotoxic chemicals [22–24]. It therefore remains to be seen if polymorphisms in susceptibility genes would affect the genotoxic response observed with methylphenidate.

In conclusion, the lack of research on the long-term effects of methylphenidate use in humans warrants great concern. At present, it is not clear what the long-term effects would be for children who took methylphenidate 10 or 20 years ago or for those who are currently being treated with this drug. Clearly, this study opens the door for further larger studies that address these issues in order to establish the safety of methylphenidate, as well as possible replacement drugs, for the treatment of ADHD.

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