Peer Review File

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Reviewer A

Comment 1: This is a well written manuscript describing MRI changes to mouse brain architecture over time after receiving intrathecal and oral chemotherapy similar to childhood leukemia chemotherapy. The authors detail these changes over the effective lifespan of the mice as a model of potential changes in humans over the lifespan after chemotherapy for childhood leukemia. This is a pilot study but shows proof of principle that this technique could be valuable for studies modeling chemotherapy effects on the brain over time.

I have no major concerns. My only minor concern is that I could not find a COI statement for the authors.

Reply 1: Thank you for your comments and apologies for the oversight regarding the COI statements. We have added the COI forms to the revision.

Reviewer B

This manuscript describes what appears to be a pilot project with the objective of demonstrating the feasibility of repeated MRI assessment of brain development after female juvenile mice are treated with methotrexate and dexamethasone, modeling chemotherapy administered to children with acute lymphoblastic leukemia. Treated and control animals demonstrated significant differences in change over time in brain structures.

Questions and comments for the authors:

Comment 1: Only female animals were used in these experiments described here, precluding any analysis of sex differences in susceptibility to the impact of chemotherapy exposure. This is fine for this feasibility manuscript, and the limitation is addressed in the discussion. I would suggest making this limitation clear to the reader by including the word "female" in the title (e.g. "… in a Female Mouse Model …) and in the abstract (methods, results, and conclusion).

Reply 1: Thank you for your suggestion. We implemented the suggested changes in the title and abstract.

Comment 2: Please elaborate on how the intrathecal dose of methotrexate (10 mg/kg) was chosen, as this appears to be substantially higher than what is used clinically, and detracts from the clinical relevance of the results. It should be noted in the discussion that children are given intrathecal doses in the range of 8 to 15 mg total, which amounts to much less per kg of body weight. Even accounting for differences in CSF volume, the dose used in this work might be expected to cause CSF methotrexate concentrations in the mice an order of magnitude greater than what is achieved in children.

Reply 2: Thank you for the opportunity to clarify. Single dose administration in mice is generally higher than the single human doses, while the cumulative mouse doses are lower than the cumulative human doses. As discussed in the limitation section, mice have a short lifespan. We therefore cannot achieve the higher cumulative dose administered in humans over a period of approximately 2 years. We give mice a higher single dose to approximate the high clinical dose in a compressed timeline.

Comment 3: The second paragraph of introduction suggests that the majority of preclinical work has been in vitro or described the cell biology observed after chemotherapy exposure. However, as noted by Tyler and Krull (Neuroscience & Behavioral Reviews, 2021) there exists a body of work describing in vivo exposure of juvenile rodents to chemotherapy, including methotrexate and corticosteroids (Mullenix et al., Pediatric Research 1994;35(2):171-8). Others have demonstrated that methotrexate exposure in juvenile animals causes cognitive deficits and neuroanatomical changes that persist into adulthood (e.g. Berlin et al., Neuro-Oncology, Volume 22, Issue 8, August 2020 and Wen et al., Neuropharmacol 2022). This prior work should be acknowledged here and differences with this work addressed in the discussion.

Reply 3: Thank you for your suggestion and apologies for the oversight. The Berlin reference was added to the third paragraph of the introduction:

Changes in text (Page 6, lines 101-103): Spencer Noakes and colleagues[1] utilized longitudinal neuroimaging to demonstrate that exposure to vincristine in young mice was associated with significant alterations in structural brain growth in several different regions at the human age equivalent of early adulthood [1]. Using ex vivo MRI, the authors also demonstrated that intra-orbitally administered methotrexate and dexamethasone resulted in neuroanatomical changes relative to saline-treated mice [1]. Likewise, Berlin and colleagues used ex vivo diffusion tensor imaging to quantify white matter in rats that received MTX via intraperitoneal injection for four weeks. Compared to controls, rats exposed to MTX exhibited lower fractional anisotropy across major white matter bundles.[2]

We added the Mullenix and Wen papers to the fourth paragraph of the discussion where we reference existing behavioral data:

Changes in text (Page 12, Lines 246-250): Second, behavioral data were not collected and as such, the functional consequences of the observed patterns of growth were not evaluated. A behavioral study in rats demonstrated that combined therapies—encompassing cranial radiation, MTX, and prednisone—caused behavioral deficits. Moreover, prednisone appeared to enhance the adverse impact of MTX and radiation.[3] More recent work by Wen and colleagues[4] showed that longitudinal exposures to systemic and IT MTX resulted in persistent cognitive deficits in rats.

The Wen 2022 study was also added to discussion in the context of differential impact of methotrexate based on sex:

Changes in text (Page 12, lines 258-259): As shown in a growing number of clinical [5,6] and animal studies,[4] sex is an important consideration when evaluating neurocognitive development in the context of chemotherapy exposures.

Comment 4: Half of the treated mice and 3 of 10 control mice did not complete all scans. Please provide more detail in the results regarding the outcome of these mice who were dropped.

Reply 4: We appreciate the reviewer's comment and apologize for the lack of detail. Death only occurred during the injection procedures. We added the following to the first paragraph of the result section for additional detail:

Changes in text (Page 9, Line 186): Of the 20 mice included in the study, five mice in the IT-MTX+DEX group died prior to scanning as a result of the injection procedure.

Comment 5: I agree that it is unfortunate that behavioral assays of cognition were not conducted among the long-term survivors of this treatment regimen. This is addressed adequately in the discussion. I bring it up only to note that I'll look forward to seeing those results in the follow-up work!

Reply 5: Thank you for the encouragement!

Comment 6: Please provide more detailed methods regarding the methotrexate administration. What was the methotrexate source. What was it dissolved in? Was the pH adjusted prior to administration? What was the total volume of administration? Regarding the intrathecal injection, how was proper placement of the needle assured? Was CSF flow observed?

Reply 6: Thank you for the opportunity to clarify. Regarding MTX administration, we used pharmaceutical-grade, preservation-free MTX injection USP from Teva pharmaceuticals, with a total volume administration of 3-4 µL depending on body weight at the time of injection. Concentration of mixture was 10 mg/L. The following was added to the methods of the revised manuscript:

Changes in text (Page 7, Lines 133-136): Pharmaceutical-grade, preservative-free MTX injection USP from Teva Pharmaceuticals. MTX or saline was slowly injected into the spinal column using pre-filled syringes with 29-gauge needles, with a total volume administration of 5-10 µL depending on body weight.

Comment 7: Please provide additional detail regarding toxicity of the methotrexate administration. Were seizures or other neurotoxicity observed as has been described after intraventricular injection? What was the cause of death of five treated animals who did not make it to their first scanning time point, and three controls who died between T1 and T2?

Reply 7: Mice were monitored by veterinarians, and there were no reports of seizures or overt toxicity. As indicated in our reply to comment 4, cause of death was primarily the injection procedure or failure of the injection procedure. Mice were sacrificed if any sign of paralysis or morbidity was observed. However, we did not conduct necropsies on mice and are not able to ascertain the exact cause of death.

Minor suggestions:

Comment 8: In the discussion, consider adding a note that while methotrexate and corticosteroids are the agents primarily blamed for altering brain development, other chemotherapy drugs used for childhood leukemia may also contribute, such as asparaginase and anthracyclines.

Reply 8: Thank you for your suggestion. We added the following to paragraph 4 of the discussion to acknowledge that other agents are also associated with neurotoxicity.

> **Changes in text (Page 12, Lines 255-256):** Third, while experiments were designed to mimic the clinical experience, treatment exposures in mice were compressed into a timeframe spanning a few weeks. The short lifespan of mice necessitates time compression and affords opportunities for efficient longitudinal assessment; however, the experimental protocol does not fully reflect the extent, intensity, and phasing of ALL therapy. Relatedly, patients with ALL require a variety of agents beyond MTX and DEX, some of which have been shown to have neurotoxic properties (e.g., vincristine[7]).

Comment 9: (very minor) consider changing the labels for the time points from T0, T1, T2 to anything different, as "T1 and T2" might get confused by some readers with MRI sequences on a quick glance. Maybe 3m, 6m, and 12m?

Reply 9: We changed the references from T0/T1/T2 to T1/T2/T3 throughout the manuscript. Note that 'Months' is annotated at the bottom of Figure 2 that shows the volumetric change data.

Comment 10: Consider omitting figure 2 – the data can be described concisely in the text and the bar graph doesn't add significantly.

Reply 10: We removed the referenced plot from the revised manuscript.