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Short communication

Ferumoxytol administration does not alter infarct volume or the inflammatory response to stroke in mice

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HIGHLIGHTS

- Ferumoxytol is an ultrasmall superparamagnetic iron oxide (USPIO) nanoparticle.
- Ferumoxytol is beginning to be used off-label as an imaging agent in stroke.
- USPIOs similar to ferumoxytol can activate peripheral macrophages.
- Ferumoxytol did not alter infarct volume or the inflammatory response to stroke.
- Ferumoxytol as a contrast agent does not adversely affect stroke outcome in mice.

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ABSTRACT

Ferumoxytol is an ultrasmall superparamagnetic iron oxide (USPIO) nanoparticle that is FDA-approved as an intravenous iron replacement therapy for the treatment of iron deficiency anemia in patients with chronic kidney disease. Ferumoxytol has also been used as a contrast agent for cerebral blood volume mapping by magnetic resonance imaging (MRI), which suggests it could be used for imaging hemodynamic abnormalities after stroke. However, circulating macrophages can internalize USPIOs, and recent data indicate that the accumulation of iron in macrophages can lead them to adopt the M1 pro-inflammatory phenotype. Therefore, the uptake of intravenously administered iron particles by circulating macrophages that home to the stroke core could potentially alter the inflammatory response to stroke. To test this possibility *in vivo* we administered a dose of ferumoxytol previously used to obtain cerebral blood volume maps in healthy humans by steady-state susceptibility contrast (SSC) MRI to BALB/cJ mice 48 h after stroke and examined cytokine levels, microglial/macrophage activation, and lesion volume in the brain 5 days later. Treatment with ferumoxytol did not lead to any differences in these parameters. These data indicate that the use of ferumoxytol as a contrast agent for brain imaging after stroke does not alter the inflammatory response to stroke in mice, and is therefore unlikely to do so in human subjects.

1. Introduction

Ferumoxytol is an ultrasmall superparamagnetic iron oxide (USPIO) nanoparticle that is FDA-approved as an intravenous iron replacement therapy for the treatment of iron deficiency anemia in patients with chronic kidney disease. Ferumoxytol has also been used for MRI cerebral blood volume mapping (CBV) and cellular imaging of inflammation in the injured CNS [4,22].

Currently, the primary method for cerebral blood volume mapping is to use dynamic susceptibility contrast (DSC) MRI following bolus administration of gadolinium-based contrast agents. However, this technique requires a fast image acquisition protocol, which limits spatial resolution [2,3]. Although ferumoxytol can also be used for DSC MRI, recently, ferumoxytol has been used to generate higher resolution CBV maps in humans by steady-state susceptibility contrast perfusion mapping (SS CBV) [3,21].

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Ferumoxytol can be used in this way because it has an intravascular half-life of ~12 h in humans [3], which contrasts with a typical intravascular half-life of 1.6 h for gadolinium-based contrast agents [22]. Therefore, ferumoxytol could be a useful contrast agent for perfusion mapping after stroke.

In addition, USPIOs such as ferumoxytol may be useful to image inflammation after stroke [24]. USPIOs can be ingested by activated peripheral monocytes and macrophages that home to the injured brain to participate in the clearance of dead cells and cellular debris after stroke [1,8,25]. Ingestion by macrophages occurs *via* both receptor-mediated endocytosis and phagocytosis [9,16], both of which are processes that activate intracellular signaling pathways. This raises the possibility that prior ferumoxytol uptake could alter the way macrophages respond to inflammation in the brain after stroke. Indeed, in support of this possibility, Sindrilaru et al. recently showed that uptake of iron by cells of the mononuclear phagocyte system can cause them to adopt a pro-inflammatory M1 phenotype [20]. In addition, iron oxide particles of various sizes and with diverse coatings have been reported to have varying effects on macrophages *in vitro*, including pro-inflammatory, anti-inflammatory, and no effects [10,13,14,19]. For ferumoxytol itself there is no available data on its effects on macrophage function.

Therefore, to test if the administration of ferumoxytol to stroke patients might alter their inflammatory responses and change stroke outcomes, we sought to test its effects on the immune response to stroke in mice. We injected wildtype BALB/cJ mice 2 days after experimental stroke with a ferumoxytol dose equivalent to that used for SS CBV in humans. We compared final (5 days post-injection) lesion size and the attendant inflammatory response to stroke between mice that received ferumoxytol and vehicle control injections.

2. Methods

Mice: We used 45 12-week-old male BALB/cJ mice for this study. All procedures met NIH guidelines with the approval of the Stanford University Institutional Animal Care and Use Committee.

Stroke surgery and ferumoxytol treatment: Distal middle cerebral artery occlusion (DMCAO) was induced as described [5]. Briefly, animals were anesthetized and the middle cerebral artery (MCA) exposed and cauterized. Mice were injected *via* the tail vein with ferumoxytol (Feraheme, AMAG Pharmaceuticals, Inc., Cambridge, MA) at a concentration of 7 mg/kg, or saline vehicle, 48 h after DMCAO. Mice were euthanized 7 days post DMCAO for elucidation of infarct volume and immunostaining ($n = 9$ per group) and multiplex immunoassay ($n = 11$–12 per group).

Immunohistochemistry and infarct volume assessment: Immunostaining and infarct assessment were performed using standard techniques on PFA-fixed 40 μm coronal brain sections. Microglial/macrophage immunostaining was performed using an anti-CD68 antibody (Serotec #MCA1957). To calculate stroke volume as a percentage of the contralateral side, combined cresyl violet and NeuN (Millipore #MAB377B) histology was used to trace the non-infarcted ipsilateral hemisphere and contralateral hemisphere in six sections per mouse spaced 640 μm apart using ImageJ software.

Multiplex immunoassay: After saline perfusion, the lesion (stroke core) was dissected, immersed in lysis buffer (Sigma Cel-Lytic MT Cell Lysis Reagent containing Sigma protease inhibitor cocktail and Sigma phosphatase inhibitor cocktail 2) at a ratio of 1:20, and sonicated. Cytokines and chemokines were detected and quantified in duplicate by multiplex immunoassay performed by the Human Immune Monitoring Center at Stanford University (himc.stanford.edu).

Perls' iron staining: A Perls' iron stain, a 1:1 mixture of 10% $K_4Fe(CN)_6$ and 20% HCl solution, was performed in conjunction with anti-CD68 immunostaining on six coronal brain sections per mouse spaced 640 μm apart. To calculate the percentage of sections with Perls' iron staining, photomicrograph images were taken of four regions of interest from each section in an unbiased, blinded, fashion. The regions of interest were the cortex adjacent to the lesion, the stroke border, the stroke core, and the ipsilateral side of the corpus callosum. Each image was assessed for the presence or absence of iron staining, and for each region the number of images with observable iron staining was divided by the total number of images.

Statistical analysis: Allocation of mice into groups was performed randomly. Data are expressed as means ± standard error of the mean (SEM) unless otherwise indicated in the figure legend. Statistical analyses were performed with Prism 5 software (GraphPad-San Diego, CA). Means between two groups were compared with a two-tailed, unpaired Student's *t*-test for cytokines, and Mann–Whitney test for iron staining.

3. Results

3.1. Administration of ferumoxytol does not alter cytokine expression in the stroke lesion

The induction of pro-inflammatory cytokines and chemokines in the stroke lesion contributes to secondary injury after stroke [6]. To investigate whether the administration of ferumoxytol after DMCAO leads to altered expression of cytokines and chemokines in the stroke lesion, ferumoxytol (7 mg/kg) or saline was intravenously administered *via* the tail vein to BALB/cJ mice 48 h after DMCAO. Mice were euthanized 5 days later and the stroke lesion was dissected and used for a multiplex immunoassay. Ferumoxytol administration did not lead to any significant changes in the expression of MCP1, MCP3, MIP1A, Eotaxin, GMCSF, GCSF, IL1A, IL2, IL5, IL6, IL10, IFNγ, TNFα, IL12p40, IL12p70, IP10, RANTES, TGFβ, or VEGF (Fig. 1). Levels of IL1β, IL3, IL4, IL17, IL23p19, KC, and IL13 were undetectable in the stroke lesion in both the ferumoxytol- and vehicle-treated mice 7 days after stroke (data not shown).

3.2. Administration of ferumoxytol does not alter CD68 expression in the stroke lesion and penumbra

Next we performed immunostaining for CD68 to determine if administration of ferumoxytol alters the monocytic response to stroke, which is composed of both activated brain resident microglia and macrophages that have migrated into the stroke core from the bloodstream [17,18]. CD68 is a lysosome membrane glycoprotein, and antibodies against CD68 are widely used to assess microglial/macrophage activation after stroke [5]. Administration of ferumoxytol did not lead to any alteration in the % area covered by CD68 immunoreactivity (Fig. 2A and B), suggesting that ferumoxytol administration does not change microglial or macrophage activation in the area of injury. Ferumoxytol administration also did not lead to any overt differences in microglial/macrophage morphology, with cells from mice in both treatment groups retaining a similar amoeboid shape and distribution in the stroke lesion and penumbra (Fig. 2C).

3.3. Effect of ferumoxytol administration on brain iron content

To determine if the dose of ferumoxytol required for SS CBV leads to a prolonged increase in iron content in the stroked brain, a Perls' iron stain was performed 7 days after stroke, 5 days after ferumoxytol administration. Overall, Perls' iron staining was similar between saline- and ferumoxytol-injected mice. Brain sections