Minocycline Protects against Neurologic Complications of Rapid Correction of Hyponatremia

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ABSTRACT

Osmotic demyelination syndrome is a devastating neurologic condition that occurs after rapid correction of serum sodium in patients with hyponatremia. Pathologic features of this injury include a well-demarcated region of myelin loss, a breakdown of the blood–brain barrier, and infiltration of microglia. The semisynthetic tetracycline minocycline is protective in some animal models of central nervous system injury, including demyelination, suggesting that it may also protect against demyelination resulting from rapid correction of chronic hyponatremia. Using a rat model of osmotic demyelination syndrome, we found that treatment with minocycline significantly decreases brain demyelination, alleviates neurologic manifestations, and reduces mortality associated with rapid correction of hyponatremia. Mechanistically, minocycline decreased the permeability of the blood–brain barrier, inhibited microglial activation, decreased both the expression of IL1α and protein nitrosylation, and reduced the loss of GFAP immunoreactivity. In conclusion, minocycline modifies the course of osmotic demyelination in rats, suggesting its possible therapeutic use in the setting of inadvertent rapid correction of chronic hyponatremia in humans.


Osmotic demyelination syndrome (ODS) is a severe neurologic condition that is characterized by severe demyelination in the central nervous system (CNS) secondary to osmotic imbalance. In a clinical setting, this syndrome often occurs after too rapid correction of chronic hyponatremia.1–5

In ODS, demyelination is widespread in the brain, with predominance in hippocampus, basal ganglia, and subcortical regions. The physiopathology of this disorder is not yet fully understood, and an experimental murine model has been developed to better understand the mechanisms leading to myelin damage after an osmotic injury.2,5,6 Previous experiments have suggested that ODS might share key characteristics with other models of central nervous system demyelination in which both opening of the blood–brain barrier (BBB) and microglia–macrophage activation are involved in the genesis of demyelinating changes.7–11

Minocycline is a second-generation tetracycline that has been well studied in various models of brain pathology including autoimmune or ischemic myelin damage, and others have reported that administration of minocycline in CNS injury was associated with a striking reduction in BBB permeability, inhibition of microglia–macrophage activation, and inflammatory

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cytokines secretion, with subsequent benefits in mortality and functional outcome.12–17 In this work, we aimed to investigate whether minocycline has a protective effect in an animal model of ODS.

We report that, in a rat model, minocycline decreases mortality and alleviates neurologic manifestations after rapid correction of chronic hyponatremia in rats. The action of minocycline was associated with restoration of normal BBB permeability, decrease in microglia–macrophage activation, reduction in glial fibrillary acidic protein (GFAP) immunoreactivity loss, and decreased expression of proinflammatory cytokines, along with significant attenuation of demyelination lesions.

RESULTS

Serum Sodium Values before and after Correction of Hyponatremia

We successfully used a previously described model (Figure 1)5,6,18 to induce chronic severe hyponatremia in all rats. In these experiments, only rats who had severe hyponatremia (SNa < 120 mEq/L) were included in the analysis. Table 1 shows the SNa value in the different groups before and after the correction for each set of experiments. SNa levels at day 4 were comparable in the different groups studied for all experiments. Administration of hypertonic saline resulted in a significant and comparable rise of SNa in all groups (mean 24-hour gradient of serum sodium of 31 mEq/L for animals treated with hypertonic saline alone and animals treated with minocycline early and 33 mEq/L for animals treated with delayed minocycline; P = not significant for ΔSNa in group 1 versus group 2 and group 3; P = 0.001 for SNa day 4 versus SNa day 5).

Improved Functional Outcome after Minocycline Treatment in ODS

After rapid correction of serum sodium, animals treated with hypertonic saline alone exhibited severe neurologic manifestations including seizures, paralysis, and coma. In contrast, animals treated with early minocycline only showed mild neurologic manifestations. The incidence and severity of these neurologic manifestations was significantly lower after treatment with minocycline throughout the course of the disease (Figure 2A). Body weight loss, which can be used as a marker of CNS pathology after induction of ODS,5 was significantly greater in animals corrected with hypertonic saline alone compared with animals treated with hypertonic saline and minocycline. (33 ± 2% in group 1 versus 17 ± 7% in group 2; P = 0.014; Figure 2B).

Minocycline Reduces Death Associated with Rapid Correction of Chronic Hyponatremia

Table 1 shows mortality in animals treated with hypertonic saline only and with minocycline before and after hypertonic saline. Eighty-four percent (26/31) of the animals treated with hypertonic saline alone died 6 days after the correction, whereas after the same amount of time, only 48% (13/27) of animals treated with hypertonic saline and minocycline before and during the correction of hyponatremia died (P = 0.0053). Survival analysis showed that treatment with minocycline was associated with significant reduction in mortality.

Delayed Administration of Minocycline Still Provides Protection in ODS

In the clinical setting, rapid correction of hyponatremia is generally inadvertent, the toxic gradient of serum sodium is achieved within 12 to 24 hours, and the symptoms are usually delayed for 48 to 72 hours after hyponatremia is corrected. Therefore, we sought to determine whether minocycline administered 18 hours after the toxic gradient of serum sodium was still beneficial. Six of 13 animals who received minocycline 18 hours after the correction survived versus 26 of 31 animals who received hypertonic saline alone (P = 0.056). In the group of animals who received delayed minocycline 18 hours after the correction of hyponatremia, all seven rats who later
Table 1. Biological and clinical parameters of animals used for all three experiments

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SNa day 4 (mEq/L) 105
SNa day 5 (mEq/L) 136
DSNa (mEq/L) 31
Mortality day 10 26/31

Early Minocycline Treatment Decreases the Permeability of BBB 24 Hours after Rapid Correction of Chronic Hyponatremia

Evans Blue Dye Analysis.

As shown in Figure 3A, rapid correction of chronic hyponatremia resulted in a significant increase in the permeability of the BBB (P < 0.01 compared with uncorrected controls). Early treatment with minocycline restored the permeability of the BBB to almost normal levels (P < 0.05 compared with group 1, P = not significant compared with uncorrected controls).

Immunostaining for IgG.

We used immunolabeling for IgG to identify regions with an increased permeability of the BBB. Representative microphotographs of the brains of animals in each group are shown in Figure 3, B and C. In animals treated with hypertonic saline alone, a massive immunoreactivity for IgG was found in cortical and subcortical areas, the basal ganglia, and hippocampus. In contrast, animals treated with minocycline showed only a faint immunoreactivity for IgG in the same areas.

Minocycline Reduces Myelin Damage and Microglia–Macrophage Accumulation after Rapid Correction of Chronic Hyponatremia

We next wanted to know whether the improvement in clinical outcome achieved by minocycline was associated with decreased demyelination in the CNS. We investigated myelin integrity by myelin basic protein (MBP) immunoreactivity and we found that animals treated with hypertonic saline alone developed large areas of demyelination in the cortex, basal ganglia, and hippocampus (Figure 4, A and C); in contrast, animals treated with hypertonic saline and minocycline had less demyelination lesions (Figure 4, B and D). Using semiquantitative image analysis to evaluate the extent of myelin pathology in the two groups of animals, we found that treatment with minocycline significantly reduced the surface area of lesions (P < 0.05 in group 1 versus group 2; Figure 4E).

Because the effects of minocycline have been reported to be partly caused by inhibition of microglial activation, we analyzed microglia–macrophage activation by immunolabeling for CD68 (clone ED1). In animals treated with hypertonic saline alone, there was a higher proportion of CD68-positive cells than in animals treated with minocycline (P < 0.05 in group 1 versus group 2). Activated microglia were seen at the border of and within demyelinating regions (Figure 5).

Treatment with Minocycline Decreases GFAP Immunoreactivity Loss in ODS

GFAP immunoreactivity loss has been shown to be affected
Minocycline Decreases Expression of IL-1α and Nitrosylation of Protein in ODS

IL-1α is involved in the pathogenesis of demyelination, and nitric oxide has been shown to be a potent inflammatory mediator in demyelinating disorders. Previous reports suggested increased production of nitric oxide synthase by activated microglia in ODS. Therefore, we use immunohistochemistry to study the production of IL-1α and the effect of nitric oxide synthase (nitrosylation of protein) after minocycline administration. Treatment with minocycline resulted in a decrease in IL-1α immunoreactivity and protein nitrosylation, as shown by a striking decrease in nitrotyrosine immunoreactivity in the CNS (Figure 7).

DISCUSSION

In this work, we studied the effect of minocycline, a second-generation tetracycline, in an animal model of ODS.

Using a very large series of animals, we found that treatment with minocycline resulted in increased survival and decreased severity of neurologic manifestations after rapid correction of chronic hyponatremia. We also found that treatment with minocycline reduces BBB permeability and reduced microglia–macrophage activation.

The doses of minocycline used in this animal study, derived from the doses used in other models of experimental CNS protection, are very large and seldom used in humans and might limit the impact of our findings in the clinical setting. However, recently it was shown that local CNS delivery of minocycline combined with an intraperitoneal dose can reduce the amount of minocycline administered for neuroprotection and increase the protective effect in another model of CNS injury. It should also be pointed out that, in our study, the gradient of serum sodium achieved by animals in all groups is very high and rarely seen in the setting of correction of hyponatremia.

Another interesting conclusion of our work comes from the fact that delayed administration of minocycline still carries some protection. Of note, all rats that died after receiving delayed minocycline (18 hours after correction) were already symptomatic by the time minocycline was given. Translated to clinical setting, it is rare to observe symptoms of ODS within the first 24 hours of correction of hyponatremia, and the fact that all of six animals who survived after delayed administration of minocycline were pauci-symptomatic or asymptomatic, suggest that this approach might still be used in clinical practice.

Our results are in line with previously published observations on the effect of minocycline on BBB in other models of CNS demyelinating and nondemyelinating pathology. Regarding the possible mechanisms associated with the protection of the BBB, previous work has suggested that minocycline acts on matrix metalloproteases and inflammatory cytokines secretion that are involved in the alteration of the BBB permeability.

Minocycline did not completely abrogate the histologic lesions of myelinolysis, and some areas of myelin loss were found in nonsymptomatic or pauci-symptomatic animals, suggesting dissociation between neurologic manifestations and myelin destruction. These findings reflect the vast spectrum of ODS and clinically asymptomatic central pontine myelinolysis found in humans. The fact that significant asymptomatic or...
pauci-symptomatic demyelination can occur despite minimal opening of the BBB together with our recent study using dexamethasone in ODS challenge the assumption that opening of the BBB in ODS plays a crucial role in the development of demyelination.8,9,23,28 The same process could be applied to the role of microglia in ODS. In accordance with previous studies,13–15,29 we found that minocycline reduced microglia–macrophage activation in CNS. Nevertheless, despite significant microglial inhibition, demyelinating lesions were still present in the brains of animals treated with minocycline. This further questions the role of microglial activation in ODS. It is possible that activated microglia–macrophages appear in the CNS after the original demyelinating insult to remove myelin debris without contributing to the initial demyelinating lesion. This was suggested by experiments showing that inhibition of microglia–macrophage activation with lovastatin23 was not associated with a net decrease in mortality after inadvertent correction of chronic hyponatremia. A very likely explanation of these findings is that the secretion of inflammatory cytokines by microglia exacerbates neurologic deficits and demyelination but has marginal contribution to the main demyelinating process.

The decrease in inflammatory marker reactivity (IL-1β and nitro-tyrosine) observed after minocycline administration further provides strong evidence that minocycline attenuates inflammation after ODS induction.

GFAP loss is an early phenomenon in diverse neurologic

Figure 3. Minocycline decreases the permeability of the BBB after rapid correction of chronic hyponatremia. (A) Evans blue dye extravasation 24 hours after the correction of chronic hyponatremia showing increase permeability in animals treated with hypertonic saline alone in contrast to animals treated with hypertonic saline and minocycline (n = 8 for NaCl alone; n = 7 for NaCl + minocycline; n = 6 for uncorrected Ctrl). P < 0.05 for group 1 versus group 2 and P < 0.01 for group 1 versus group 3. Group 2 versus group 3, P = not significant. (B–E) Immunohistochemistry for IgG. Increase deposition of IgG in subcortical regions and basal Ganglia (C) in animals treated hypertonic saline alone compared with animals who also received minocycline (B). At higher magnification, IgG are seen intraparenchymally in animals treated with NaCl alone (D), whereas mainly intravascular deposition is visible in animals treated with minocycline (E). Scale bar: 200 μm.

Figure 4. Minocycline reduces myelin loss after rapid correction of chronic hyponatremia. Immunohistochemistry for MBP showing large area of demyelination in cortex, hippocampus, and basal ganglia (arrows) after correction of hyponatremia with NaCl alone (A); less and smaller lesions are found after administration of minocycline (B). Image quantification of MBP lacking area (C) showing that treatment with minocycline significantly attenuated the demyelination (P < 0.05 compared with animals treated with NaCl alone) but did not suppress the myelin loss. (C and D) Higher magnification of hypothalamus in animals treated with hypertonic saline alone (C) or hypertonic saline and minocycline (D) showing massive and well-delineated loss of myelin on axons bundles (*). (E) Represents the quantification of myelin loss in the two groups compared with uncorrected control. Computerized quantification was applied on MBP-stained images (as in A and B) (n = 4 for NaCl alone; n = 12 for NaCl + Minocycline; n = 5 for uncorrected controls). Scale bar = 200 μm.
disorders.\textsuperscript{30,31} Previous work reported a beneficial effect of minocycline after CNS injury in relation to decreased GFAP loss,\textsuperscript{19} and \textit{in vivo} studies showed that minocycline prevents astrocyte cell death.\textsuperscript{32} Here, we found that experimental ODS is associated with massive loss of GFAP reactivity and that treatment with minocycline significantly reduced GFAP staining loss. The full significance of GFAP loss with regard to astrocyte viability and the role in ODS is yet to be determined.

After rapid correction of chronic hyponatremia, few therapeutic options are available to prevent the development or limits the consequences of ODS. Thus far, re-lowering serum sodium is the most beneficial treatment when the initial osmotic injury has already occurred.\textsuperscript{6,33–35} Our results suggest that, in clinical practice, early administration of minocycline might have a role in preventing neurologic damage in hyponatremic patients who underwent rapid correction of their sodium deficit. Nevertheless, re-lowering of serum sodium should remain the treatment of choice when a toxic gradient is achieved until further work is done to fully explore the possibilities of minocycline in management of rapid correction of chronic hyponatremia in terms of the minimal effective dose and possible combination with re-lowering of serum sodium.

In summary, this work addressed the effect of minocycline...
on ODS. We found that, in rats, minocycline decreased the neurologic manifestations and increased the survival after rapid correction of chronic hyponatremia. These effects were associated with a striking reduction in BBB permeability, a decrease in microglia–macrophage activation and GFAP immunoreactivity loss, and a decrease in inflammatory protein expression.

CONCISE METHODS

Animals
Male Wistar rats (250 to 300 g) were used for all experiments. They were housed in individual cages under conditions of constant temperature (23°C) and a 12-hour/12-hour light/dark cycle and used after a brief period of adaptation (3 to 4 days), during which ad libitum water and standard pelleted chow was provided. All procedures were performed in accordance with guidelines for animal care at Université Libre de Bruxelles.

Induction of Hyponatremia
As described previously, chronic hyponatremia was induced by 1-deamino-[8-D-arginine] vasopressin infusion and liquid diet.3,6,18 An osmotic minipump (Model 2001; Alzet, Palo Alto, CA) was filled with 4 µg/ml 1-deamino-[8-D-arginine] vasopressin (Minirin, Ferring, Sweden) and inserted in the back of the animal under light halothane anesthesia at the beginning of the experiment (day 0). On the day of insertion, rats were switched to a liquid diet with low sodium content (AIN 76; Technilab BMI). The liquid diet was maintained until the morning of day 5 after light halothane anesthesia for serum sodium analysis. In animals of group 2, blood samples were taken after the first injection of minocycline hydrochloride (Duchefa) at a dose of 60 mg/kg of body weight. At time of correction (injection of hypertonic saline), the same dose was repeated, and minocycline injections were continued at a decreased dose of 45 mg/kg of body weight every 12 hours for 3 days (until day 7 of experiment). Group 1 served as controls animals and received only intraperitoneal injection of sterile saline instead of minocycline.

To determine whether delayed administration of minocycline impacts survival, we used a second administration scheme in another group of 13 animals (group 3), where minocycline (60 mg/kg of body weight) was given 18 hours after the correction of hyponatremia when the toxic gradient was already achieved. This dose was repeated 12 hours later, and, as in group 2, a reduced dose of 45 mg/kg of body weight every 12 hours was then given until day 7.

The minocycline dosage and administration scheme were derived from previous reports studying the effects of that drug in CNS injury.13,25

Correction of Hyponatremia
After 4 days of liquid feeding (day 4 of experiment), hyponatremia was rapidly corrected by intraperitoneal administration of 1 M NaCl in a single dose.6,37 Pelleted chow was given to the animal on day 5 of the experiment, after the blood sampling for the determination of final 24-hour SNa; to avoid incidental re-lowering occurring at the day 5, water was given only at day 6 of the experiment.5,33

Blood Measurements
Blood samples (0.3 ml) were collected via tail transection at days 4 and 5 after light halothane anesthesia for serum sodium analysis. In animals of group 2, blood samples were taken after the first injection of minocycline. Electrolytes measurements were performed using MODULAR p800 (Roche).

Evaluation of Neurologic Manifestations and Mortality
A first set of experiments was designed to assess mortality and neurologic manifestations. Animals were corrected with 2 ml hypertonic NaCl/100 g of body weight, and they were allowed to survive until day 10 (6 days after the correction) of the experiment.

A total of 31 rats were included in the group of animals treated with hypertonic saline alone; in a second group of 27 rats, minocycline was started 12 hours before the beginning of the correction, and in a third group of 13 animals, minocycline was given 18 hours after correction of hyponatremia.

Rats treated with early minocycline and hypertonic saline were observed daily for occurrence of neurologic manifestations. Using a previously described scoring system,22 neurologic manifestation were scored on a daily basis as follows: 6 = no neurologic manifestations;
5 = slow or awkward gait; 4 = limb weakness and/or paralysis; 3 = seizures; 2 = severe motor deficits; 1 = complete inability to move; and 0 = death.

In a second set of experiments (experiment 2), weight loss was evaluated in animals of group 1 and group 2 (early minocycline). Because a correction gradient of >25 mEq/L per 24 hours has been associated with increased mortality, we specifically aimed to reach a lower gradient of correction to induce ODS with decreased mortality. These animals were given 1 ml/100 g of body weight of hypertonic saline, and weight was recorded before the correction of hyponatremia and 3 days after the correction (day 7 of experiment). For this experiment, we used an independent set of rats divided into two groups: animals treated with hypertonic saline alone (n = 9) and animals treated with minocycline before the correction (n = 12).

Immunohistochemistry and Immunofluorescence
At day 10 of the experiment, surviving animals treated with NaCl or early minocycline were killed by decapitation. The brain was divided along the midline into its two hemispheres and was fixed overnight in formalin 10% before rinsing with PBS. Immunohistochemistry was performed on 7-μm-thick paraffin section using anti-myelin basic protein for myelin (anti-MBP 1/1000; Abcam, Cambridge, UK), anti-CD68–clone ED1 for microglia (1/200; AbDserotec, Dusseldorf, Germany), and anti-GFAP for astrocytes (1/1000; Dako, Brussels, Belgium). Anti-IL-1α and anti-nitrotyrosine were purchased from Pierce Endogen and Millipore (Brussels, Belgium). Fluorescent conjugated secondary antibodies used were goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 594 (1:100), both from Invitrogen. Technical procedures have been described elsewhere. IgG immunohistochemistry was performed as an assessment of BBB reactivity as described previously, using one-step detection with biotinylated anti-rat IgG.

Myelin, Microglia–Macrophage Activation, and Astrocyte Loss Quantification
Image quantification of areas of demyelination and GFAP loss was performed as described previously in animals treated with early minocycline and hypertonic saline. Briefly, macro-images of stained sagittal sections of brain were obtained with a Nikon camera fitted with a Nikon macro-objective. Three sets of two adjacent slides for each animal (six slides/animals) labeled for myelin (anti-MBP) and astrocytes (anti-GFAP) were analyzed blindly using Image J software (National Institutes of Health). Each set of slides was separated by at least five slides. The three sets of two slides were taken at a similar sagittal level in all of the groups studied. The regions with loss of myelin and astrocytes were identified as MBP and GFAP unstained areas with light microscope and were measured by the software after gray level thresholding of the corresponding image. Results were reported over the total brain surface area and expressed as percentage of demyelinated or astrocyte loss area.

Microglia-macrophage activation was quantified on sagittal sections stained for CD68. As described by others, area of CD68 immunoreactivity was measured on non counterstained slides using image J software and the ratio of CD68 stained area over total brain surface was calculated.

Evaluation of BBB by Evans Blue Dye Extravasation
In another set of experiments (experiment 3), Evans blue was used to determine the BBB permeability 24 hours after the correction of hyponatremia in animals treated with hypertonic saline and early minocycline (n = 8 for each group) and in a third control group of uncorrected hyponatremic rats (n = 6). In each animal, Evans blue solution (Sigma Chemical, St. Louis, MO) (2% in saline; 3 ml/kg of body weight) was injected intravenously in the tail vein 24 hours after rapid correction of chronic hyponatremia and allowed to circulate for 40 minutes. Under deep anesthesia, the chest was dissected, and the left ventricle was perfused with saline for 20 minutes to remove any circulating dye. The skull was dissected to extract the brain; brain tissue was weighed and allowed to incubate in formamide for 72 hours at 50°C. The formamide solution containing extracted Evans blue was centrifuged at 20,000 × g for 15 minutes, and the absorbance of the supernatant was analyzed spectrophotometrically at 610 nm. The content of Evans blue, expressed per gram of brain tissue, was calculated using a linear standard curve built after absorbance of known amounts of dye in formamide.

Statistical Analysis
Results were expressed as mean ± SEM. Paired and unpaired t tests were used as a parametric test for normally distributed variables. Otherwise, Mann-Whitney nonparametric tests were used. Fischer exact test on proportions and Kaplan-Meir survival analysis were also used to compared mortality and survival in both groups. One-way ANOVA followed by Bonferonni least significant difference was used for Evans blue dye contains analysis. Tests were run using statdirect software. P ≤ 0.05 was considered significant.

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DISCLOSURES
None.

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