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Methotrexate causes persistent deficits in memory and executive function in a juvenile animal model

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ABSTRACT

Methotrexate is a dihydrofolate reductase inhibitor widely employed in curative treatment for children with acute lymphoblastic leukemia (ALL). However, methotrexate administration is also associated with persistent cognitive deficits among long-term childhood cancer survivors. Animal models of methotrexate-induced cognitive deficits have primarily utilized adult animals. The purpose of present study is to investigate the neurotoxicity of methotrexate in juvenile rats and its relevant mechanisms. The doses and schedule of systemic and intrathecal methotrexate, given from post-natal age 3–7 weeks, were chosen to model the effects of repeated methotrexate dosing on the developing brains of young children with ALL. This methotrexate regimen had no visible acute toxicity and no effect on growth. At 15 weeks of age (8 weeks after the last methotrexate dose) both spatial pattern memory and visual recognition memory were impaired. In addition, methotrexate-treated animals demonstrated impaired performance in the set-shifting assay, indicating decreased cognitive flexibility. Histopathological analysis demonstrated decreased cell proliferation in methotrexate-treated animals compared to controls, as well as changes in length and thickness of the corpus callosum. Moreover, methotrexate suppressed microglia activation and RANTES production. In conclusion, our study demonstrated that a clinically relevant regimen of systemic and intrathecal methotrexate induces persistent deficits in spatial pattern memory, visual recognition memory and executive function, lasting at least 8 weeks after the last injection. The mechanisms behind methotrexate-induced deficits are likely multifactorial and may relate to suppression of neurogenesis, alterations in neuroinflammation and microglial activation, and structural changes in the corpus callosum.

1. Introduction

Methotrexate is a dihydrofolate reductase inhibitor widely employed for the treatment of children with acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma, and osteosarcoma. Curative regimens for patients with these conditions typically include both systemic (oral, intramuscular, and/or intravenous) administration, as well as repeated intrathecal doses to bypass the blood brain barrier and prevent central nervous system relapse. Because the most common age for diagnosis with ALL is 2–4 years, and treatment regimens have a two to three-year duration, children with ALL are repeatedly exposed to methotrexate during a period of ongoing brain development. As a result, as many as 50–70% of pediatric ALL survivors experience long-term irreversible deficits in attention, working memory and executive function (Hearps et al., 2017; Jacola et al., 2016b; Pierson et al., 2016; van der Plas et al., 2015). Unfortunately, these behavioral abnormalities generally persist into adulthood.

The pathophysiology of methotrexate-induced neurotoxicity is multifactorial and incompletely defined (Cole and Kamen, 2006; Vezmar et al., 2003). Animal modeling has shown promise in elucidating the mechanisms underlying cognitive dysfunction following both systemic and intrathecal administration of methotrexate (Li et al., 2010; Seigers et al., 2009; Thomsen et al., 2018).

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Data from these models suggest methotrexate can induce deficits through induction of oxidative stress (Caron et al., 2009), modulation of the immune system (Cutolo et al., 2001; Phillips et al., 2003; Zhang et al., 2009), inhibition of neurogenesis (Seigers et al., 2009), altered neurotransmission through the NMDA receptor (Cole et al., 2013; Vijayanathan et al., 2011) and/or induction of structural brain alterations (Seigers et al., 2009). Other studies point toward the alpha-7 nicotinic acetylcholine receptor, since positive modulators of this receptor, such as cotinine, will improve spatial memory and decrease depressive-like behavior in rats treated with methotrexate, cyclophosphamide and 5-flurouracil (Iarkov et al., 2016).

However, most animal models do not specifically address the effects of repeated systemic and intrathecal methotrexate administration in juvenile animals. Experiments in adult animals may not be relevant to understanding the impact of chemotherapy on the developing brain. The experiments described here were designed to address this gap, and further explore the mechanisms of methotrexate-induced cognitive deficits in juvenile subjects. The methotrexate regimen we employed was designed to model treatment for young children with ALL. Treatment included repeated administration of systemic and intrathecal methotrexate, at clinically relevant doses, over a five-week period extending from three to seven weeks of age. Similarly, the behavioral battery chosen to assess cognitive function was designed to probe those domains that are most frequently described as impaired among cancer survivors. The object placement test of pattern recognition and the novel object recognition test of recognition memory assess components of memory most frequently, albeit not exclusively, associated with hippocampal function (Rubin et al., 2014). A set shifting assay was designed to probe executive function and cognitive flexibility, domains associated with cortical function (Euston et al., 2012).

Finally histopathological examination at two time points was undertaken to describe biomarkers that correlate with methotrexate exposure, and may begin to explain the pathophysiologic mechanisms.

2. Materials and methods

2.1. Animals and reagents

Long Evans rats (evenly split between females and males) were purchased from Charles River Laboratories (Wilmington, MA) at 1 week of age and were habituated to the vivarium for one week before the experiments. Rats were housed in groups of two or three with a 12/12 h light/dark cycle and ad lib. access to food (LabDiet 5001) and water. All experiments were approved by the Animal Institute and Use Committee of the Albert Einstein College of Medicine (Bronx, NY) and were conducted following the “Guide for the Care and Use of Laboratory Animals”. The NC3R's ARRIVE guidelines were followed in the conduct and reporting of all experiments described here.

Methotrexate (USP grade), phosphate buffered saline (PBS) and other chemicals were purchased from Sigma (Saint Louis, MO) unless otherwise stated. Methanol and water (HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA). All injected solutions were sterilized by filtering through 0.22 μm syringe filters (Millipore, Billerica, MA).

2.2. Injection schedule and CSF collection

The detailed injection schedule is illustrated in Fig. 1. Briefly, at 3 weeks old, rats received two IP injections (0.5 mg/kg methotrexate or PBS) one week apart. This was followed by 4 intrathecal injections within a two week period (1 mg/kg methotrexate or artificial cerebrospinal fluid [aCSF] at 4–5 weeks old, and IP injections once every week at 6 and 7 weeks of age.

Intrathecal injections were carried out as previously described (Li et al., 2010) by transcutaneous cisterna magna puncture with a 25 gauge butterfly needle, with inhaled isofluorane anesthesia (2–5%). Correct insertion of the needle was verified by outflow of CSF, which was collected in isovolumetric amounts (i.e., volume of CSF removed was equivalent to volume of drug to be administered) prior to IT injection. All animals were monitored for signs of acute toxicity under direct visualization for 1 h after injection and subsequently on a daily basis for evidence of abnormal behaviors. Intrathecal injection of methotrexate or aCSF (aCSF, Na^+ 150 mM, K^+ 3 mM, Ca^{2+} 1.4 mM, Mg^{2+} 0.8 mM, P 1.0 mM, and Cl^- 155 mM, in double distilled water) was conducted after CSF collection from cisternae magna. CSF with visible contamination by blood (approximately 10% of samples) was discarded. CSF samples were centrifuged, cell pellets were discarded and the supernatants were stored at −80 °C until analysis.

2.3. Behavioral testing schedule

Behavioral assessments of cognitive function were conducted at two time points (Fig. 1) in order to assess acute effects (9 weeks of age; 1 week after the last methotrexate exposure and persistent effects (15 weeks of age; 8 weeks after the last methotrexate exposure). The battery included the following: open field (OF), object placement (OP) (a.k.a object location) and novel object recognition (NOR)(Ennaceur and Meliani, 1992), conducted as previously published (Li et al., 2010; Thomsen et al., 2018) and described briefly below. A modified set shifting assay, described below, was done at a single time point (16–19 weeks of age; 9–12 weeks following the last methotrexate exposure).

2.3.1. Open field

The open field test was used to evaluate locomotor activity and thigmotaxis, an indicator of anxiety-like behavior (Treit and Fundytus, 1988). The assay was carried out in an arena (69 × 69 × 69 cm) with visual cues for 6 min. Total track length, center track length, center time, and center entries, were recorded and analyzed by Viewer III software (Biobserve, Bonn, Germany).

2.3.2. Object placement (OP) and object recognition (NOR)

Both the OP and NOR test rely on the innate preference of rodents to preferentially explore novel environmental stimuli. Intact pattern recognition in the object placement test is indicated by a preference for an object that has been moved to a novel location. Intact recognition memory in the novel object recognition test is indicated by a preference for a novel object over the familiar one previously encountered. Briefly, during training, animals are exposed to a pair of identical objects. After a defined retention interval in their home cages (20–180 min depending on the task), rats were presented with one unmoved and one relocated object (OP) or one old and one novel object (NOR) in a testing trial. Total activity, assessed by track length, and total object exploration times were recorded manually in seconds, using stopwatches. Exploration was defined as any physical contact with the object (sniffing, whisking, or touching). Data from subjects with less than 4 s of total exploration time were excluded from analysis of preference scores (less than 2% of subjects). A preference score was determined by the ratio of time exploring the relocated or novel object to total exploration time during the testing trial, and recorded as a percentage. For each cohort of identically treated animals, intact memory was demonstrated by a group mean preference score significantly higher than 53%. Our previously published data