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 IL1β 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.

corticosteroids, accompanied by subsequent improvements in mortality and functional outcome. 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) 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, 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 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

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
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<th>Experiment 2</th>
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<th>Experiment 3</th>
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<tbody>
<tr>
<td></td>
<td>Group 1 (NaCl Alone)</td>
<td>(n = 31)</td>
<td>Group 2 (NaCl + Early Minocycline)</td>
<td>(n = 27)</td>
<td>Group 3 (NaCl + Delayed Minocycline)</td>
<td>(n = 13)</td>
</tr>
<tr>
<td>SNa day 4 (mEq/L)</td>
<td>105 ± 1</td>
<td>105 ± 1*</td>
<td>106 ± 2*</td>
<td>106 ± 2*</td>
<td>107 ± 2*</td>
<td>107 ± 2*</td>
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<tr>
<td>SNa day 5 (mEq/L)</td>
<td>136 ± 1</td>
<td>135 ± 1*</td>
<td>138 ± 1*</td>
<td>130 ± 2*</td>
<td>132 ± 3*</td>
<td>24 ± 2*</td>
</tr>
<tr>
<td>DSNa (mEq/L)</td>
<td>31 ± 1</td>
<td>31 ± 1*</td>
<td>33 ± 1*</td>
<td>24 ± 2*</td>
<td>26 ± 2*</td>
<td>NA</td>
</tr>
<tr>
<td>Mortality day 10</td>
<td>26/31</td>
<td>13/27*</td>
<td>7/13*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Percent of weight loss day 7</td>
<td>NA</td>
<td>NA</td>
<td>33 ± 2c</td>
<td>17 ± 6c</td>
<td>NA</td>
<td>NA</td>
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Experiment 1 was designed to address mortality on day 10 (6 days after correction of chronic hyponatremia). Experiment 2 was designed to address the weight loss on day 7, and experiment 3 addressed the BBB permeability 24 hours after the correction of hyponatremia. NA, not applicable.

a P not significant for group 1 versus group 2 in all three experiments.

b P = 0.056 for group 1 versus group 3 and P = 0.0026 for group 1 versus group 2.

c P = 0.014 for group 1 versus group 2.
NaCl + Minocycline 12hrs before correction

Figure 2. Minocycline reduces mortality and morbidity after rapid correction of chronic hyponatremia. (A) Treatment with minocycline decreases neurologic manifestations in ODS. Less severe neurologic manifestations were observed in the group of animals treated with minocycline (*P ≤ 0.05; **P < 0.01). (B) Animals treated with minocycline also showed a decrease in weight loss 3 days after ODS induction (P = 0.014 for group 1 versus group 2). (C) Kaplan-Meyer survival curve showing a decreased mortality in animals treated with minocycline (P = 0.011 by log-rank test).

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.

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 that this approach might still be used in clinical practice.

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 ODS6 challenge the assumption that opening of the BB 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.

GFAP loss is an early phenomenon in diverse neurologic
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 a 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...
4. Infusion was maintained until the end of the experiment. Technilab BMI). The liquid diet was maintained until the morning of day 0. Rats were switched to a liquid diet with low sodium content (AIN 76; NaCl alone

**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. 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 4. Infusion was maintained until the end of the experiment.

**Drug Treatment**

Three groups of animals were studied. Group 1 consisted of controls animals with no minocycline treatment, and group 2 received early minocycline; 12 hours before the correction of hyponatremia, group 2 received intraperitoneal 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.

**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. Pelleted chow was given to the animal on day 5 of the experiment, after the blood sampling for the determination of final 24-hour SNAs; to avoid incidental re-lowering occurring at the day 5, water was given only at day 6 of the experiment.

**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, neurologic manifestation were scored on a daily basis as follows: 0 = no neurologic manifestations; 1 = slight neurologic manifestations; 2 = moderate neurologic manifestations; 3 = severe neurologic manifestations; and 4 = death.

**Figure 7.** Minocycline decreases IL-1α and nitrotyrosine immunoreactivity in ODS. Representative images of nitrotyrosine (A and B) and IL-1α (C and D) immunoreactivity. Intense immunoreactivity for nitrotyrosine and IL-1α was found in brain of animals submitted to ODS (A and C, respectively). In contrast, moderate immunoreactivity was found after treatment with minocycline (B and D).
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 described by others, 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 CD 68. 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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