

Additive Neuroprotective Effects of Minocycline with Creatine in a Mouse Model of ALS

Wenhua Zhang, MD, PhD, Malini Narayanan, MS, MD, and Robert M. Friedlander, MD, MA

The known neuroprotective effects of minocycline and creatine in animal models of amyotrophic lateral sclerosis (ALS) led us to examine whether the combination of these agents would result in increased neuroprotection. As previously reported, we confirmed in ALS mice that either minocycline or creatine treatment results in improvement in motor performance and extended survival. We report that combination of minocycline and creatine resulted in additive neuroprotection, suggesting this to be a novel potential strategy for the treatment of ALS. To our knowledge, this is the first report demonstrating additive neuroprotection of a combinatorial approach in a mouse model of ALS. Adding relevancy to our findings, minocycline and creatine, are relatively safe, cross the blood-brain barrier, and are currently available for human evaluation.

Ann Neurol 2003;53:267–270

Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative disease characterized by progressive motor weakness resulting from selective motor neuron cell death.¹ Mortality is seen on the average 4 years after disease onset. Proven therapeutic options are limited to riluzole, which extends survival by an average of 3 months.¹ It is therefore of utmost importance to develop novel and more effective therapeutics for this universally fatal devastating disease. Because of the multifactorial downstream pathogenic pathways activated in ALS, improving therapeutic efficacy will depend on implementing a strategy addressing multiple components of the pathogenesis. Combinatorial therapeutics have been implemented with varying degrees of

From the Neuroapoptosis Laboratory, Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.

Received Jul 17, 2002, and in revised form Nov 5. Accepted for publication Nov 9, 2002.

Address correspondence to Dr Friedlander, Neuroapoptosis Laboratory, Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.
E-mail: rfriedlander@rics.bwh.harvard.edu

success in complex human diseases such as human immunodeficiency virus.² To determine whether such an approach might be effective in ALS, we evaluated the combination of two agents currently available for human use. Experimental administration of either minocycline or creatine has been demonstrated to delay disease onset and extend survival in transgenic mouse models of ALS.^{3–6} Given that these two compounds act by different mechanisms of action, we evaluated whether the combination of minocycline with creatine might result in additive neuroprotective effects in ALS mice. We report that the combination of minocycline and creatine results in an additive effect, delaying disease onset, slowing progression, and delaying mortality. Because these two compounds are currently available for human use, their combined use may be evaluated in patients with ALS.

Materials and Methods

Mice and Treatment Regimen

Mice carrying the human *SOD1*^{G93A} mutant gene were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were crossbred and genotyped as previously described.⁷ Mice were fed a diet supplemented with 2% creatine beginning at 3 weeks of age (courtesy of Dr R. Kaddurah-Daouk). Minocycline (22mg/kg body weight/day) was injected intraperitoneally beginning at 4 weeks of age (Sigma, St. Louis, MO). Minocycline was prepared fresh in 0.9% saline daily. The monotherapy and untreated groups were fed control diet or injected with saline as appropriate in the same schedule as the treated groups. Experiments were conducted in accordance with protocols approved by the Harvard Medical School Animal Care Committee.

The selection of 10 mice per group was determined from our previous experience evaluating minocycline in ALS mice.³ In that study, using 10 mice per group, we detected 10% protection mediated by minocycline. Given the above-mentioned information, as well as our hypothesis that the combinatorial regimen would result in improved protection, we decided to evaluate a group size of 10.

Evaluation of Motor Function

Motor strength and coordination were evaluated with a Rotarod (Columbus Instruments, Columbus, OH), beginning at 10 weeks of age. Mice were evaluated at 5 and 15rpm. The time each mouse remained on the rod was registered automatically. If the mouse remained on the rod for 7 minutes, the test was completed and scored as 7 minutes. Mice were tested weekly until they could no longer perform the task. Two investigators performed the experiment, one evaluated Rotarod performance and was blinded to mice treatments (M.N.), and the other treated the mice (W.Z.).

Onset and Survival

Disease onset was defined as the first day that a mouse could not remain on the Rotarod for 7 minutes at 15rpm. Mortality was scored as the age of death or the age when the mouse was unable to right itself within 30 seconds.

Statistical Analysis

The findings were reported as the mean \pm standard deviation. Statistical comparisons between control group and treated groups were performed using one-way analysis of variance.

Results

As previously reported, mutant *SOD1^{G93A}* mice treated with either intraperitoneal injections of minocycline or 2% oral creatine demonstrated a significant delay of disease onset and increased survival as compared with mice fed unsupplemented diets and injected with saline (Table).^{3,4,5,6} Disease onset was delayed to 113 and 111 days, respectively, in the minocycline- and creatine-treated groups as compared with 94 days in the control group ($p < 0.05$). Survival was similarly delayed in the minocycline and creatine groups to 142 and 141 days, respectively, as compared with 126 days in the control group ($p < 0.05$). Minocycline-creatine combination resulted in additive neuroprotection, with disease onset detected at 122 days and mortality at 157 days. The differences of onset and survival between the monotherapy groups and the minocycline-creatine group were significantly different ($p < 0.05$). Survival was extended by 13%, 12%, and 25% in the minocycline, creatine, and minocycline-creatine groups, respectively, as compared with the control group. Kaplan–Meier curves of disease onset and survival as well as plots of Rotarod performance clearly demonstrate the additive protection of the minocycline-creatine combination (Fig). At the doses and schedule tested, there were no significant differences in motor performance or disease progression between the minocycline and creatine groups.

Discussion

Successful combinatorial approaches rely on the use of agents with different but complementary mechanisms of action. This approach is of particular importance when treating diseases with complex and multiple pathologic abnormalities. A broad variety of cellular abnormalities have been described in human specimens and mouse models of ALS. Broadly, documented abnormalities in ALS include apoptotic, bioenergetic, excitotoxic, and mitochondrial pathways.^{1,3,4,7–9} Many of

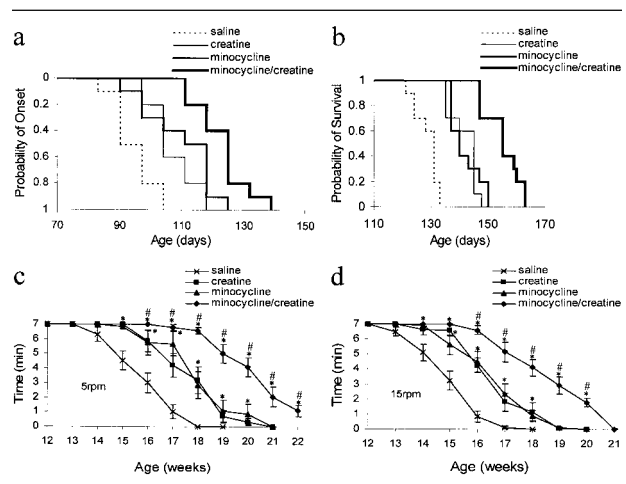


Fig. Cumulative probability of onset of Rotarod deficits (a) and survival (b) in amyotrophic lateral sclerosis (ALS) mice. Survival was significantly prolonged and the onset of Rotarod deficit was significantly delayed in ALS mice treated with minocycline and/or creatine when compared with saline-treated transgenic littermates. Combination of minocycline with creatine significantly delayed onset and mortality as compared with minocycline or creatine alone. Motor function was tested with the Rotarod at 5 (c) and 15rpm (d). Testing was terminated either when the mouse fell from the rod or at 7 minutes if the mouse remained on the rod. Mice treated with the minocycline-creatine combination performed significantly better than mice treated with either compound alone ([pound sign] $p < 0.05$). Mice treated with either minocycline or creatine performed significantly better than control mice ([asterisks] $p < 0.05$, $n = 10$ per group).

these abnormalities are interrelated, although a direct cause and effect relationship cannot always be clearly delineated. Therapeutic approaches targeting only one of the above-described abnormalities is likely to have only a very limited impact on the progression of the disease. Similar to what has been successfully executed in cancer and human immunodeficiency virus, a combination of drugs targeting different components of the particular pathophysiology of the disease will result in enhanced effects as compared with monotherapeutic approaches.² Using the incremental knowledge we have acquired regarding the mechanisms of pathogenesis in ALS, we are in the position to design rational thera-

Table. Onset of Motor Deficits and Mortality of ALS Mice Treated with Minocycline and/or Creatine (mean \pm SD)

	Saline ($n = 10$)	Minocycline ($n = 10$)	Creatine ($n = 10$)	Minocycline/creatine ($n = 10$)
Onset	94.2 \pm 6.8	113.1 \pm 8.8 ^a	111 \pm 4.7 ^a	122 \pm 8.9 ^{a,b}
Mortality	126.3 \pm 4.2	142.2 \pm 4.9 ^a	141.9 \pm 4.3 ^a	157.2 \pm 4.1 ^{a,b}

^a $p < 0.05$ compared with saline group.

^b $p < 0.05$ compared with minocycline and with creatine group.

ALS = amyotrophic lateral sclerosis; SD = standard deviation.

peutics, targeting different components of this disease. The result of these experiments is the first demonstration of the efficacy of this approach in ALS. Furthermore, many patients with ALS are likely taking combinations of medications, with no objective evidence of any benefit, and exposing themselves to potential for harmful interactions or additive side effects.

Minocycline is a second-generation tetracycline, currently used in the treatment of certain infections, acne, and rheumatoid arthritis. Minocycline is used as a chronic medication in the latter two diseases. Currently, the mode of action of minocycline appears to be multifactorial. We recently have demonstrated that a direct action of minocycline is inhibition of mitochondrial permeability transition-mediated release of cytochrome *c*.³ Given that release of cytochrome *c* is a critical component of the apoptotic cascade, inhibition of cytochrome *c* release results in inhibition of cell death. Additional activities have been associated with minocycline-mediated neuroprotection including inhibition of reactive microgliosis, of caspase-1, caspase-3, and nitric oxide synthase transcriptional upregulation and of p38 MAPK activation.^{9–13} Whether any of these properties of minocycline are direct or reactive in nature remains to be elucidated. In addition to ALS, minocycline thus far has been demonstrated to be effective in experimental models of stroke, Huntington's disease, Parkinson's disease, and trauma.^{10–12,14}

Creatine is an amino acid endogenously produced from glycine, methionine, and arginine in the liver, kidney, and pancreas. It has remarkable neuroprotective qualities in models of ALS, traumatic brain injury, Huntington's disease, and Parkinson's disease.^{4,15–17} Currently, the mechanism of creatine-mediated neuroprotection is not well understood. Creatine kinase along with its substrates creatine and phosphocreatine constitute key components of cellular bioenergetic pathways.⁴ The mechanistic basis for the neuroprotective effects of creatine may involve alterations of the insult-induced depletion of cellular ATP, because chronic ingestion of creatine results in increased brain levels of phosphocreatine. To this regard, creatine protects mitochondrial creatine kinase activity against peroxynitrite-induced inactivation, which may play a role in tissue damage in ALS.¹⁸ Most recently, direct antioxidant properties of creatine have been described that may be relevant to explain some of its neuroprotective effects.¹⁹ Creatine has been evaluated in human ALS trials. Preliminary results demonstrated that creatine supplementation temporarily increases maximal isometric power in ALS patients.²⁰

This study does not address neuropathological changes in combined therapy compared with monotherapy. However, neuropathological studies may help elucidate the mechanism of additive neuroprotection using minocycline and creatine. Neuropathological

studies have been done in single therapy of creatine⁴ and minocycline⁵ in *mSOD1*^{G93A} transgenic mice. Klivenyi and colleagues demonstrated a remarkable inhibition of motor neuron cell loss in creatine-treated ALS mice as compared with control ALS littermate mice.⁴ Furthermore, Van den Bosch and colleagues reported decreased microglial activation in spinal cord.⁵ Synergistic protection of motor neurons could likely explain the additive neuroprotection detected in this study using minocycline and creatine.

For the first time to our knowledge, we report effective additive neuroprotection in a therapeutic trial using ALS mice. Note that the ability to rationally design such studies has depended on incremental understanding of the mechanisms of disease progression in ALS, as well as the mechanisms of action of minocycline and creatine. Even greater neuroprotection than that demonstrated from the minocycline-creatine combination might result from including additional compounds with complementary mechanisms of action. In light of the above described results, the relative safety record of minocycline and creatine, and their ability to cross the blood-brain barrier, their combined use may be evaluated in human ALS clinical trials.

This work was supported by grants from the NINDS (NS41635 and NS39324, R.M.F.).

References

1. Rowland LP, Shneider NA. Amyotrophic lateral sclerosis. *N Engl J Med* 2001;344:1688–1700.
2. Temesgen Z, Wright AJ. Antiretrovirals. *Mayo Clin Proc* 1999; 74:1284–1301.
3. Zhu S, Stavrovskaya IG, Drozda M, et al. Minocycline inhibits cytochrome *c* release and delays progression of amyotrophic lateral sclerosis in mice. *Nature* 2002;417:74–78.
4. Klivenyi P, Ferrante RJ, Matthews RT, et al. Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. *Nat Med* 1999;5:347–350.
5. Van Den Bosch L, Tilkin P, Lemmens G, Robberecht W. Minocycline delays disease onset and mortality in a transgenic model of ALS. *Neuroreport* 2002;13:1067–1070.
6. Kriz J NM, Nguyen M, Julien J. Minocycline slows disease progression in a mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis* 2002;10:268.
7. Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 1994;264:1772–1775.
8. Friedlander RM, Brown RH, Gagliardini V, et al. Inhibition of ICE slows ALS in mice. *Nature* 1997;388:31.
9. Martin LJ. Neuronal death in amyotrophic lateral sclerosis is apoptosis: possible contribution of a programmed cell death mechanism. *J Neuropathol Exp Neurol* 1999;58:459–471.
10. Yrjanheikki J, Keinanen R, Pellikka M, et al. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. *Proc Natl Acad Sci USA* 1998;95:15769–15774.
11. Chen M, Ona VO, Li M, et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat Med* 2000;6:797–801.

12. Du Y, Ma Z, Lin S, et al. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. *Proc Natl Acad Sci USA* 2001;98:14669–14674.
13. Almer G, Vukosavic S, Romero N, Przedborski S. Inducible nitric oxide synthase up-regulation in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J Neurochem* 1999;72:2415–2425.
14. Sanchez Mejia RO, Ona VO, Li M, Friedlander RM. Minocycline reduces traumatic brain injury-mediated caspase-1 activation, tissue damage, and neurological dysfunction. *Neurosurgery* 2001;48:1393–1399; discussion, 1399–1401.
15. Sullivan PG, Geiger JD, Mattson MP, Scheff SW. Dietary supplement creatine protects against traumatic brain injury. *Ann Neurol* 2000;48:723–729.
16. Ferrante RJ, Andreassen OA, Jenkins BG, et al. Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. *J Neurosci* 2000;20:4389–4397.
17. Matthews RT, Ferrante RJ, Klivenyi P, et al. Creatine and cyclocreatine attenuate MPTP neurotoxicity. *Exp Neurol* 1999;157:142–149.
18. Wendt S, Dedeoglu A, Speer O, et al. Reduced creatine kinase activity in transgenic amyotrophic lateral sclerosis mice. *Free Radic Biol Med* 2002;32:920–926.
19. Lawler JM, Barnes WS, Wu G, et al. Direct antioxidant properties of creatine. *Biochem Biophys Res Commun* 2002;290:47–52.
20. Mazzini L, Balzarini C, Colombo R, et al. Effects of creatine supplementation on exercise performance and muscular strength in amyotrophic lateral sclerosis: preliminary results. *J Neurol Sci* 2001;191:139–144.

Oculobulbar Involvement Is Typical with Lambert–Eaton Myasthenic Syndrome

Ted M. Burns, MD,¹ James A. Russell, MD,¹
Daniel H. LaChance, MD,²
and H. Royden Jones, MD¹

Oculobulbar symptoms and/or signs were present in 18 of 23 (78%) of Lambert–Eaton myasthenic syndrome (LEMS) patients evaluated at the Lahey Clinic (Table). Sixty-five percent (15 of 23) of our patients had ptosis and/or diplopia, each present in 11 individuals. Bulbar signs and symptoms, including dysarthria in 10 and dys-

phagia in 8 patients, also were observed among our LEMS population. More than one prereferral oculobulbar feature occurred in 13 of our LEMS patients. Prereferral diagnostic considerations included myasthenia gravis, myopathies, and psychiatric disorders. These findings suggest that these atypical characteristics served to dissuade some colleagues from a diagnosis of LEMS. Thus, the presence of oculobulbar symptoms and signs cannot be used to exclude LEMS from the differential diagnosis.

Ann Neurol 2003;53:270–273

The Lambert–Eaton myasthenic syndrome (LEMS) is an autoimmune disorder of neuromuscular transmission.^{1–3} Typically when LEMS is related to a malignancy, it is almost always a small cell lung cancer (SCLC). Although LEMS is sometimes associated with other neoplasms, 15% of patients with other SCLC-related autoimmune disorders have a coexisting, unrelated, and often obvious neoplasm.^{4–13} When there is no paraneoplastic mechanism identified, LEMS usually has a primary autoimmune basis sometimes associated with other immunological disorders.^{7,10} The neurophysiological characteristics of LEMS represent the clinical prototype of a presynaptic neuromuscular transmission disorder.^{1,4–6} The voltage-gated calcium channel of motor nerve terminals is the primary site of immune attack.^{3,8,9}

Classically, LEMS presents with fatigability, proximal weakness, hyporeflexia or areflexia of the muscle stretch reflexes, and cholinergic dysautonomia.^{4–7,14} Oculobulbar symptoms are reported to be less common and mild and therefore have not received much attention.^{6,7,14} The diagnosis of LEMS is sometimes quite challenging. This is because of its relative rarity, the nonspecificity of symptoms with fatigue as a primary clinical complaint, often the generally mild nature of objective weakness, and because the symptoms of dysautonomia are often overlooked. Last, the protean clinical presentations of LEMS are not always appreciated. Because of the therapeutic importance inherent in the early recognition of LEMS, it is incumbent on the neurologist to be aware of these variable clinical presentations. Because it is our clinical experience that LEMS may be underrecognized and its diagnosis is often delayed, we have reviewed our series of 23 LEMS patients diagnosed at the Lahey Clinic. These data demonstrate that oculobulbar involvement, so typical of myasthenia gravis (MG), is also a more common clinical finding of LEMS than is emphasized in the classic teaching of this entity. Recognition of this observation may enhance an earlier diagnosis of the LEMS.

From the ¹Department of Neurology, Lahey Clinic, Burlington, MA, and the ²Department of Neurology, Mayo Clinic, Rochester, MN.

Received Jun 14, 2002, and in revised form Nov 11. Accepted for publication Nov 11, 2002.

Address correspondence to Dr Burns, Lahey Clinic, 41 Mall Road, Burlington, MA 01805. E-mail: tmb8r@virginia.edu