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No Increases in Biomarkers of Genetic Damage or Pathological Changes in Heart and Brain Tissues in Male Rats Administered Methylphenidate Hydrochloride (Ritalin) for 28 Days

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Abstract

Following a 2005 report of chromosomal damage in children with attention deficit/hyperactivity disorder (ADHD) who were treated with the commonly prescribed medication methylphenidate (MPH), numerous studies have been conducted to clarify the risk for MPH-induced genetic damage. Although most of these studies reported no changes in genetic damage endpoints associated with exposure to MPH, one recent study (Andreazza et al. 2007) reported an increase in DNA damage detected by the Comet assay in blood and brain cells of Wistar rats treated by intraperitoneal injection with 1, 2, or 10 mg/kg MPH; no increases in micronucleated lymphocyte frequencies were observed in these rats. To clarify these findings, we treated adult male Wistar Han rats with 0, 2, 10, or 25 mg/kg MPH by gavage once daily for 28 consecutive days and measured micronucleated reticulocyte (MN-RET) frequencies in blood, and DNA damage in blood, brain, and liver cells 4 hr after final dosing. Flow cytometric evaluation of blood revealed no significant increases in MN-RET. Comet assay evaluations of blood leukocytes and cells of the liver, as well as of the striatum, hippocampus, and frontal cortex of the brain showed no increases in DNA damage in MPH-treated rats in any of the three treatment groups. Thus, the previously reported observations of DNA damage in blood and brain tissue of rats exposed to MPH for 28 days were not confirmed in this study. Additionally, no histopathological changes in brain or heart, or elevated serum biomarkers of cardiac injury were observed in these MPH-exposed rats.

Keywords

genotoxicity; ADHD; chromosomal damage; Comet assay; micronuclei; troponin

INTRODUCTION

Methylphenidate hydrochloride (MPH; Ritalin) is one of the most commonly prescribed stimulant medications used to treat children and adults diagnosed with attention deficit/

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hyperactivity disorder (ADHD) (Safer, 2000; Zito et al., 2000). Exposure to MPH is widespread, with annual prescriptions in the United States alone exceeding five million. The overall safety profile of MPH has been favorable (Biederman et al., 2006), and in terms of its genotoxicity, results from a variety of standard genetic toxicity assays have provided little evidence that treatment with MPH poses a hazard (Mortelmans et al., 1986; NTP, 1995; Teo et al., 2003; Suter et al., 2006). In 2005, however, a report of chromosomal damage in ADHD children following 3 months of treatment with MPH (El Zein et al., 2005) prompted a number of follow-up studies designed to clarify the genotoxic potential of MPH in humans (Suter et al. 2006; Walitza et al., 2007; Manjanatha et al., 2008; Witt et al., 2008b; Walitza et al., 2009). Results from all these studies, which investigated several chromosomal and mutagenic endpoints in humans, animals, and *in vitro* human cell systems, were uniformly negative.

In contrast to the overwhelmingly negative genetic toxicity data for MPH, Andreazza et al. (2007) reported increased levels of DNA damage, measured by the Comet assay, in peripheral blood leukocytes, and in cells of the striatum and hippocampus tissues of the brain in young (25 days old) and adult (60 days old) male Wistar rats following exposure to MPH via intraperitoneal (IP) injection. Rats received either a single injection or 28 consecutive daily injections of 1, 2, or 10 mg MPH /kg/day; positive controls were not included. The authors indicated that the lower two doses were within the human equivalent dose range, and the high dose represented an exposure that might be achieved by “recreational” use of MPH. The observations of increased DNA damage in striatum and hippocampus at human equivalent doses were particularly concerning because these brain regions are the site of MPH’s therapeutic action in humans (Gray et al., 2007). In addition to DNA damage, Andreazza et al. (2007) measured the frequency of micronuclei (biomarkers of structural chromosomal damage and chromosome loss) in lymphocytes of MPH-treated rats and found no effect on this endpoint at any dose level, following single or repeated dosing, in either of the two age groups they treated.

Because of the widespread exposure to MPH, particularly within pediatric populations, we designed a study to attempt to confirm the findings of DNA damage reported by Andreazza et al. (2007). We introduced several key modifications to the original protocol. First, we focused only on the adult rats and the 28-day exposure period, since the increases in DNA damage reported by Andreazza et al. were stronger and more consistent in this group of animals. In addition, we included a third brain section (frontal cortex) for analysis of DNA damage, selected gavage as the route of administration to better approximate the oral exposure route in humans, and measured frequency of micronuclei in blood reticulocytes using a flow cytometry-based assay, rather than evaluating blood leukocytes for MN frequency. We added a higher dose of MPH (25 mg/kg/day) to increase our chances of detecting DNA damage, particularly with the change in route of administration from IP injection to gavage, and we included a positive control group to establish the sensitivity of our assay protocols to detect an effect. To obtain additional information on the effects of MPH in the rat brain, we conducted detailed neurohistopathological examinations of the three brain sections evaluated for DNA damage. Finally, because adverse cardiovascular events have been reported, although rarely, in children treated with MPH (Biederman et al., 2006), we conducted histological examination of the hearts and measured cardiotoxicity biomarkers in serum.

METHODS and MATERIALS

Animal Husbandry

All animal experiments were conducted at ILS, Inc. (Research Triangle Park, NC). Male Wistar Han rats were obtained from the supplier (Charles River Laboratories, Raleigh, NC)

at approximately 8 weeks of age. All animals were housed singly in polycarbonate cages with absorbent hardwood bedding (Beta-Chip Hardwood Laboratory Bedding, Northeastern Products Corp, Warrensburg, NY) in an AAALAC-accredited Specific Pathogen Free facility with a 12-hr light /12-hr dark cycle. Temperature and humidity were monitored continuously. Temperature ranged from 20°C–24°C and relative humidity ranged from 17%–69%. Food (Purina Pico Chow No. 5002, Ralston Purina Co., St. Louis, MO) and water were provided ad libitum. All procedures were completed in compliance with the Animal Welfare Act Regulations, 9 CFR 1–4, and animals were handled and treated according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). The ILS, Inc., Institutional Animal Care and Use Committee approved this study.

Experimental Procedures

Following one week acclimatization, 10 rats per treatment group (randomized using a body weight stratification scheme) were dosed by oral gavage with aqueous solutions of D,L-methylphenidate HCl (MPH, 99.3% pure; Research Biochemicals International, Natick, MA) once daily for 28 consecutive days. Doses of MPH were 2, 10, and 25 mg/kg/day; concurrent negative (purified, deionized water) and positive (ethyl methanesulfonate, 100 mg/kg/day; EMS; Sigma-Aldrich Co., St. Louis, MO) control groups were also included. The vehicle control was administered daily for 28 days to each of 10 control rats and EMS, prepared fresh each day in deionized water, was administered by gavage the final three days of the study to each of 5 animals.

Genotoxicity Studies

Erythrocyte Micronucleus (MN) Assay: Five animals each from the vehicle control, MPH, and positive control groups were sacrificed 4 hr after the final treatment. This 4 hr sample time was used to obtain tissues for DNA damage assessment (see below) within the recommended time frame (3–6 hr) following gavage exposure (Burlinson et al., 2007). Peripheral blood samples were processed for flow cytometric evaluation of micronucleated reticulocytes (MN-RET) (Witt et al., 2008a), and blood and tissue samples (liver; frontal cortex, hippocampus, striatum of brain) were processed for DNA damage assessment using the Comet assay (Tice et al., 2000; Ghanayem et al., 2005; Burlinson et al., 2007). Briefly, blood samples for MN-RET analyses were processed using MicroFlow^{PLUS} Kit (Rat) reagents (Litron Laboratories, Rochester, NY) according to the kit's instructional manual. For each peripheral blood sample, 20,000 (± 2000) immature CD71-positive RET were analyzed using a FACSCalibur™ (Becton-Dickinson Biosciences, San Jose, CA) flow cytometer to determine the frequency of MN-RET. Typically, more than 10^6 mature erythrocytes were enumerated concurrently during MN-RET analysis, and the percentage of RET (%RET) among total erythrocytes was calculated, as a measure of bone marrow toxicity.

DNA Damage - Comet Assay: Blood samples, 50 μL per animal collected during exsanguination, were placed into vials containing 1 mL of mincing solution (Mg^{+2} and Ca^{+2} free Hank's buffer containing 20 mM EDTA and 10% DMSO, pH 7.4–7.7), flash frozen in liquid nitrogen, and stored below −60°C until processed. A portion of each solid tissue (liver, brain) to be analyzed for DNA damage was added to a vial containing 1 mL of mincing solution and kept on ice until processed. After tissues were rapidly minced, all vials were flash frozen in liquid nitrogen and stored below −60°C until processed. For processing, cells were removed from the −80° freezer and placed into a warm water bath. When partially thawed (determined by a small amount of ice remaining in the tubes), the tubes were removed from the water bath and once again placed on ice until slide preparation. For slide preparation, cells were diluted to a total volume of 50 μL with PBS as necessary,

mixed with 500 μL of 0.5% low melting point agarose (Molecular Biology grade, Cat. #9414, Sigma-Aldrich, St. Louis, MO) at 37± 2°C and 50 μL of the cell/agarose suspension was layered onto each well of a 2-well CometSlide™ (Trevigen, Gaithersburg, MD). Slides were placed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, with 10% DMSO and 1% Triton X-100, added fresh; (NaCl, Na_2EDTA, and Triton X-100 were from Sigma-Aldrich). After incubating at least 1 hr, one slide per sample was rinsed with 0.4 M Trizma base (pH 7.5; Sigma-Aldrich), treated with alkali (300 mM NaOH, 1 mM Na_2EDTA; pH>13) for 20 min, and then electrophoresed at room temperature for 20 min at 1.0 V/cm, 300 mA. After electrophoresis, slides were neutralized with 0.4 M Trizma base (pH 7.5) for 3–5 min, incubated for 3–5 min in ice-cold 100% ethanol (McCormick Distilling Co., Inc., Weston, MO) and allowed to air-dry in an environment with a relative humidity (RH) of <60%. Slides were stored at room temperature in a desiccator (RH<60%) until stained and scored.

After staining slides with SYBR Gold™ (Molecular Probes, Invitrogen, Carlsbad, CA), 100 cells were scored per sample at magnification 200x using the Comet Assay IV Imaging Software, Version 4.11 (Perceptive Instruments, Ltd., Suffolk, UK). Slides were scored without knowledge of their identity. The extent of DNA migration was evaluated according to the following endpoint measurements:

% Tail DNA: intensity of all tail pixels divided by the total intensity of all pixels in the comet, expressed as a percentage

Tail Length: the horizontal distance from the center of the head (start of tail) to the end of the tail

Olive Tail Moment (OTM): the distance between the center of gravity of the DNA distribution in the tail and the center of gravity of the DNA distribution in the head, multiplied by the fraction of DNA in the tail.

DNA Damage – Neutral Diffusion Assay: To assess for the presence of cells with low molecular weight (LMW) DNA, which is potentially indicative of apoptosis or necrosis (Tice et al., 2000; Burlinson et al., 2006), one slide for each sample was removed from lysis buffer after 1–2 hr. The slide was neutralized with 0.4 M Trizma base (pH 7.5), fixed in 100% ethanol, and air-dried and stored in a desiccator at room temperature at a RH of <60%. After staining slides with SYBR Gold™, 100 cells per slide were scored microscopically as having either condensed DNA or diffused DNA (LMW DNA).

Cardiotoxicity and Brain Neurohistopathology Studies—Hearts from each of the 5 rats per treatment group (MPH and control groups) used in the genotoxicity studies were collected and preserved in 10% neutral buffered formalin (Surgipath Medical Industries, Inc., Richmond, IL), transferred to 70% histology grade alcohol 18–24 hr later, then routinely processed and embedded in paraffin within 48 hr of collection. 5-μm sections were prepared and stained with hematoxylin and eosin (H&E; Surgipath Medical Industries, Inc.) and a complete microscopic evaluation was performed on each heart.

Cardiac perfusion was performed on the remaining 5 animals in the vehicle control and MPH-treated groups for the collection of brain tissues for histology studies. Animals were anesthetized with isoflurane (Halocarbon Products Corporation, River Edge, NJ) and perfused via the left ventricle with an initial flushing solution of 0.9% sodium chloride containing 1000 units/L heparin sodium and 1 mL/L of 1% sodium nitrite (approximately one minute) followed by McDowell Trumps fixative (at room temperature) (Electron Microscopy Services, Hatfield, PA) at a rate of approximately 30 mL/min for 10–15 min. Pressure was by gravity from approximately 1 meter above the table level. The brains were

collected and stored in McDowell Trumps fixative and sent to the pathology laboratory (Pathology Associates, Inc., Durham, NC) for histological analysis by a veterinary pathologist. Thirty subanatomic sites, including both grey and white matter, within three brain sections (hippocampus, frontal cortex, striatum) were examined in each animal using routine H&E (Surgipath Medical Industries, Inc., Richmond, IL) and Fluorojade B (Schmued and Hopkins, 2000) staining.

Cardiac Injury Biomarker Assay—Blood serum was prepared from each of the 5 rats per treatment group used in the genotoxicity studies for assessment of cardiotoxicity biomarkers (Gaze and Collinson, 2005). Circulating serum levels of cardiac Troponin T (TnT), Troponin I (TnI), and Fatty Acid Binding Protein-3 (FABP3) were measured using a rat MULTI-SPOT cardiac injury panel assay kit and SECTOR™ Imager 2400 electrochemiluminescence detection platform (Meso Scale Discovery (MSD™), Gaithersburg, MD). The criteria for acceptance of calibration curve data points was ≤20% Coefficient of Variation and 100 ± 20% recovery. Serum sample measurements outside the quantitation range for a biomarker were excluded from the data analysis.

Data Analysis

Body Weight—Body weight changes in relation to dose were assessed for normality using a Shapiro-Wilk test (Shapiro and Wilk, 1965) and then, when determined to be normally distributed, evaluated using one-way ANOVA analysis followed by a two-tailed t-test. If the data were not normally distributed, nonparametric statistical tests (e.g., Mann-Whitney) were used.

MN Assay—The frequency of MN-RET was determined in 20,000 RET per animal. These measurements are typically normally distributed within each dose group. Levene's test was applied to test for equal variances among the treatment groups. If the variances were equal, simple linear regression was used to test for a dose-related trend and pairwise differences between each dosed group and the controls were tested using Williams' test (Williams 1971, 1972). If the variances were not equal, Jonckheere's test (Jonckheere 1954) was used to test for a dose-related trend and pairwise differences with the control group were tested using Dunn's test (Dunn 1964). To maintain the overall significance level at 0.05 with multiple tests, the trend is declared statistically significant if one-tailed $P < 0.025$, and pairwise differences from the control group are declared statistically significant if one-tailed $P < 0.025$, as Williams' and Dunn's tests already correct for multiple comparisons.

Comet and Neutral Diffusion (LMW DNA) Assays—Data from 100 cells per animal were collected and assessed for a significant ($p < 0.05$) increase in DNA migration or the frequency of cells with LMW DNA. Using individual animal data, the Shapiro-Wilk test was used to assess normality of the negative control group. Data that were not normally distributed were analyzed by the Mann-Whitney test (Mann and Whitney, 1947) comparing each dose level to the concurrent control, followed by the Kendall rank correlation test (Kendall, 1938) to determine the presence of a dose response. Data that were normally distributed were analyzed using the F test to determine homogeneity of variances, an independent two-sample t-test comparing each dose level to the concurrent control, and linear regression to determine the presence of a dose response.

RESULTS

Clinical and Necropsy Observations

All animals survived to the end of the study; no clinical signs of toxicity were apparent with the exception of sporadic occurrences of hyperactivity noted in a total of eight animals one

hour after dose administration. Findings were not consistent with day of treatment or any other study parameter. All animals treated with MPH showed percent body weight gains similar to controls, whereas EMS-treated rats lost weight during the three days they were dosed with the positive control chemical (Table I). No gross findings were noted at necropsy.

Histology of the Brain and Heart

No MPH-related lesions were detected in the hearts or in any of 30 subanatomic sites in the brain (including both grey and white matter within the frontal cortex, hippocampus, or striatum) of male rats. Cardiomyopathy was identified in the left ventricles of five rats (3 in the vehicle control group and 2 in the high dose MPH group). This lesion was characterized by scattered rare foci of lymphocytic and histiocytic inflammatory infiltrate, sometimes associated with fragmented and/or hyalinized, necrotic myocytes and occasional Anitschkow cells, which may represent regenerating myocytes in rodents. The lesions varied from minimal to mild in severity and from focal to multifocal. This myocardial lesion is typical of ‘spontaneous cardiomyopathy’ in rats (Boorman et al., 1990) and was not considered to be treatment-related.

Cardiac Injury Biomarker Assays

Duplicate measurements of serum troponin levels were made for each animal. Most MPH-treated rats (17/20) had one or both measurements of TnT below detectable limits. For TnI, 7/20 animals had one or both measurements below the detection level. Serum FABP3 levels were above the limits of detection for 2 of 20 animals; no FABP3 measures fell below detection limits. Measurements of serum levels of cardiac TnT, TnI, or FABP3 falling within the quantifiable ranges showed no significant change as a result of MPH treatment (Table II). No MPH-treated rats had measurable serum TnT or FABP3 levels that exceeded the highest concentration measured in vehicle control animals. Serum TnI levels were elevated (1.6-fold increase over the vehicle control value) in one animal in the 2 mg/kg dose group and one in the 10 mg/kg dose group (data not shown). No remarkable histopathological lesions and no cardiomyopathy were observed in either of these two animals.

MN Test

No significant or dose-related increases in MN-RET were seen in Wistar Han rats treated with MPH (Table III). Although the frequencies of MN-RET are rather low in the rats in this study, they are all within 1–2 standard deviations of the laboratory historical control mean value for Wistar Han rats (0.93 ± 0.37%). The %RET was unchanged over the dose range, indicating an absence of MPH-related bone marrow toxicity.

Comet Assay

Under the conditions used in the neutral diffusion assay, no increase in the frequency of cells with LMW DNA was observed in blood leukocytes, liver, striatum, hippocampus, or frontal cortex of male Wistar Han rats exposed to MPH (Table IV). These data suggest that exposure to MPH did not induce cytotoxicity (necrosis or apoptosis) in male rats at the concentrations tested.

No significant increases in OTM were observed in blood leukocytes or in cells of the liver, or of the striatum, hippocampus, or frontal cortex of the brain (Table V), suggesting the absence of MPH-associated increases in DNA damage in these tissues. The positive control chemical, EMS, induced significant increases in the amount of DNA damage in blood leukocytes, in liver cells and in cells of the striatum, hippocampus, and frontal cortex of the

brain in treated rats (Table V). However, EMS-associated cytotoxicity (LMW DNA) was only detected in blood leukocytes (Table IV).

DISCUSSION

The findings reported by Andreazza et al. (2007) of elevated levels of DNA damage, measured by the Comet assay, in cells of the blood and brain of adult rats exposed to MPH for 28 days were not confirmed in our study. Our findings are consistent with the accumulating evidence that MPH does not induce genetic damage *in vitro* or *in vivo* (Mortelmans et al., 1986; Teo et al., 2003; Suter et al., 2006, Walitza et al., 2007; Manganatha et al., 2008; Witt et al., 2008b; Walitza et al., 2009; Morris et al., 2009).

A number of factors may be responsible for the differences in results between our study and that reported by Andreazza et al. (2007). One difference is the method of scoring DNA damage (Comet figures). Andreazza et al. (2007) visually evaluated 100 randomly selected cells and subjectively scored the images according to tail intensity (determined by the size and shape of the tail). In contrast, we used an automated system that sequentially evaluated well-defined Comet figures using objective quantification of image pixels that were converted into measurements of tail length and % tail DNA; we then quantified DNA damage using the OTM (Olive Tail Moment) measure, which factors in both tail length and % tail DNA (Burlinson et al., 2007). In addition, cells containing LMW DNA (Table IV) were evaluated separately in our study to assess any possible impact of cytotoxicity on interpretation of the Comet data. It should be noted that we used flash freezing of tissue samples in DMSO to carefully control the time interval from obtaining tissue until lysing. The low frequency of cells with LMW or fragmented DNA (*e.g.* <2% in the liver of vehicle control mice and no apparent increase in MPH exposed mice) demonstrates that our procedure for flash freezing produces little nuclear DNA degradation. This observation is consistent with an earlier report of a direct comparison of fresh and cryopreserved lymphocyte samples in which there was no difference in the amount of DNA damage detected by the Comet assay (Duthie et al., 2002). Moreover, there is no expectation that the extent of DNA damage induced by snap freezing will vary among samples that are processed similarly. Thus, our data benefit from the careful control of the time between tissue harvest and lysis, a critical factor for obtaining consistent, reliable data using the Comet assay.

The study protocol we employed differed in certain other key respects from the one used by Andreazza et al. (2007). Rather than administering MPH by intraperitoneal injection, which avoids first-pass metabolism, we employed oral gavage, a route that better mimics the route of exposure (oral) and metabolism of MPH in humans. Recent publications provide evidence that metabolism of MPH may be strongly influenced by route and treatment regimen (e.g., oral versus dermal patch, and extended release formulations versus single daily bolus dosing), and therefore, using a route (gavage) that more closely approximates human oral dosing might be expected to yield results more reflective of events that occur in humans (Teicher et al., 2006; Devilbliss and Berridge, 2008).

In regards to dose selection, we used the two highest doses administered in the Andreazza study (2 and 10 mg/kg/day), and we added a third, higher dose (25 mg/kg/day). This higher dose was used to compensate for any reduced bioavailability of MPH administered via the gavage route (our study) compared with IP injection (Andreazza et al. study), and enhance our ability to detect DNA or chromosomal damage, were either to occur as a consequence of MPH exposure. With regards to bioavailability, Gerasimov et al. (2000) measured concentrations of MPH (a racemic mixture of *d-threo*- and *l-threo*-MP) in plasma, and in striatum and cerebellum of Sprague-Dawley rats 20 minutes after either intraperitoneal or

intragastric administration of a single dose of 5 mg/kg MPH and found higher concentrations of MPH (approximately 2.5-fold) in all three tissues following intraperitoneal dosing compared with the oral route. Studies by Wargin et al. (1983) comparing bioavailability of MPH in rats and monkeys following a single intravenous or oral gavage administration indicated that both routes had similar bioavailability values of approximately 20% in both species, based on area-under-the-curve measurements in plasma over an 8 hour period of time (Wargin et al., 1983). Based on this limited information, use of the gavage route in our study was unlikely to have appreciably decreased the total exposure of the rats in our study to MPH, although the kinetics of exposure may have been markedly different than the kinetics in the Andreazza et al. (2007) study. Our use of the higher 25 mg/kg dose would be expected to compensate to some degree for a decrease in MPH bioavailability from oral administration.

In another point of contrast, we used only adult male Wistar Han rats rather than adults and juveniles, because increases in DNA damage reported in the earlier study were stronger and more consistent in the adult animals. Furthermore, we used only the 28-day repeated exposure protocol, because Andreazza et al. (2007) reported more pronounced increases in DNA damage in 28-day exposed rats compared with rats that received a single injection of MPH. Another justification for using the repeated dosing protocol is the closer similarity to human exposures to MPH, which are typically chronic rather than single administration.

Because Andreazza et al. (2007) reported DNA damage in the hippocampus and striatum of the brain following 28 days of exposure to MPH, we added a histology component to our protocol to look for pathological changes associated with MPH exposure in tissues from these brain regions, where MPH is believed to exert its clinical effects in humans (Biederman and Faraone, 2005; Dommett et al., 2008). No evidence of histopathological changes were seen in either region in any of the dose groups in our study, and likewise, no histopathological changes were noted in tissues of the frontal cortex, another brain region believed to be a site of MPH therapeutic activity (Biederman and Faraone, 2005; Devilbiss and Berridge, 2008).

In addition to the absence of primary DNA damage in our study, no significant increases in MN-RET (micronucleated reticulocytes) were seen in MPH-treated rats, a result that is consistent with the negative results reported by Andreazza et al. (2007) and others for induction of micronuclei either in lymphocytes or in erythrocytes of MPH-treated rats, mice, or nonhuman primates (NTP, 1995; Suter et al., 2006; Teo et al., 2003; Manjanatha et al., 2008; Morris et al., 2009). Assessment of MN frequencies in rat RET is a relatively new procedure (Torous et al., 2000), and was facilitated by the advent of flow cytometry for evaluating micronucleated erythrocyte frequencies in peripheral blood (MacGregor et al., 2006). Thus, although the rat spleen rapidly and efficiently removes micronucleated erythrocytes from circulation, evaluating damage in very young RETs, newly emerged from the bone marrow compartment, provides an accurate assessment of recently acquired chromosomal damage (Witt et al., 2008a). Using flow cytometry, RETs are identified by the presence of an active transferrin receptor (CD71+) on the cell surface; newly emerged RETs have the highest CD71 expression levels, and mature erythrocytes are CD71−. The negative micronucleus test results in MPH-treated rats in the study reported here support the results of studies conducted in humans in which no increase in the frequency of micronucleated lymphocytes was observed following exposure to therapeutic doses of MPH for periods of time ranging from 1 month to 2 years (Walitza et al., 2007; Walitza et al., 2009; Witt et al., 2008b; Tucker et al., in press). Although the numerous MN studies conducted on humans and animals treated with MPH have used varied exposure times and experimental protocols, and have assessed damage in different cell types (reticulocytes, erythrocytes, or

lymphocytes), the consistency of the negative responses across all studies strongly suggests that MPH does not induce numerical or structural chromosomal damage *in vivo*.

Rarely, adverse cardiovascular events (tachycardia, sudden death) have been reported in children taking MPH and other stimulant medications for treatment of ADHD (Beiderman et al., 2006). Therefore, in addition to the genetic toxicity and brain histopathology studies, we examined biomarkers of cardiac injury including serum levels of cardiac Troponin T, Troponin I, and Fatty Acid Binding Protein-3 in rats exposed to MPH. Cardiac troponins are sensitive biomarkers of cardiac injury, and have recently gained increasing importance as part of an overall assessment of cardiac toxicity (Gaze and Collinson, 2005). No evidence of cardiac injury was observed in any of the MPH-treated rats using these endpoints of damage, and histological examination of the hearts revealed no treatment-related effects.

The negative results reported here from the MN and Comet assays add to the rapidly growing body of evidence that MPH, at clinically relevant and higher doses, after acute or prolonged exposure periods, in a variety of in vivo test systems, does not induce cytogenetic damage. Together, results of all these recently conducted studies should alleviate concerns regarding the potential for genetic damage from MPH and other stimulant drugs used in the treatment of ADHD. Answers to questions regarding the possibility of changes in normal patterns of behavior, growth and maturation, or learning associated with chronic exposure to MPH during development await further investigation.

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Table I

Body Weight Changes for Rats Following Treatment with MPH for 28 Days by Gavage[a]

Treatment	Dose (mg/kg/day)	Body Weight Change in grams (%)
Water[b]		62.3 ± 14.55 (22.5)
MPH	2	62.6 ± 10.13 (22.7)
	10	62.1 ± 12.29 (22.6)
	25	62.4 ± 15.87 (22.3)
EMS[b]	100	−14.3 ± 7.07 [c] (−4.1)

[a] Data presented as mean ± standard deviation. Body weight changes were measured from the beginning of dosing to the end of study (28 days for vehicle and MPH-treated rats; 3 days for positive control rats).

[b] Vehicle, deionized water; positive control, EMS (ethyl methanesulfonate).

[c] $p<0.05$ compared to vehicle control.

Table II

Cardiac Injury Biomarkers in Serum of Male Wistar Han Rats Treated with MPH for 28 Days By Gavage[a]

Dose (mg/kg/day)	TnT		TnI		FABP3	
	N[b]	ng/ml	n	ng/ml	n	ng/ml
0[c]	3	0.132	4	0.035	5	3.82
2	4	0.116	4	0.035	4	1.72
10	4	0.122	5	0.040	4	2.25
25	2	0.119	5	0.028	5	2.04

[a] TnT, serum troponin T; TnI, serum troponin I; FABP3, Fatty Acid Binding Protein-3. Biomarkers were measured using the MesoScale Discovery (MSD®) rat MULTI-SPOT™ cardiac injury panel assay kit and SECTOR Imager 2400 electrochemiluminescence detection platform.

[b] Number of animals providing measurable levels of cardiac biomarkers.

[c] Vehicle control, deionized water.

Table III

Frequency of MN-RET in Male Wistar Han Rats Treated with MPH for 28 Days[a,b]

Treatment	Dose (mg/kg/day)	MN-RET/1000	p[c]	%RET
Water[d]	0	0.54 ± 0.11		1.11 ± 0.12
MPH	2	0.89 ± 0.19	*0.090*	0.81 ± 0.10
	10	0.78 ± 0.09	*0.109*	1.21 ± 0.19
	25	0.76 ± 0.13	*0.115*	0.93 ± 0.12
	trend p[e]	*0.697*		*0.853*
EMS[f]	100	4.89 ± 0.43	*<0.001*	0.876 ± 0.13

[a] Data presented as mean ± SE, based on 20,000 cells per animal, 5 animals per treatment group.

[b] MN-RET, micronucleated reticulocytes; %RET, percent reticulocytes among total erythrocytes.

[c] Pairwise p-value, treated group versus concurrent control; significant at p<0.025.

[d] Vehicle

[e] One-tailed trend test, significant at p<0.025.

[f] Positive control, EMS (ethyl methanesulfonate).

Table IV

LMW DNA in Male Wistar Han Rats Treated with MPH by Gavage for 28 Days[a,b]

Treatment	Dose (mg/kg/day)	% Cells with LMW DNA	p[c]
		Blood	
MPH	0	3.2 ± 2.33	
	2	1.8 ± 0.66	0.421
	10	1.8 ± 0.49	0.345
	25	1.4 ± 0.40	0.345
	trend p[d]	*0.916*	
EMS	100	18.0 ± 5.45	0.016
		Liver	
MPH	0	1.6 ± 0.60	
	2	1.0 ± 0.45	0.777
	10	1.2 ± 0.20	0.728
	25	0.4 ± 0.24	0.949
	trend p	*0.928*	
EMS	100	0.6 ± 0.24	0.919
		Frontal Cortex	
MPH	0	1.2 ± 0.20	
	2	2.0 ± 0.84	0.345
	10	1.2 ± 0.20	0.579
	25	1.4 ± 0.68	0.579
	trend p	*0.803*	
EMS	100	1.4 ± 0.24	0.345
		Hippocampus	
MPH	0	1.2 ± 0.73	
	2	1.0 ± 0.32	0.596
	10	0.8 ± 0.58	0.660
	25	0.8 ± 0.37	0.680
	trend p	*0.392*	
EMS	100	0.6 ± 0.24	0.770
		Striatum	
MPH	0	0.8 ± 0.49	
	2	1.4 ± 0.24	0.210
	10	1.2 ± 0.80	0.500
	25	0.6 ± 0.40	0.655
	trend p	*0.589*	
EMS	100	1.4 ± 0.40	0.210

[a] LMW, low molecular weight; MPH, methylphenidate; EMS, ethylmethanesulfonate (positive control); vehicle, deionized water.

[b] Data are mean ± SE, based on 100 cells per animal, 5 animals per treatment group.

[c] Pairwise comparison of each treated group to the concurrent vehicle control.

[d] One-tailed linear regression or Kendall Rank correlation tests to assess dose-related increases in cells with LMW DNA; significant at $p<0.05$.

Table V

Comet Assay Results in Male Wistar Han Rats Treated with MPH by Gavage for 28 Days[a]

Treatment	Dose (mg/kg/day)	Olive Tail Moment	p[b]	% Tail DNA	Tail Length (microns)
		Blood			
MPH	0	0.4 ± 0.08		3.0 ± 0.44	23.0 ± 0.64
	2	0.5 ± 0.17	0.358	3.1 ± 0.89	24.7 ± 1.01
	10	0.6 ± 0.08	0.098	3.5 ± 0.31	23.3 ± 0.61
	25	0.3 ± 0.06	0.747	2.1 ± 0.40	24.6 ± 0.77
	trend p[c]	*0.448*			
EMS	100	9.2 ± 0.56	<0.001	41.4 ± 1.85	54.8 ± 1.20
		Liver			
MPH	0	5.6 ± 1.30		25.1 ± 4.46	54.3 ± 2.24
	2	7.1 ± 1.49	0.231	29.3 ± 5.04	60.8 ± 2.01
	10	6.7 ± 1.24	0.282	28.7 ± 4.57	56.3 ± 3.20
	25	7.3 ± 0.53	0.126	30.6 ± 1.70	62.2 ± 1.45
	trend p	*0.464*			
EMS	100	14.3 ± 3.13	0.017	50.6 ± 7.50	66.7 ±4.57
		Frontal Cortex			
MPH	0	3.7 ± 0.56		18.6 ± 2.23	51.3 ± 1.65
	2	4.7 ± 0.65	0.157	21.0 ± 2.70	58.7 ±1.67
	10	3.9 ± 0.28	0.429	19.0 ± 1.11	49.8 ± 1.82
	25	3.9 ± 0.65	0.409	18.8 ± 2.62	53.2 ± 2.63
	trend p	*0.244*			
EMS	100	10.2 ± 0.47	<0.001	40.6 ± 1.28	63.5 ± 1.64
		Hippocampus			
MPH	0	4.3 ± 0.58		21.9 ± 2.29	50.6 ± 1.33
	2	3.5 ± 0.52	0.842	18.5 ± 2.17	52.9 ± 2.01
	10	4.1 ± 0.71	0.603	20.2 ± 2.61	54.5 ± 2.17
	25	3.6 ± 0.21	0.866	17.6 ± 0.90	56.9 ± 2.61
	trend p	*0.411*			
EMS	100	12.9 ± 0.97	<0.001	49.7 ± 2.26	67.5 ± 2.48
		Striatum			
MPH	0	4.0 ± 0.40		20.9 ± 1.13	60.3 ± 1.29
	2	3.9 ± 0.26	0.632	20.7 ± 1.14	57.3 ±0.98
	10	3.9 ± 0.36	0.592	21.1 ± 1.55	56.3 ± 2.37
	25	3.7 ± 0.30	0.724	19.7 ± 0.84	58.1 ± 1.18
	trend p	*0.449*			
EMS	100	10.4 ± 0.65	<0.001	43.8 ± 2.25	68.8 ± 1.44

[a]Data presented as mean ± SE, based on 100 cells per animal, 5 animals per treatment group; ; MPH, methylphenidate; EMS, ethylmethanesulfonate (positive control); vehicle, deionized water.

[b] One-tailed pairwise comparison using Student's t-test, for OTM data.

[c] One-tailed linear regression testing for a dose-related increase in DNA damage; significant at $p<0.05$.