

## Combined Treatment with Anisodamine and Neostigmine Inhibits Joint Inflammation in Collagen-Induced Arthritis Mice

Jv-Xiang Zhou, Ping Ke, Gui Huan, Bo-Zong Shao & Chong Liu

Department of Pharmacology, Second Military Medical University, Shanghai, China

### Correspondence

C. Liu, Department of Pharmacology,  
Second Military Medical University,  
325 Guo He Road,  
Shanghai 200433, China.

Tel.: +86-21-8187-1280-802;

Fax: +86-21-6549-3951;

E-mail: wanlc2004@gmail.com

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The first two authors contributed equally to this work.

Rheumatoid arthritis (RA) is a complicated and treatment refractory autoimmune disease that is characterized by a chronic inflammatory infiltration of immune cells [1]. The central nervous system plays an important role in limiting inflammatory responses via an inflammatory reflex of the vagus nerve termed as “the cholinergic antiinflammatory pathway” [2]. Acetylcholine, the principal neurotransmitter of the vagus nerve, interacts with  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7$ nAChR) expressed on macrophages and other cells and could inhibit the production of proinflammatory cytokines such as TNF $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6. Decreased level of vagus nerve activity is correlated with increased serum concentration of late-phase inflammation cytokine high-mobility group box chromosomal protein 1 in patients with RA. Stimulation of nicotinic acetylcholine receptors attenuates collagen-induced arthritis (CIA) in mice. Lack of  $\alpha 7$ nAChR aggravates the development of CIA [3].

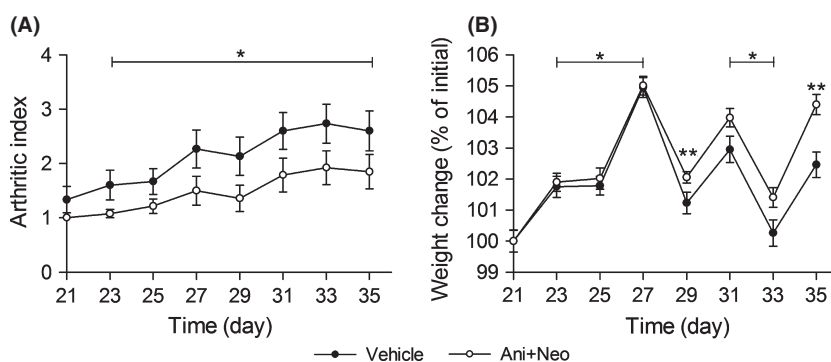
Our previous studies showed that anisodamine blocks muscarinic receptors, and thus promotes more endogenous acetylcholine to bind with  $\alpha 7$ nAChR [4]. Neostigmine, a cholinesterase inhibitor, produces antiinflammatory actions through increasing the concentration of endogenous acetylcholine [5]. A combination of anisodamine and neostigmine augments the antishock efficacy through activating the  $\alpha 7$ nAChR-dependent cholinergic antiinflammatory pathway and reduces the infarct size in rats subjected to middle cerebral artery occlusion [6].

In this study, we evaluated the therapeutical value of anisodamine/neostigmine combination in CIA model [7]. CIA was induced in DBA/1 mice which were immunized with 100  $\mu$ g bovine type II collagen (Chondrex, Redmond, WA, USA) and emulsified with an equal volume of complete Freund's adjuvant (Chondrex). The day of the first immunization was defined as day

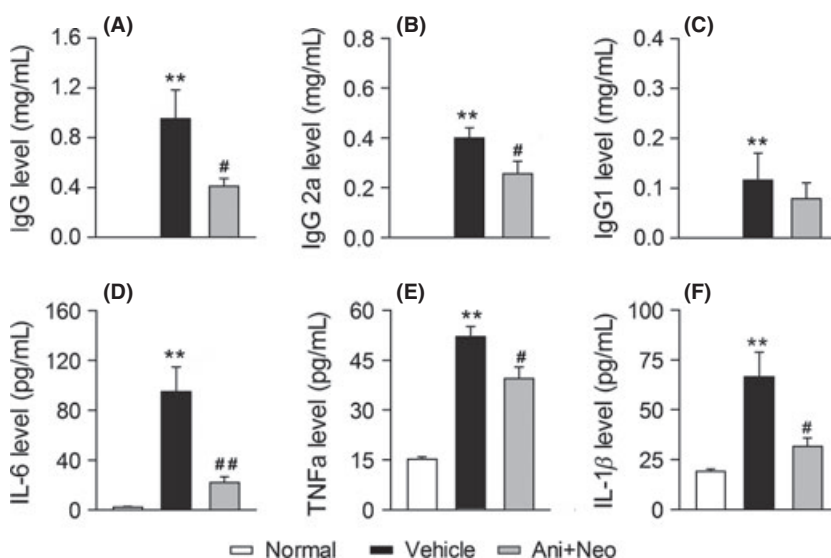
0. A boost injection of bovine type II collagen of the same amount was carried out on day 21. Mice received daily i.p. injection of anisodamine (25 mg/kg) and neostigmine (50  $\mu$ g/kg) from the day of the boost immunization for 10 days. Immunized mice receiving daily saline injection were used as a control. Arthritic symptoms were evaluated according to previously reported [8]. The sum of the scores in the two hind limbs was used as the arthritic score. Blood samples were collected 5 days after the last treatment, and type II collagen-specific antibodies and inflammatory cytokines were measured using commercial ELISA kits (R&D system, Research & Development, San Diego, CA, USA) [9,10]. All results are expressed as the mean  $\pm$  SD. The Mann-Whitney *U*-test was used to analyze the arthritic severity. Cytokines and antibody levels were analyzed using the Student's *t*-test. Statistical significance was set at  $P < 0.05$ .

A combined treatment significantly reduced arthritis score and joint swelling ( $P < 0.05$  vs. vehicle; Figure 1A), starting from day 23 and throughout the evaluation period. Combined treatment also significantly inhibited weight loss (Figure 1B). The immunization increased the serum concentration of anti-type II collagen-specific antibodies (IgG, IgG<sub>1</sub>, and IgG<sub>2a</sub>). Combined treatment significantly decreased the serum levels of IgG and IgG<sub>2a</sub>, but not IgG<sub>1</sub> (Figure 2A–C). Combined treatment also significantly decreased the serum level of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 (Figure 2D–F).

Collectively, a combination of anisodamine and neostigmine could decrease arthritic index, reduce joint swelling, and inhibit weight loss. Combined treatment also decreased the serum levels of anti-type II collagen-specific antibodies and inflammatory cytokines in CIA mice. Our findings may have important implications toward the development of new treatment strategies for RA, but such a possibility requires further investigation.



**Figure 1** The therapeutic effects of anisodamine/neostigmine combination in collagen-induced arthritis (CIA) mice. CIA was induced in DBA/1 mice by a tail intradermal injection of 100  $\mu$ g of bovine type II collagen and was again given a booster injection of 100  $\mu$ g of bovine type II collagen 3 weeks after. Vehicle or a combination of anisodamine (25 mg/kg, i.p.) and neostigmine (50  $\mu$ g/kg, i.p.) was given intraperitoneally for 10 consecutive days after the secondary immunization. Arthritic symptoms and the change of body weights were evaluated. Combination therapy significantly (A) decreased arthritis score and (B) inhibited weight loss.  $n = 13$  per group. \* $P < 0.05$  versus vehicle, \*\* $P < 0.01$  versus vehicle.



**Figure 2** Effects of anisodamine/neostigmine combination on serum anti-CII antibodies and inflammatory cytokines in CIA mice. (A–C) Combination therapy significantly decreased the levels of IgG, and IgG<sub>2a</sub>, but had no significantly effect on IgG<sub>1</sub> (D–F). Combination therapy also significantly decreased the levels of inflammatory cytokines such as IL-6, TNF $\alpha$ , and IL-1 $\beta$ .  $n = 13$  per group. \*\* $P < 0.01$  versus Normal, # $P < 0.05$  versus vehicle, ## $P < 0.01$  versus vehicle. CIA, collagen-induced arthritis.

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## Conflict of Interest

The authors declare no conflict of interest.

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