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Restorative effect and mechanism of mecobalamin on sciatic nerve crush injury in mice

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Abstract

Mecobalamin, a form of vitamin B_{12} containing a central metal element (cobalt), is one of the most important mediators of nervous system function. In the clinic, it is often used to accelerate recovery of peripheral nerves, but its molecular mechanism remains unclear. In the present study, we performed sciatic nerve crush injury in mice, followed by daily intraperitoneal administration of mecobalamin (65 μg/kg or 130 μg/kg) or saline (negative control). Walking track analysis, histomorphological examination, and quantitative real-time PCR showed that mecobalamin significantly improved functional recovery of the sciatic nerve, thickened the myelin sheath in myelinated nerve fibers, and increased the cross-sectional area of target muscle cells. Furthermore, mecobalamin upregulated mRNA expression of growth associated protein 43 in nerve tissue ipsilateral to the injury, and of neurotrophic factors (nerve growth factor, brain-derived nerve growth factor and ciliary neurotrophic factor) in the $L_{4–6}$ dorsal root ganglia. Our findings indicate that the molecular mechanism underlying the therapeutic effect of mecobalamin after sciatic nerve injury involves the upregulation of multiple neurotrophic factor genes.

Key Words: *nerve regeneration; peripheral nerve injury; mecobalamin; sciatic nerve; nerve repair; neurotrophic factor; neuroprotective effect; vitamin B_{12}; molecular mechanism; gene expression; neural regeneration*

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Introduction

Peripheral nerve injury is commonly caused by accidental trauma, acute compression or iatrogenic injury (Wu et al., 2012). Such injury can induce temporary or permanent neurapraxia, and may seriously affect a patient's quality of life and ability to work. Drug treatment can improve neurological function after peripheral nerve damage, especially crush injury (Jacob et al., 2000; Xie et al., 2001; Gu et al., 2011). After peripheral nerve injury in adult mammals, a slow increase in the neuronal expression of neurotrophic factor can be observed from 7 days after injury. Axonal regeneration at the proximal stump of the injury site is also very slow, and even after nerve suturing, axons may take up to 1 month to extend across the region of damage (Gordon, 2009; Unezaki et al., 2009). Therefore, even when the nerve stump is promptly sutured, additional measures are needed to support satisfactory neural regeneration. At present, such measures include gene therapy, cell therapy, and administration of neurotrophic factors. In experimental animals, gene and cell therapy successfully promote neuronal repair after peripheral nerve injury (Wang et al., 2012); however, there are several difficulties with the use of these approaches in humans, including finding an appropriate donor, continued elevated expression of the exogenous gene, high treatment costs, transplantation challenges, and a need for long-term efficacy evaluation. Therefore, a considerable amount of further research into the safety and efficacy of these methods is needed before they can be relied on in the clinic (Cai et al., 2011; Chen et al., 2011; Dadon-Nachum et al., 2011; Hoyng et al., 2011). Neurotrophic factors promote neuronal survival and regeneration, but their purification is complicated and costly, and doubts remain about their clinical efficacy (Rizos et al., 2014; Valiente-Gomez et al., 2014; Wang et al., 2014a, b).

Mecobalamin is a form of vitamin B_{12} that contains cobalt (Yang et al., 2013). It is currently used to treat diabetic peripheral neuropathy (Huang et al., 2011; Izumi et al., 2013). As a cofactor of the methyltransferase enzyme, mecobalamin is an essential vitamin for nervous system functioning. It contributes to the synthesis of methionine and thymine, increases the uptake of folic acid, and protects its transfer and storage within the cell, activates amino acids, contributes to the biosynthesis of nucleic acid and proteins, and is involved in the formation of nerve tissue lipoprotein (Matsushita et al., 2009; Kocaoglu et al., 2014; Meziere et al., 2014). However, the molecular mechanism by which mecobalamin promotes functional nerve recovery remains unclear.

In the present study, we evaluated the effects of mecobalamin on the morphological and functional recovery of nervous tissue, and its effects on target muscle atrophy, in a mouse model of sciatic nerve injury. In addition, we used real-time PCR to measure the expression of genes for various

neurotrophic factors associated with nerve growth, in order to examine the molecular mechanisms by which mecobalamin may promote peripheral nerve regeneration.

Materials and Methods

Animal surgery and treatment

Sixty adult male ICR mice, weighing 22–25 g, were provided by the Experimental Animal Center of Soochow University (Suzhou, Jiangsu Province, China). All experimental procedures involving animals were carried out in accordance with the US National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Administration Committee of Experimental Animals, Jiangsu Province, China.

All animals were deeply anesthetized with an intraperitoneal injection of a cocktail of xylazine (10 mg/kg), ketamine (95 mg/kg) and acepromazine (0.7 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA). A10 mm long incision was made in the left hindlimb to expose the sciatic nerve, and 2 mm of nerve was crushed by clamping for 30 seconds with smooth-jaw forceps. The distal end of the crush site was marked with a 9-0 nylon suture. After the surgical incisions were closed, the animals were randomly divided into three groups (n = 20 per group), to receive daily intraperitoneal injections of 65 μg/kg (low-dose) mecobalamin (Eisai, Tokyo, Japan), 130 μg/kg (high-dose) mecobalamin, or equivalent volumes of saline. The treatment lasted for 21 days.

Walking track analysis

Walking track analysis was performed 1, 5, 10, 15 and 20 days after sciatic nerve injury to examine motor function recovery in the mice. Paw length (PL) and toe spread (TS) were measured. Sciatic functional index was calculated using the following formula: 118.9 [(ETS − NTS)/NTS] − 51.2 [(EPL − NPL)/NPL] − 7.5, where E represents the experimental side and N refers to the normal control side. An sciatic functional index value of 0 indicates normal nerve function, and −100 indicates total impairment (Rustemeyer and Dicke, 2009).

Histomorphological examination

Twenty-one days after surgery, two mice were chosen at random from each group. Approximately 3 mm of nerve was obtained from the distal segment of the injury site after mice were sacrificed by cervical dislocation under anesthesia, fixed in glutaraldehyde, and embedded in Epon 812 epoxy resin, and cut into ultrathin (3 nm) sections. The sections were contrasted using uranium-lead and viewed under a transmission electron microscope (JEOL USA Inc., Peabody, MA, USA).

The remaining eight mice were sacrificed by cervical dislocation under anesthesia, fixed with 4% paraformaldehyde. Gastrocnemius muscle on the ipsilateral side was embedded in paraffin, sectioned (section thickness, 10 μm), and stained with hematoxylin and eosin. Myocyte cross-sectional area was determined using the Leica QWin image analysis system (Leica Imaging Systems Ltd., Munich, Germany).

Real-time PCR

Oligonucleotide primers were designed using Primer Version 4.0 software (Whitehead Institute, Cambridge, MA, USA), and synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA). Primer sequences are listed in **Table 1**.

At 5, 10, 15 and 20 days postoperatively, three mice were chosen at random from the physiological saline group and from the high-dose mecobalamin group. Total RNA was harvested from nerve tissue on the injured side and from $L_{4–6}$ segments of the ipsilateral spinal cord dorsal root ganglia using Trizol (Invitrogen). Total RNA was purified using an RNeasy Mini Kit, and cDNA was synthesized using an Omniscript RT Kit (both from Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

Quantitative real-time PCR was conducted using a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and a SYBR Green RT-PCR system (FastStart Universal SYBR Green Master (ROX) for quantitative PCR; Roche, Mannheim, Germany). Each 20 mL of reaction mixture contained 0.5 mL cDNA from each sample that was mixed with 12.5 mL of 1 × FastStart Universal SYBR Green Master (ROX; Roche), 0.5 mL forward primer, 0.5 mL reverse primer and 6 mL of PCR-grade water. Real-time PCR conditions were as follows: pre-denaturation at 95°C for 2 minutes, then 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The housekeeping gene GAPDH served as the internal reference. Each sample was tested in triplicate and the $2^{-\Delta\Delta Ct}$ method was used to analyze relative transcription data (Bijwaard et al., 2001; Livak and Schmittgen, 2001).

Statistical analysis

All data were presented as the mean ± SD. Data were compared by one-way analysis of variance and Scheffe *post hoc* test, using SPSS 11.5 software package (IBM, San Francisco, CA, USA). A $P < 0.05$ level was considered statistically significant.

Results

Mecobalamin promoted the recovery of sciatic nerve function in mice

All mice recovered consciousness after surgery. Mice walked with their hindlimbs on the injured side dragging on the ground. Walking track analysis demonstrated that sciatic functional index value in each group increased with time (**Figure 1**). One day after the surgery, no significant difference in sciatic functional index value was detectable among groups, but by 10 days, sciatic functional index was significantly better in the high-dose mecobalamin group than in the saline group ($P < 0.05$). At 15 and 20 days, sciatic functional index was significantly better in both mecobalamin groups than in the saline group ($P < 0.01$).

Mecobalamin contributed to sciatic nerve regeneration and prevents target muscle atrophy in mice

At 21 days, ultrathin sections of the crushed portion of the nerve were observed in two mice from each group under a transmission electron microscope. The crushed nerve in

Table 1 Real-time PCR oligonucleotide primers

Gene	Gene locus	Sequence (5′–3′)	Product size (bp)
GAPDH	NM 008084	Forward: GTG GCA AAG TGG AGA TTG TT Reverse: CCT CAC CCC ATT TGA TGT TA	195
GAP43	NM 008083	Forward: AGC TTC CGT GGA CAC ATA AC Reverse: TCG GTA GTA GCA GAG CCA TC	149
NGF	NM 013609	Forward: GCC TCA AGC CAG TGA AAT TA Reverse: AGA CAC TGA GGT GAG CTT GG	125
BDNF	NM 007540	Forward: CAA AGC CGA ACT TCT CAC AT Reverse: TTG TCC GTG GAC GTT TAC TT	220
CNTF	NM 170786	Forward: GCA AGG AAG ATT CGT TCA GA Reverse: TTG GTT AAC ATC CCT TGG AA	197

GAP43: Growth associated protein 43; NGF: nerve growth factor; BDNF: brain-derived nerve growth factor; CNTF: ciliary neurotrophic factor; GAPDH: glyceraldehyde phosphate dehydrogenase.

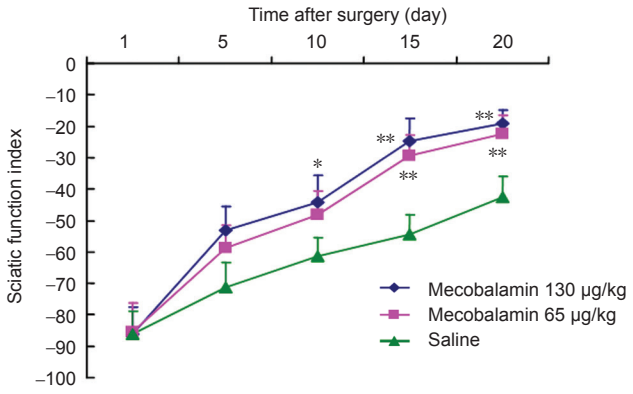

Figure 1 Walking track analysis after sciatic nerve crush.
Sciatic function index value at different time points after surgery in mice receiving daily intraperitoneal injections of saline or mecobalamin (65 or 130 μg/kg). Data are represented as the mean ± SD (n = 8). *P < 0.05, **P < 0.01, *vs.* saline group (one-way analysis of variance and Scheffe *post hoc* test).

animals that had received saline showed abundant axonal degeneration, and the regenerated myelinated nerve fibers were arranged sparsely and with thinned myelin. Abundant, densely arranged myelinated fibers with mature and thick myelin sheaths were observed in the mecobalamin groups (**Figure 2A–C**). The myelin sheath in regenerated myelinated nerve fibers was significantly thicker in the high-dose mecobalamin group than in the saline group (P < 0.05; **Figure 2D**).

At 21 days, hematoxylin-eosin staining of gastrocnemius muscle on the injured side in the saline group showed thin, widely-spaced myocytes, whereas in the high-dose mecobalamin group, muscle cells were plump and regularly arranged (**Figure 3A–C**). The cross-sectional areas of muscle cells in mice in the high-dose mecobalamin group were significantly greater than in the saline group (**Figure 3D**; P < 0.05).

Mecobalamin upregulates gene expression of growth associated protein 43 in nerve tissue, and of neurotrophic factors in the dorsal root ganglion

Ten days after surgery, growth associated protein 43 mRNA expression in the L_{4-6} segments of the crushed nerve was significantly greater in the high-dose mecobalamin group than in the saline group (P < 0.05), and remained elevated until 15 days postoperatively (P < 0.05). Nerve growth factor, brain-derived nerve growth factor and ciliary neurotrophic factor mRNA levels in ipsilateral dorsal root ganglia were also significantly greater in the high-dose mecobalamin group than in the saline group at 5 and 10 days (P < 0.05; **Figure 4**).

Discussion

Axonal regeneration is not always accompanied by functional motor and sensory recovery after peripheral nerve injury (Allodi et al., 2012; Daly et al., 2012). Mecobalamin, a coenzyme of vitamin B_{12}, promotes the metabolism of nucleic acids, proteins and lipids *via* a methyl conversion reaction. Mecobalamin readily enters nerve tissues and promotes restoration of injured nervous tissue, but the underlying mechanisms remain poorly understood (Matsushita et al., 2009).

In the present study, we used mouse models of sciatic nerve crush injury, and investigated the restorative effects of mecobalamin after peripheral nerve injury using behavioral and histomorphological analyses. We show that mecobalamin improved motor function after sciatic nerve injury, contributed to neural regeneration, and prevented target muscle atrophy. To date, few studies have explored the molecular mechanisms of mecobalamin in peripheral nerve regeneration. We therefore analyzed the expression of genes for a variety of proteins associated with nerve growth after daily mecobalamin injections in mouse models of peripheral nerve injury. Growth associated protein 43 is strongly associated with nervous system development and plasticity, and during neuronal development or after injury, levels of growth associated protein 43 expression are elevated up to a hundredfold (Chen et al., 2012). Significant upregulation of growth associated protein 43 expression contributes to the growth of nervous processes and activates neurite growth cone movement, and these changes are particularly evident during nerve regeneration (Zhou et al., 2009; Tsai et al., 2011; Zhang et al., 2014). Here, we demonstrated that mecobalamin significantly upregulates growth associated protein 433 mRNA levels in nervous tissue after sciatic nerve injury.

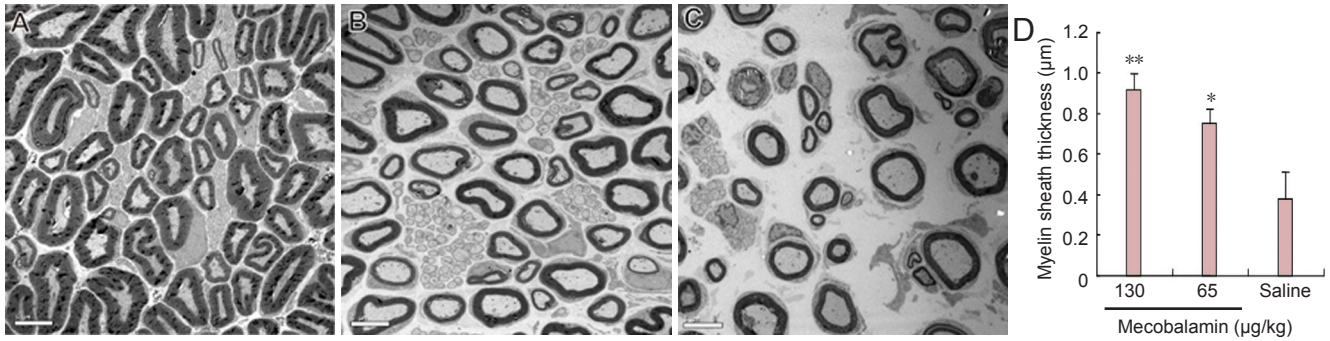

Figure 2 Transmission electron micrographs and quantification of myelinated fibers after sciatic nerve crush.
(A–C) Transmission electron micrographs of ultrathin sciatic nerve sections obtained 21 days after nerve crush surgery in mice that received daily intraperitoneal injections of 130 μg/kg mecobalamin (A), 65 μg/kg mecobalamin (B) or saline (C) for 21 days. Scale bars: 5 μm. (D) Statistical analysis of myelin sheath thickness in the three groups (data are represented as the mean ± SD). $*P < 0.05$, $**P < 0.01$, *vs.* saline group (one-way analysis of variance and Scheffe *post hoc* test).

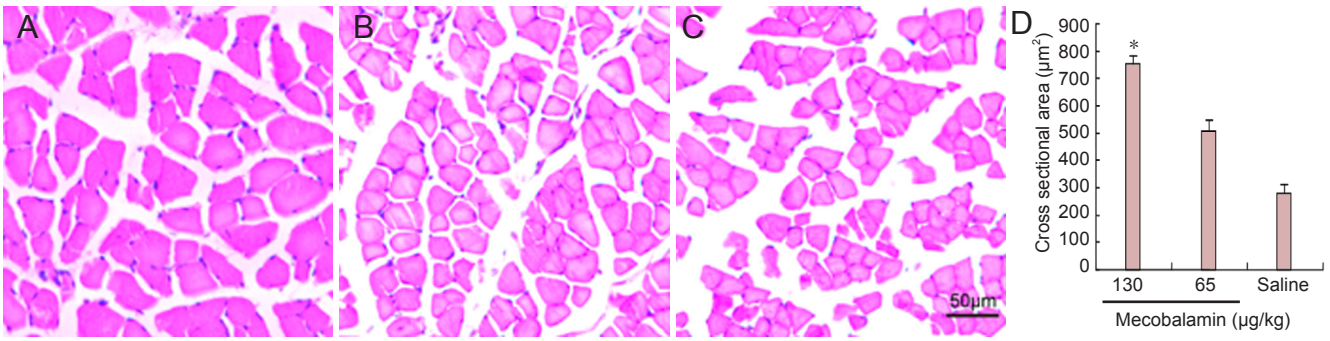

Figure 3 Hematoxylin-eosin staining and cross-sectional area analysis of gastrocnemius muscle fibers after sciatic nerve crush.
(A–C) Representative light micrographs of transversely sectioned gastrocnemius muscle ipsilateral to the nerve injury in mice that received daily intraperitoneal injections of 130 μg/kg mecobalamin (A), 65 μg/kg mecobalamin (B) or saline (C) for 21 days after surgery. Scale bar: 50 μm. (D) Statistical analysis of the cross-sectional area of the fibers (data were represented as the mean ± SD; $n = 8$). $*P < 0.05$, *vs.* saline group (one-way analysis of variance and Scheffe *post hoc* test).

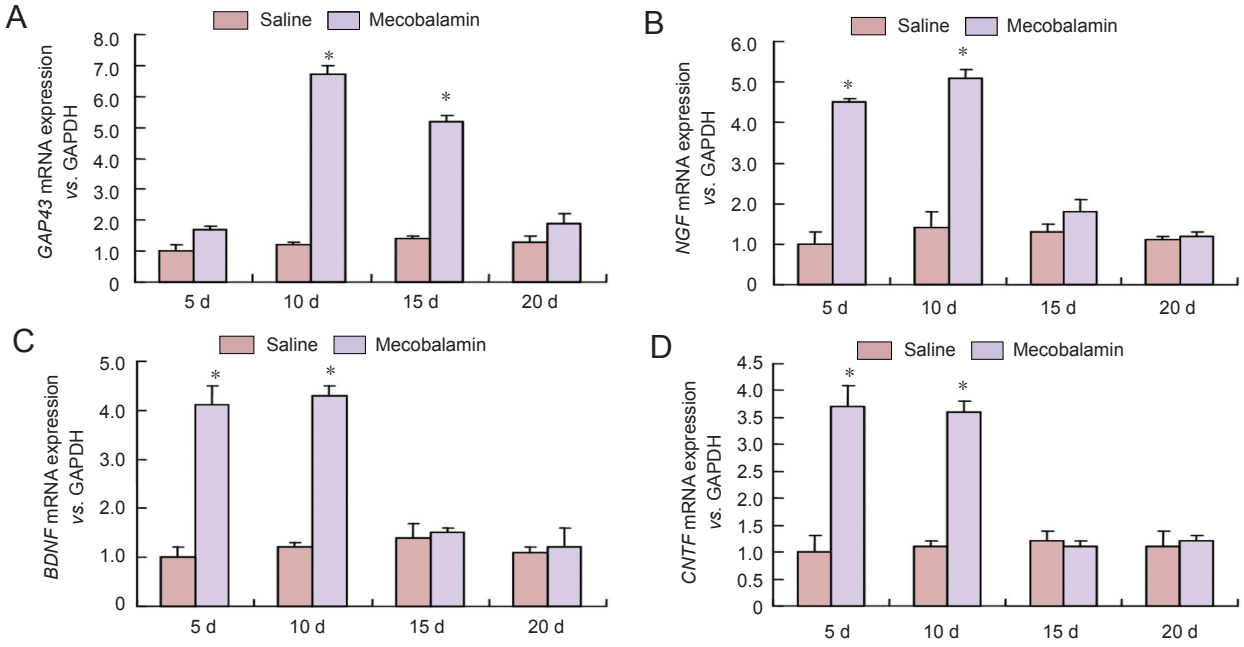

Figure 4 The mRNA expression in crushed nerve and L_{4-6} dorsal root ganglia (real-time RT-PCR) 5, 10, 15 and 20 days after surgery.
The mRNA expression of *GAP43* in the crushed nerve (A), and *NGF* (B), *BDNF* (C) and *CNTF* (D) in the dorsal root ganglia, after intraperitoneal injections of high-dose mecobalamin (130 μg/kg) or saline, following sciatic nerve crush. Data are represented as the mean ± SD ($n = 3$). $*P < 0.05$, *vs.* saline group (one-way analysis of variance and Scheffe *post hoc* test). GAP43: Growth associated protein 43; NGF: nerve growth factor; BDNF: brain derived nerve growth factor; CNTF: ciliary neurotrophic factor; GAPDH: glyceraldehyde phosphate dehydrogenase; d: day.

Dorsal root ganglia contain cell bodies of peripheral nerves, and are readily cultured *in vitro* (Saijilafu and Zhou, 2012). Cultures of dorsal root ganglia are frequently used to study the growth, development and regeneration of neurons in the peripheral nervous system (Johnson and Sears, 2013). An *in vitro* study highlighted the sensitivity of neuronal survival and regeneration in the dorsal root ganglion to neurotrophic factors, such as nerve growth factor, brain-derived nerve growth factor and ciliary neurotrophic factor (Atlasi et al., 2009; Xiao, 2009). Over a third of dorsal root ganglion neurons with broken axons die (Burland et al., 2014). After peripheral nerve injury, neurotrophic factor expression is upregulated in adult mammals, but the response is very slow (Wan et al., 2010; Saleh et al., 2013; Xu et al., 2013), beginning 7 days after injury (Grumbles et al., 2009; Ziv-Polat et al., 2014). Accordingly, axonal regeneration at the proximal end of the injured nerve stump is also slow (Cui, 2006; Grumbles et al., 2009), requiring additional measures for adequate neuronal survival. Although neurotrophic factors promote the survival and regeneration of neurons, their purification for clinical use is complicated and expensive, and clinical outcomes remain debated (Rizos et al., 2014; Wang et al., 2014b). In the present study, we have shown that the use of 130 μg/kg mecobalamin for 5 days after nerve injury upregulates gene expression of nerve growth factor, brain-derived neurotrophic factor and ciliary neurotrophic factor in the L_{4-6} segments of the ipsilateral spinal cord dorsal root ganglion.

In summary, mecobalamin promotes functional and morphological recovery after peripheral nerve injury. The molecular mechanism underlying the restorative effects of mecobalamin on injured nerves may involve upregulation of the genes for multiple neurotrophic factors. The signaling pathway through which mecobalamin acts to promote peripheral nerve regeneration remains to be investigated in the future.

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Author contributions: *Gan L and Zhu GX conceived and designed the study. Gan L, Qian MQ, Shi KQ, and Chen G collected the data. Du W and Zhu GX revised the manuscript. Gan L wrote the paper. All authors approved the final version of the manuscript.*

Conflicts of interest: *None declared.*

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