Published in final edited form as:

Brain Res. 2008 December 3; 1243: 146–151. doi:10.1016/j.brainres.2008.09.047.

A Re-assessment of Minocycline as a Neuroprotective Agent in a Rat Spinal Cord Contusion Model

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Abstract

This study was initiated due to an NIH “Facilities of Research-Spinal Cord Injury” contract to support independent replication of published studies that could be considered for a clinical trial in time. Minocycline has been shown to have neuroprotective effects in models of central nervous system injury, including in a contusive spinal cord injury (SCI) model at the thoracic level. Beneficial effects of minocycline treatment included a significant improvement in locomotor behavior and reduced histopathological changes (Lee et al., 2003). To verify these important observations, we repeated this study in our laboratory. The NYU (MASCIS) Impactor was used to produce a moderate cord lesion at the vertebral level T9–T10 (height 12.5 mm, weight 10 g), (n=45), followed by administration of minocycline, 90mg/kg (group 1: minocycline IP, n=15; group 2: minocycline IV, n=15; group 3: vehicle IP, n=8; group 4: vehicle IV, n=7) immediately after surgery and followed by two more doses of 45 mg/kg/IP at 12 hrs and 24 hrs. Open field locomotion (BBB) and subscores were examined up to 6 weeks after SCI and cords were processed for quantitative histopathological analysis. Administration of minocycline after SCI did not lead to significant behavioral or histopathological improvement. Although positive effects with minocycline have been reported in several animal models of injury with different drug administration schemes, the use of minocycline following contusive SCI requires further investigation before clinical trials are implemented.

Keywords

spinal cord injury; neuroprotection; minocycline; behavioral outcome

1. Introduction

Minocycline, a chemically modified tetracycline, exerts both anti-apoptotic and anti-inflammatory effects (Stirling et al., 2005) in models of CNS injury. Minocycline can penetrate the blood-brain barrier (BBB) and has been shown to have protective effects in cerebral ischemia (Yrjanheikki et al., 1998; Fox et al., 2005; Nagel et al., 2008), traumatic brain injury (Sanchez Mejia et al., 2001) and spinal cord injury (SCI) (Hoang et al., 2008; Lee et al.,

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2003; Stirling et al., 2004, 2005; Teng et al., 2004; Yune et al., 2007). In these published studies, minocycline treatment has been reported to protect motor neurons, oligodendrocytes, and white matter structures. Thus, the promise of minocycline as a treatment in several neurological disorders has been discussed recently (Yong et al., 2004).

Minocycline has been shown in several studies to target various secondary injury mechanisms considered to participate in the devastating consequences of brain and SCI (Stirling et al., 2005). In the study by Sanchez and colleagues (2001), minocycline was reported to reduce tissue injury through a caspase-1 dependent mechanism. Minocycline may help prevent inflammatory induced apoptotic effects by inhibiting caspase-1 and caspase-3 gene expression (Chen, et al, 2000). Following SCI, minocycline treatment has also been shown to reduce proinflammatory cytokine levels and DNA laddering (Lee et al., 2003). In several injury models, minocycline treatment inhibited cytochrome c release from mitochondria that was associated with decreased apoptosis of cells (Teng et al., 2004; Zhu et al., 2002). In a study by Yrjanheikki and colleagues (1999), neuronal cell death induced by glutamate toxicity was attenuated by minocycline administration. Minocycline has also been reported to provide neuroprotection by inhibiting the p38 mitogen-activated protein kinase pathway in microglial cells, thereby inhibiting the interaction between excitotoxicity and inflammation (Piao et al., 2006; Tikka, et al., 2001; Yune et al., 2007). Thus, there is ample evidence that minocycline's beneficial effects may be multi-factorial and independent of its anti-microbial actions. Importantly, the ability of a single compound to target multiple injury pathways may be advantageous in terms of promoting long-term functional improvements after injury (Dirnagl et al., 1999).

Recently, it has been shown that the use of minocycline as a cytoprotective agent following SCI can improve locomotor recovery and histopathological outcome (Lee et al., 2003; Wells et al., 2003; Sitriling et al., 2004; Saganova et al., 2008; Teng et al., 2004). Since minocycline is a medication currently approved by the FDA, and has well-known pharmacological properties of low toxicity, low side effects and safety, it would seem promising for human use. Thus, it seemed worthwhile to replicate a study in an independent laboratory to corroborate the use of minocycline as a neuroprotective agent in a model of acute contusive SCI. To this end, attempts were made to replicate the experimental conditions described by Lee and colleagues (2003) utilizing the same injury model, injury severity and behavioral outcome measures.

2. Results

Locomotor scores

The open field locomotor scores and subscores of the corresponding groups are presented in Figures 1 and 2, respectively. As demonstrated, all traumatized rats exhibited a severe BBB score at 1 day after SCI. During subsequent scoring periods, BBB scores increased and plateaued around 1 week after SCI. Two-way repeated measures ANOVA for BBB scores were not significant for group ($F_{3, 41} = 0.734, p > 0.5$) or group x time ($F_{21, 269} = 0.757, p > 0.7$) but time ($F_{7, 269} = 550.37, p < 0.001$) was significant. The animals did improve over time but there was no significant difference at any point in time among the four groups. The highest average BBB score at the end of the study was 12.1 ± 0.18 for the minocycline IP-treated group and the lowest score was for the saline control group with a score of 11.8 ± 0.2 (Mean $\pm$ SEM). The subscore analysis of the BBB showed similar findings with no significance for group ($F_{3, 41} = 1.51, p > 0.2$) or group x time ($F_{21, 269} = 0.862, p > 0.6$) but time ($F_{7, 269} = 550.37, p < 0.001$) was significant.

Histology

Histopathological analysis was based on area values from horizontal central cord sections calculated by computer contour analysis (Fig. 3). Data from these 20 mm segmental cord sections are presented in Figures 4 and 5. One-way ANOVA for differences between spared tissue area were not significant for group (F_{3, 39}=1.04, *p*>0.39). The greatest amount of spared tissue was obtained in group 3 (Control IP; spared tissue, 35.35 ± 2.17 (mm²; Mean ± SEM); Fig. 4), although this value was not significantly different from the other groups. The total cavity area (Figure 5) was also not significantly different for group (F_{3, 39}=0.33, *p*>0.80). The group 4 (control IV) had the smallest area of cavitation (1.19 ± 0.36 (mm²; Mean ± SEM).

3. Discussion

Recently, minocycline has been shown to have neuroprotective effects in models of central nervous system injury (for review see Yong et al., 2004). For SCI, it was reported that a significant improvement in functional outcome was obtained with the use of minocycline using a moderate contusion injury similar to that produced in the present replication study (Lee et al., 2003). In both studies, the force-calibrated weight-drop device developed at New York University was utilized (Gruner, 1992). Also, a similar anesthetic protocol including chloral hydrate administration was delivered. In the current study, no significant differences at any point in time between the four groups were found in terms of locomotor function as assessed by the BBB test. It should be noted that in the Lee et al., 2003 paper, at 38 days after injury, BBB scores in the vehicle-treated rats were 15 ± 0.5. In our study, the highest average BBB score at the end of the study was 12.1 for the minocycline IP-treated group and the lowest score was for the saline control group with a score of 11.8. Thus, although a similar injury device and injury severity was used in the two studies, there was a difference in BBB scores for the vehicle treated groups. Also, pathological analysis based on area values from horizontal central cord sections showed no significant differences between the different experimental groups in spared tissue and cavity areas when analyzing the epicenter section in the contusion model. Thus, in contrast to published data using this SCI model, minocycline did not improve behavioral or histopathological outcome.

It is important to mention that there were some differences in the present study design with respect to the original study. For example, the chloral hydrate used to anesthetize the animals in the original study was reported to be 50 mg/kg IP (Lee et al., 2003). Because we found that this concentration of the drug was insufficient for anesthetic induction in our hands, 300 mg/kg IP of chloral hydrate was found to be optimal and used in the present study. Subsequent discussions with the Lee laboratory indicated that the dose of drug was incorrectly reported in the published manuscript (Oh, personal communication). In fact, 500 mg/kg IP was used in the original study but this dose resulted in high mortality in our hands. Whether these differences in the anesthesia dosage explain the different outcomes between the two experiments is a possible consideration. An additional difference between this study and the Lee paper was the animal vendor. The Lee paper obtained their animals from a vendor in Korea while our rats were obtained from Charles River Laboratories. It is difficult to determine what potential effects these different animal sources may have on SCI outcome studies. It is clear however, that we used a similar impact model, similar surgical procedures and injury severity described by Lee and colleagues but obtained different behavioral outcomes even within the nontreated group.

Recently the importance of the method of minocycline treatment has been emphasized. In a study by Fagan and colleagues (2004), intravenous administration of minocycline was reported to be a reliable method when neuroprotection was required. These investigators found that the intraperitoneal route led to the drug being incompletely and erratically absorbed. In the present

study, we compared IP with IV injections of minocycline and neither treatment route improved outcome after SCI.

In addition to the beneficial effects reported for minocycline treatment, studies have shown recently that minocycline may worsen or not improve outcome under specific experimental conditions (Tsuji et al., 2004; Yang et al 2003; Diguët et al 2004). In a model of MPTP-induced dopaminergic damage to neurons, minocycline treatment was reported to worsen outcome in mice and cynomolgus monkeys (Yang et al 2003; Diguët et al 2004). Also, in an animal model of Huntington's disease, minocycline aggravated motor scores and produced neuronal loss in the striatum (Diguët et al 2004). Finally, in a recent study where minocycline treatment following a balloon-compression SCI was investigated, limited sparing of tissue and no significant effects on BBB locomotor recovery were reported (Saganova et al., 2008). Thus, it is clear that this treatment may have a wide range of consequences on functional outcome effects depending on the species and model of CNS injury.

In summary, no behavioral or histopathological improvements were observed in the present study within any of the experimental groups administered minocycline in the contusion SCI model. While these results cannot exclude possible beneficial effects of minocycline in other models of CNS injury or with different treatment protocols, the present study does question the benefit of this treatment in traumatic SCI. Thus, the replication of positive findings in independent laboratories should be emphasized before the implementation of clinical trials in SCI are undertaken.

4. Experimental Procedure

Spinal Cord Injury

Adult male Sprague-Dawley rats (220–280g; n=45) were housed according to National Institutes of Health and United States Department of Agriculture guidelines. The Institutional Animal Care and Use Committee of the University of Miami approved all animal procedures. Animals were divided into 4 groups. Anesthetic induction with inhaled halothane was followed by injection of chloral hydrate (300 mg/kg IP). Verification of an adequate level of anesthesia was first determined by assessing the corneal reflex and withdrawal reflex to painful stimuli for the hindlimbs. All animals underwent a T9–T10 spinal laminectomy. During surgery, the rats were placed on a warming pad to maintain the body temperature at $37 \pm 0.5^{\circ}\text{C}$. Briefly, the rat was placed ventrally on top of a small bed of sterile gauze to elevate the surgical site, presenting an adequate exposure of the back anatomy. A 2 cm longitudinal skin incision was centered over the T9 spinous process along the midline. Para-spinal muscles and ligaments were laterally dissected and retracted, followed by removal of bony elements of the posterior spine (i.e. spinous process and laminae) using a micro-rongeur. Without disrupting the dura mater, the ninth thoracic (T9) spinal segment was exposed by removing the dorsal part of the vertebra.

The exposed cord was next contused by a 10 gm weight dropped from a height of 12.5 mm. The contusion injury was induced by the weight drop device developed at New York University (Gruner, 1992). The impact velocity and compression were monitored and recorded to guarantee consistency between animals. This type of injury corresponds to a moderate lesion in the spinal cord. After injury, the muscles were sutured in layers and the skin was closed. The rats were returned to their cages with the temperature regulated for the following 24h, with ad libitum access to water and food.

Drug Treatment and Behavioral Assessment

Animals were categorized into 4 groups: In group 1 (n=15), rats received a dose of 90 mg/kg of minocycline (Sigma, St. Louis, MO) IP immediately after surgery and 45 mg/kg at 12 and 24 hr after injury. This treatment group reflects the procedure conducted by Lee and colleagues (2003). In group 2 (n=15), rats received a dose of 90 mg/kg of minocycline IV through a jugular canula, and also 45 mg/kg/IP at 12 and 24 hr after injury. Groups 3 (n=8) and 4 (n=7) were control groups where only an equal volume of saline was administered with the same scheme as each correspondent minocycline treated group. Behavioral assessment was performed in all groups by two blinded examiners at 24 hrs, 72 hrs and then weekly after the procedure with the open field locomotor test (Basso et al., 1996) as well as the BBB subscore (Popovich et al., 1999).

Histology

At 6 weeks, all animals were anesthetized and intracardially perfused with normal saline solution followed by 4% paraformaldehyde solution. Cords (n=45) were removed and the tissue embedded in paraffin. Cord segments of 20 mm were dissected and 8 μ m sections cut serially horizontally throughout the sample. A section containing the central canal for each animal was then stained with Cresyl violet and analyzed. For the present study, remaining normal cord tissue, total abnormal cord tissue and cavities were acquired using a Zeiss Axiovert 200 M microscope and Stereo Investigator and Neurolucida Software (MicroBright Field Inc). Abnormal cord tissue was defined as areas containing an alteration in size and number of neurons and their normal distribution in the gray matter, small cell infiltration and cavitation (lack of tissue). Data are reported as area of spared tissue and cavitation.

Statistical Analysis

Data are expressed as mean $\pm$ SEM of spared tissue and cavity areas. Statistical analysis of behavioral and histological data among groups was performed using repeated measures ANOVA and ANOVA followed by Dunnett's test, respectively. Statistical significance was considered for $p < 0.05$.

Acknowledgements

We would like to thank Diana Ospina, Ileana Oropesa, Denise Koivisto, Andres Maldonado, Rosa Abril, and Monica Stagg for animal care and behavioral analysis; Paulo Diaz and Michael Shumm for performing the contusion injuries; Gladys L. Ruenes and Lyudmila Rusakova for tissue processing; Robert Camarena for photography; Charla Rowlette and Jeremy Lytle for expert editorial assistance and word processing. Supported by funds from the National Institute of Neurological Disorders and Stroke, National Institutes of Health, Facilities of Research Excellence in Spinal Cord Injury (FORE-SCI) under contract No. N01-NS-3-2352, and The Miami Project to Cure Paralysis.

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Locomotor Scores (BBB test)

Figure 1.

Graph of the locomotor scores obtained with the BBB test (Mean $\pm$ SEM). The groups were not significantly different at any point in time. In fact, by day 7 post-injury, all groups have achieved similar mean scores and no additional functional improvement is observed.

Figure 2.

Graph of the BBB subscores of the 4 groups (Mean $\pm$ SEM). There is no significant difference between groups using this behavioral assessment strategy that focuses on fine motor coordination including paw position and toe clearance.

Figure 3.

Photomontage of a representative horizontal histological section from the injured spinal cord (IV minocycline group). Upper: The quantitative assessment parameters for area of spared tissue and cavity are shown (green = total cord area; red = total contusion area; blue = cavity; pink = central canal for landmark purposes). Lower: magnification at the epicenter of the injured region shows fluid-containing cavities surrounded by inflammatory and necrotic tissue.

Figure 4.

Graph of mean total spared tissue (mm²; ± SEM) in 20-mm horizontal sections obtained from cords containing the central canal. The highest preservation corresponds to the group 3 (control IP), however, there was no statistically significant difference between groups.

Figure 5.

Graph of the mean cavity area (mm²; ± SEM) obtained from horizontal sections containing central canal. There is no significant difference between groups.