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Methylphenidate clinically oral doses improved brain and heart glutathione redox status and evoked renal and cardiac tissue injury in rats

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ABSTRACT

Methylphenidate (MPH) is a first-line stimulant drug to treat attention deficit hyperactivity disorder (ADHD). Overdiagnosis of ADHD and MPH abuse lead to serious concerns about the possible long-term adverse consequences of MPH in healthy children and adolescents. We aimed to evaluate MPH effects in adolescent male Wistar rats (postnatal day 40) using an oral dose scheme (2 daily MPH doses 5 mg/kg in a 5% sucrose solution, 5 h apart, for 7 days) that mimics the therapeutic doses given to human adolescents. Twenty-four hours after the last MPH administration, rats were sacrificed and brain areas [cerebellum, prefrontal cortex (PFC), hippocampus, and striatum], peripheral organs (liver, heart, and kidneys), and blood were collected for biochemical and histological analysis.

MPH treatment did not alter rats' body temperature or weight, neither food or water intake throughout the experiment. The ratio of reduced glutathione/oxidized glutathione (GSH/GSSG) significantly increased in the PFC and hippocampus of MPH-treated rats, meanwhile protein carbonylation remained unchanged in the brain. In the heart, the GSH/GSSG ratio and GSH levels were significantly increased, with decreased GSSG, while histology revealed significant damage, namely interstitial edema, vascular congestion, and presence of a fibrin-like material in the interstitial space. In the kidneys, MPH treatment resulted in extensive necrotic areas with cellular disorganization and cell infiltration, and immunohistochemistry analysis revealed a marked activation of nuclear factor-κB.

This study showed that clinically relevant oral MPH doses improve the GSH redox status in the brain and heart, but evoke heart and kidney tissue damage to adolescent rats.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is one of the most common neurobehavioral disorder in school-aged children and adolescents and it often persists into adulthood. In the US, approximately 10% of children aged between 3–17 (more than 5 million) have been diagnosed with ADHD in 2012 [1]. Also, the American Psychiatric Association states that 5% of children have ADHD [2]. Increased rates of ADHD diagnosis and treatment throughout the past few decades have fueled concerns about whether the true prevalence of the disorder has increased over time, or if overdiagnosis is occurring. In fact, the amount of children estimated to have ADHD has changed over time and has been raising exponentially in the US [3] and other countries. Countries like the United Kingdom, Germany, France, Italy, and Brazil are also having a huge expansion in diagnosis [4]. Overdiagnosis is possibly resulting in the unnecessary treatment of otherwise healthy children.

Abbreviations: ADHD, attention deficit hyperactivity disorder; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine 5'-triphosphate; CK-MB, creatine-kinase MB; DA, dopamine; GSH, reduced glutathione; GSHt, total glutathione; GSSG, oxidized glutathione; $HClO_4$, perchloric acid; i.p, intraperitoneal; min, minutes; MPH, methylphenidate; NaCl, sodium chloride; NF-κB, nuclear factor-κB; NE, norepinephrine; OD, optical density; PBS, phosphate buffered saline solution; PFC, prefrontal cortex; PND, postnatal days; SD, standard deviation; SDS, sodium dodecyl sulphate; Total-CK, total creatine kinase

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There are both stimulant and non-stimulant options for pharmacological treatment of ADHD [5]. Stimulants are considered first-line agents and include methylphenidate (MPH), which is a first-choice drug for treating ADHD [6,7]. MPH interacts directly with the dopaminergic and noradrenergic pathways by blocking the reuptake of the neurotransmitters and, consequently, increasing dopamine (DA) and norepinephrine (NE) levels in the synaptic and extracellular space [8,9].

Despite the well described benefits of MPH treatment in ADHD, concerns have been raised regarding the possible consequences of chronic MPH exposure during childhood and adolescence. In 2009, the Committee for Medicinal Products for Human Use of the European Medicines Agency concluded that there was a lack of information on the long-term effects of MPH, and that further research would be important to focus on the neurological and cardiovascular effects of patients that took or were taking MPH for the treatment of ADHD [10]. Studies in humans, although scarce, have given critical data regarding the neurological and cardiovascular effects associated to early-age MPH use. So far, attenuation of specific structural brain abnormalities related to ADHD were verified in MPH-treated patients [11,12]. Additionally, children and adolescents treated with MPH had minor but significant increases in heart rate or blood pressure [13], while arrhythmias and/or sudden death have been rarely documented. MPH usually affects systolic blood pressure and some studies found that the increase on heart rate may be transient [14–16].

Although behavioral and neurochemical changes promoted by MPH have been the focus of many studies, less attention has been given to peripheral organs effects, namely the heart and kidneys, which are determinants in cardiovascular regulation. Additionally, the future consequences in medicated patients without being, actually, affected with ADHD are overlooked [17]. If concerns regarding overdiagnosis are correct, the impact of stimulant medication on the healthy subject in crucial development stages might lead to serious consequences in adulthood. Thus, our main aim was to assess the adverse effects of MPH in an adolescent healthy Wistar rat model [postnatal day (PND) 40], after a one-week exposure to a dose scheme that mimics the therapeutic doses taken orally by adolescents. The effects were evaluated in four different brain areas [cerebellum, prefrontal cortex (PFC), hippocampus, and striatum] and in three peripheral organs (liver, heart, and kidneys) 24 h after the last administration. Several determinations were done, including adenosine 5'-triphosphate (ATP), redox status, quinoproteins formation, and protein carbonylation. Additionally, histological analyses on the three peripheral organs were performed to assess the effects of MPH.

2. Materials and methods

2.1. Materials

Threo-methylphenidate hydrochloride was purchased from Tocris Bioscience (Bristol, UK). Ethylenediaminetetraacetic acid (EDTA), perchloric acid ($HClO_4$), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), disodium phosphate (Na_2HPO_4), copper (II) sulphate ($CuSO_4$), potassium bicarbonate ($KHCO_3$), potassium dihydrogen phosphate (KH_2PO_4), magnesium sulphate ($MgSO_4$), and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). Phosphate buffered saline solution (PBS) was purchased from Biochrom (Berlin, Germany), sodium chloride (NaCl), and sodium dodecyl sulphate (SDS) from VWR (Leuven, Belgium), potassium sodium tartrate from Fluka (Buchs SG, Switzerland), methanol, and xylene from Fisher Scientific (Loughborough, UK). Harris hematoxylin was purchased from Harris Surgipath (Richmond, IL, USA), eosin 1% aqueous from Biostain (Traralgon, Australia), and Histofluid from Marienfeld (Lauda-Königshofen, Germany). EMLA® Lidocaine 25 mg/g + Prilocaine 25 mg/g was obtained from AstraZeneca (London, UK). Isoflurane (Isoflo®) was obtained from Abbott Animal Health (North Chicago, IL, USA). ABX Pentra reagents were purchased from HORIBA (Kyoto, Japan). DC™ Protein Assay kit and the Clarity™ Western ECL Substrate were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Dinitropenhyl-KLH rabbit IgG antibody was purchased from Invitrogen/Life Technologies (Grand Island, NY, USA). Horseradish peroxidase (HRP) conjugated anti-rabbit antibody, the slot blot apparatus, and 0.45 µm Amersham Protran nitrocellulose blotting membrane were purchased from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA). Nuclear factor-κB (NF-κB) p50 rabbit polyclonal IgG and goat anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). All the other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

Fourteen adolescent male Wistar rats at PND 40 and weighing an average 120 g were born in the animal facilities of the Institute for Biomedical Sciences Abel Salazar, University of Porto (ICBAS-UP). Animals were kept in a controlled environment (23 °C, 55% humidity, 12 h light/dark cycles) with food and water available *ad libitum*. All procedures were performed in order to minimize animal suffering and stress. Housing and experimental treatment were based in the guidelines defined by the European Council Directive (2010/63/EU) transposed into the Portuguese law (Decreto-Lei n. º 113/2013). Additionally, the experiments were approved by the Ethical Committee of the Faculty of Pharmacy, University of Porto (process number 17/03/2014) and the Portuguese National Authority for Animal Health (General Directory of Veterinary Medicine) (process number 0421/000/000/2015).

2.3. Experimental protocol

One-week prior to MPH administration, the dorsocervical region of each animal was trichotomised and anesthetized locally with a topic anesthetic (EMLA® Lidocaine 25 mg/g + Prilocaine 25 mg/g), applied approximately 60 min [14] before the transponder insertion. Under a brief anesthesia with isoflurane, a temperature transponder (BioMedic Data Systems Inc., Seaford, DE) was subcutaneously inserted to allow precise core body temperature measurements throughout the experimental period, as we previously reported [18]. In the following days, animals were maintained in groups to socialize. Every day, animals were given a positive reinforcement to get them acclimatize to: a) researchers' manipulation; b) the sound of the temperature measurement device, and c) the oral administration of sweet solutions [5% sucrose (w/v)] with a syringe, to ensure an easy, complete and rapid drug intake during the treatment period. Twenty-four hours before the experiment and for the next seven days, the animals were individually housed with *ad libitum* access to food and water.

At PND 40, the fourteen male Wistar rats were randomly assigned to the two experimental groups: control (n = 7) and MPH-treated (n = 7). The MPH-treated group received two oral doses (at 9 a.m. and 2 p.m.) of 5 mg/kg MPH, previously prepared in a 5% sucrose solution. Controls received an equal volume of 5% sucrose solution, in the same dosage scheme. The oral administration of low doses of MPH, twice a day, was selected to simulate the clinical use of this drug, based on pharmacokinetic modeling to achieve peak plasma levels near the clinical range [19,20]. We wanted to focus our analysis in the adolescent period of animals, and therefore we performed an exposure of seven days to PND 40 animals. We did not perform longer periods of exposure or analyzed older animals to avoid the young adult or the adult stage of animals' life.

Before each morning dose, animals were weighted to adjust the individual dose and to assess the animal's general welfare. Body temperature was monitored and registered before and 30 min after oral dose, and then every 15 min until reaching 2.5 h post administration. Food and water intake were also measured daily, throughout the entire experimental protocol. Twenty-four hours after the last administration,