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Systemic iron chelation results in limited functional and histological recovery after traumatic spinal cord injury in rats

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ABSTRACT

Excess iron accumulation within the spinal cord is thought to exacerbate tissue damage and limit functional recovery after traumatic spinal cord injury (SCI). An optimal treatment to reverse or prevent damage would be to deliver an iron chelator systemically. Thus, we tested oral delivery of deferasirox (Exjade) in multiple studies using a rat model of mid-thoracic spinal contusion. Female Sprague-Dawley rats received a moderate contusion at vertebral level T8 and were given daily deferasirox for the first 7 or 14 days post-injury. The first two studies showed modest improvements in hindlimb function with limited improvement in tissue sparing. Two subsequent experiments to assess chronic functional changes and test longer-duration treatments failed to produce significant improvements. Testing a 2-fold higher deferasirox dose resulted in toxic side effects. To verify iron chelation treatment was effective, hepatic iron levels were measured which revealed that deferasirox robustly and significantly reduced systemic iron levels. Overall, this study suggests that oral iron chelation with deferasirox may lead to small but significant improvements in locomotor recovery or tissue sparing. However, given the lack of robust beneficial effects combined with potentially detrimental side effects such as exacerbated systemic anemia, oral administration of iron chelators may not be ideal for minimizing intraspinal iron-mediated pathology after SCI.

Introduction

Every year in the United States there are ~11,000 new spinal cord injuries (SCI), which result in more than 250,000 people currently living with the chronic pathology associated with SCI. The initial insult leads to significant functional deficits, which have a devastating impact on the individual's health and quality of life. Even though substantial improvements in care have increased survival rates, people with SCI now live with significant deficits for many decades. The average lifetime cost for treating SCI can vary between $1,400,000–$4,300,000 depending on the severity and spinal level of the injury (NSCISC, 2011). Thus, given the financial obligations and the permanently reduced quality of life after SCI, finding effective therapeutic agents is critically needed.

Following SCI, damage occurs not only to nervous tissue but also to the surrounding vasculature, which results in intraspinal hemorrhage (Noble and Wrathall, 1985, 1989a, 1989b) accompanied by deficits in tissue perfusion (Mautes et al., 2000). The presence of intraparenchymal blood plays a significant role in secondary injury processes further exacerbating tissue loss (Mautes et al., 2000). As hemoglobin from the extravasated blood is broken down, iron is released into the spinal cord leading to the production of damaging free radicals and propagation of secondary injury cascades (Liu et al., 2003, 2004; Zhang et al., 1996). For instance, iron is directly involved in the catalytic production of potent hydroxyl radicals through the Fenton reaction. Hydroxyl radicals can kill neurons (Bao and Liu, 2004) and also lead to progressive expansion of membrane damage by lipid peroxidation (Liu et al., 2004). Increased lipid peroxidation, evident by 3 h post-injury and persistent at the epicenter for at least 2 weeks (Carrico et al., 2009), demonstrates how a rapid rise in intraspinal iron can lead to long-lasting degradative cascades.

The role of iron in the progression of the secondary injury following SCI is further supported through work using iron chelators that can reduce tissue damage and promote functional improvement (Klapka et al., 2005; Paterniti et al., 2010; Rathore et al., 2008; Schultke et al., 2003, 2010a, 2010b). Although iron chelation therapy improved recovery, differences in study designs and drugs necessitate further investigation before studies should be initiated in humans. Previous work using the FDA approved iron chelator deferoxamine showed that targeting iron can attenuate post-SCI pathology (Paterniti et al., 2010). A caveat, however, is that the drug was given 30 min prior to injury. The iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) given intraperitoneally induced a delayed improvement in locomotor function in mice after SCI, suggesting that targeting chronically elevated

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intraspinal iron may be therapeutic (Rathore et al., 2008). In a different approach, a single injection of the iron chelator 2,2′-bipyridine (BPY) or BPY-5,5′-dicarboxylic acid directly into the spinal cord after spinal transection reduced the formation of the glial scar (Klapka et al., 2005; Weidner et al., 1999). However, these same studies show conflicting results as to whether treatment leads to axon regeneration and functional improvement (Klapka et al., 2005; Weidner et al., 1999). Unfortunately, SIH and BPY-DCA are not FDA approved and would require additional hurdles before being translated to human usage. Treatment with the flavonoid quercetin starting 1 h after compression SCI in rats had a variable effect on improving locomotor function in that a moderate dose improved stepping ability but higher doses had no effect (Schultke et al., 2003, 2010a, 2010b). Thus, given the diverse nature of the pre-clinical research on post-SCI iron chelation, determining the best candidate drug and treatment paradigm is essential before trials can be considered for humans. Further, our previous work showed that iron is essential for intraspinal oligodendrocyte genesis following spinal inflammation (Schonberg and McTigue, 2009; Schonberg et al., 2007). Therefore, it is equally important to ensure that iron chelation after SCI does not adversely affect anatomical or functional recovery by limiting the endogenous replacement of oligodendrocytes.

In the present studies, we tested the efficacy of the FDA approved iron chelator deferasirox after a clinically relevant spinal contusion injury. This drug is appealing because it can be given orally and is known to cross the blood–brain barrier. Therefore, we tested different administration protocols to determine if post-SCI treatment with deferasirox improved locomotor recovery and reduced tissue loss. Furthermore, given the necessity of iron for oligodendrocyte genesis, we also determined if oligodendrocyte numbers were altered by post-SCI iron chelation.

Methods

Injury and drug treatment

Spinal cord contusions were performed using standardized protocols as previously described (Almad et al., 2011; Almad and McTigue, 2010). All procedures conformed to NIH and The Ohio State University animal care guidelines. Briefly, adult female Sprague-Dawley rats (250 g; Harlan, Houston, TX) were anesthetized with ketamine (80 mg/kg, i.p) and xylazine (10 mg/kg, i.p.), and a dorsal laminectomy was performed at the T8 vertebral level. Rats then received a moderate spinal contusion injury using the Infinite Horizons device (Precision Systems and Instrumentation) with a preset force of 200 kD (actual forces ranged from 200 to 219 kD with an average force of 205 kD). The muscles overlying the spinal cord were sutured and the skin was closed with surgical clips. Animals were given 5 ml of saline and placed into warm recovery cages immediately following the injury. Postsurgical care included 5 days of antibiotic treatment (gentamicin, 5 mg/kg) and saline to maintain hydration, and twice-a-day manual bladder expression until spontaneous voiding returned. Animals were randomly assigned to drug, vehicle, or control groups prior to beginning the treatments. Deferasirox (Exjade, Novartis, Basel, Switzerland) was obtained as 500 mg tablets and manually crushed into a fine powder prior to being dissolved in water at a concentration of 160 or 320 mg/kg in 0.5 ml water. Drug was delivered by oral gavage. Vehicle control animals received 0.5 ml of water by oral gavage. To control for any effect of gavage and extra handling, injury control animals receiving no gavage treatment were included in most studies. Administration of deferasirox or vehicle was begun at 1.5 h post-injury and continued once daily for the first week post-injury. In humans, deferasirox has a circulating half-life greater than 10 h for doses over 80 mg/kg (Galanello et al., 2003). Though an accurate half-life for orally administered deferasirox has not been well established in rats, oral administration of 100 mg/kg results in a plateau of peak serum levels for up to 8 h (Bruin et al., 2008), indicating that the half-life in rodents for doses over 100 mg/kg is greater than 8 h. Thus, rats were given one treatment per day. Over the course of the studies, some deaths were linked to treatment with deferasirox. In total, 6 of 52 rats died after treatments at 160 mg/kg/day, and 5 of 8 rats died after treatments at 320 mg/kg/day. No deaths occurred in control animals or those treated with vehicle.

Behavioral analysis

Animals were assessed for baseline locomotor function prior to surgery. After SCI, two reviewers blinded to study groups simultaneously evaluated each animal for 4 min using the BBB locomotor rating scale (Basso et al., 1995). Animals were assessed on 1, 3, 7 and 10 days post-injury (dpi) and then once a week thereafter. Exclusion criteria based on BBB scores were set prior to beginning the experiments to ensure only animals with similar injury severities were compared. Rats with BBB scores >3 at 1 dpi or >10 at 7 dpi were excluded for being too mild, and BBB scores <5 at 7 dpi were excluded as too severe. Across all of the studies, 2 of 14 controls, 3 of 32 vehicle, and 5 of 30 deferasirox animals were excluded.

Urinalysis

Iron content in the urine was assayed using the liquid ferrozine method (Iron Reagents Kit, Thermo Scientific). Urine was collected from awake animals and frozen at −80 °C until analysis. Background absorbance was obtained at 560 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, California). A final absorbance reading was then taken at 560 nm in the presence of ferrozine. The concentration of iron was determined by subtracting the initial background absorbance for each sample from the final absorbance reading and then determining sample concentrations by comparing them with the standard curve based on known concentrations of ferrous iron.

Histological analysis

At the appropriate time post-injury, animals were given a lethal dose of ketamine (120 mg/kg, i.p) and xylazine (15 mg/kg, i.p.) and then transcardially perfused with 0.1 M phosphate buffered saline (PBS) until the tissue was cleared of blood. Next, animals were perfused with 400 ml of 4% paraformaldehyde (PFA). The spinal cord and liver were removed and post-fixed in 4% PFA for 2 h followed by phosphate buffer overnight. The next day, the tissue was transferred to 30% sucrose for 3 days prior to freezing and blocking for tissue sectioning. Tissue sections were cut at 10 μm using a cryostat and slide mounted (Superfrost Plus Slides, Fisher Scientific); slides were stored at −20 °C until used. For tissue analysis, the following targets were visualized using immunohistochemistry: neurofilament (DSHB, RT97, 1:2000), neurons (Chemicon, NeuN, MAB377 1:50,000), macrophages (Serotec, OX42 MCA275, 1:2000), ferritin (Abcam, L-ferritin, ab69090 1:1000; H-ferritin ab65080 1:500), and oligodendrocytes (Abcam, CC1 ab16794, 1:800). Eriochrome Cyanine was used to visualize myelin for white matter sparing and the Perls Prussian Blue stain (Polysciences #24199-1) was used with DAB intensification to visualize iron.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 (San Diego, California). For behavioral and histological analysis a two-way repeated measures ANOVA was performed followed by post-hoc analysis to determine between group differences. For analysis of urine, liver, and spinal cord iron content, a one-way ANOVA was performed followed by post-hoc analysis to determine group differences.