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7, 8-dihydroxycoumarin improves neurological function in a mouse model of sciatic nerve injury☆

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Abstract

In the present study, a mouse model of sciatic nerve injury was treated with intraperitoneal injection of 7, 8-dihydroxycoumarin (10, 5, or 2.5 mg/kg per day). Western blot and real-time PCR results showed that growth associated protein 43 expression was significantly increased in the L_{4-6} segments of the spinal cord. The amplitude and velocity of motor nerve conduction in the sciatic nerve were significantly increased in model mice. In addition, the appearance of the myelin sheath in the injured sciatic nerve was regular, with an even thickness and clear outline, and the surrounding fibroplasia was not obvious. Our results indicate that 7, 8-dihydroxycoumarin can promote the repair of injured nerve by upregulating growth associated protein 43 expression in the corresponding spinal cord segments of mice with sciatic nerve injury.

Key Words: 7, 8-dihydroxycoumarin; growth associated protein 43; sciatic nerve; peripheral nerve injury; neural regeneration

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INTRODUCTION

After cerebral ischemia/reperfusion in rats, growth associated protein 43 (GAP-43) expression gradually increased in the ischemic hemisphere, indicating the repairing effects of GAP-43 in nerve injury[1-3].
A previous study indicated that 7, 8-dihydroxycoumarin, an effective monomer isolated from thymelaeceae, can protect the cardiovascular system and permeate the blood-brain barrier[4]. 7, 8-dihydroxycoumarin can increase GAP-43 expression in ischemia/reperfusion regions in the early stage after ischemia and play a positive role in neuronal function recovery by removing necrotic substances, improving the water-electrolyte balance, enhancing neurotransmitter and energy metabolism, and secreting neurotrophic factors[4-6]. However, the effects of 7, 8-dihydroxycoumarin treatment on peripheral nerve regeneration remains poorly understood.
The present study used a BALB/c mouse model of sciatic nerve injury to investigate the role of 7, 8-dihydroxycoumarin in peripheral nerve regeneration and functional recovery.

RESULTS

Quantitative analysis of experimental animals

A total of 169 BALB/c mice were studied. Nine died due to anesthetic accidents, while the remaining mice were used to establish models of unilateral sciatic nerve injury. The model mice were randomly assigned to injury or high, medium or low dose 7, 8-dihydroxycoumarin groups, with 40 animals in each group. The mice were intraperitoneally injected with 1 mL per day normal saline, or 10, 5 or 2.5 mg/kg per day 7, 8-dihydroxycoumarin, respectively. Totally 160 mice were included in the final analysis.

7, 8-dihydroxycoumarin upregulated GAP-43 protein expression in the L_{4-6} segments of the injured side in mice with sciatic nerve injury

Western blot detection showed that GAP-43 reached peak levels at 1 week after injury, which gradually decreased after 1 week, and finally decreased to normal levels at 2 weeks[7-10]. High, medium and low dose 7, 8-dihydroxycoumarin increased GAP-43 protein expression in the L_{4-6} segments compared with the injury group ($P < 0.05$; Figure 1). GAP-43 protein expression was greater in the high and medium dose 7, 8-dihydroxycoumarin groups compared with low dose 7, 8-dihydroxycoumarin except for the high dose 7, 8-dihydroxycoumarin group at 8 weeks ($P < 0.05$; Table 1).

7, 8-dihydroxycoumarin upregulated GAP-43 mRNA expression in the L_{4-6} segments of the injured side in mice with sciatic nerve injury

There was only a small amount of GAP-43 mRNA in the corresponding spinal cord segments of sciatic nerve in normal Balb/c mice[10-15]. Real-time PCR results in this study showed that the GAP-43 mRNA level

increased in the corresponding spinal cord segments after sciatic nerve injury.

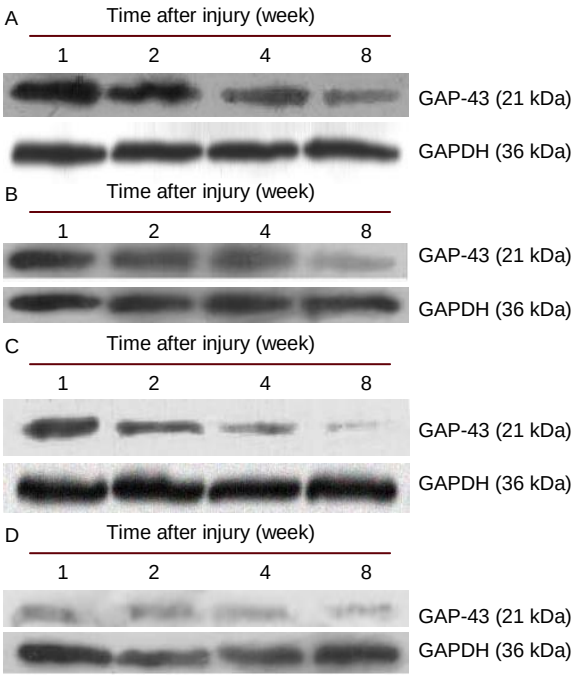


Figure 1 Growth associated protein 43 (GAP-43) expression in the L_{4-6} segments of mice with sciatic nerve injury. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

High (A), medium (B) and low (C) dose 7, 8-dihydroxycoumarin increased GAP-43 protein expression in the L_{4-6} segments compared with the injury group (D).

Table 1 Growth associated protein 43 (GAP-43) expression in the L_{4-6} segments of mice with sciatic nerve injury treated with 7, 8-dihydroxycoumarin (Western blot)

Group	Time after injury (week)	
	1	2
7, 8-dihydroxycoumarin		
High dose	0.593±0.021[ab]	0.461±0.032[ab]
Medium dose	0.512±0.020[ab]	0.432±0.019[ab]
Low dose	0.413±0.036[a]	0.342±0.040[a]
Injury	0.268±0.029	0.201±0.026

Group	Time after injury (week)	
	4	8
7, 8-dihydroxycoumarin		
High dose	0.415±0.031[a]	0.214±0.038[ab]
Medium dose	0.371±0.007[ab]	0.212±0.024[ab]
Low dose	0.317±0.029[a]	0.120±0.026
Injury	0.193±0.021	0.123±0.023

[a]$P < 0.05$, *vs*. injury group; [b]$P < 0.05$, *vs*. low dose 7, 8- dihydroxycoumarin group. The results are represented by the absorbance ratio of the target protein to GAPDH. The results are expressed as mean ± SD of 10 mice in each group. Intergroup differences were compared using one way analysis of variances and two-sample *t*-tests. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Also, the GAP-43 mRNA level was significantly greater in the high dose 7, 8-dihydroxycoumarin group compared with the medium and low dose 7, 8-dihydroxycoumarin groups at 1, 2 and 4 weeks after injury ($P < 0.05$; Figure 2).

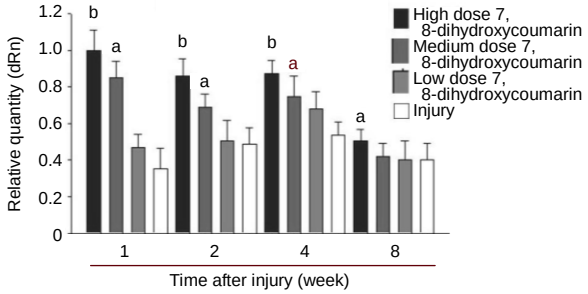


Figure 2 Relative quantity of growth associated protein 43 (GAP-43) mRNA expression 1 to 8 weeks after sciatic nerve injury in mice treated with 7, 8-dihydroxycoumarin. [a]$P < 0.05$, [b]$P < 0.01$, *vs*. injury group. The results are expressed as the mean ± SD of 10 mice in each group. Intergroup differences were compared using one way analysis of variances and two-sample *t*-tests.

7, 8-dihydroxycoumarin promoted neurological function in mice with sciatic nerve injury

Electrophysiological detection showed that high and medium dose 7, 8-dihydroxycoumarin increased the amplitude and velocity of motor nerve conduction in the sciatic nerve at 1, 2, 4, and 8 weeks after injury ($P < 0.05$), but low dose 7, 8-dihydroxycoumarin did not (Tables 2, 3).

Table 2 The amplitude of motor nerve conduction in mice with sciatic nerve injury (mV) treated with 7, 8-dihydroxycoumarin (neuroelectrophysiology method)

Group	Time after injury (week)	
	1	2
7, 8-dihydroxycoumarin		
High dose	2.08±0.21[ab]	4.42±0.15[ab]
Medium dose	2.07±0.18[ab]	4.29±0.17[ab]
Low dose	1.32±0.13	2.51±0.05
Injury	1.19±0.06	2.17±0.14

Group	Time after injury (week)	
	4	8
7, 8-dihydroxycoumarin		
High dose	24.98±0.15[ab]	26.11±0.91[ab]
Medium dose	21.28±0.23[ab]	22.14±0.29[ab]
Low dose	16.66±0.30	18.71±0.30
Injury	12.19±0.06	16.78±0.71

[a]$P < 0.05$, *vs*. injury group; [b]$P < 0.05$, *vs*. low dose 7, 8- dihydroxycoumarin group. The results are expressed as mean ± SD of 10 mice in each group. Intergroup differences were compared using one way analysis of variances and two-sample *t*-tests.

7, 8-dihydroxycoumarin improved the histological changes in the injured region in mice with sciatic nerve injury

Luxol fast blue staining showed that at 8 weeks after injury, the appearance of the myelin sheath in the injured

sciatic nerve was regular, with an even thickness and clear outline, and the surrounding fibroplasias was not obvious in the high and medium dose 7, 8-dihydroxycoumarin groups. In the low dose 7, 8-dihydroxycoumarin group, the appearance and thickness of the myelin sheath was irregular, but the outline was clear, and fibrous connective tissue hyperplasy was observed. In the injury group, the appearance of the myelin sheath was irregular and fibrous connective tissue hyperplasy was observed (Figure 3).

Table 3 The velocity of motor nerve conduction in mice with sciatic nerve injury (m/s) treated with 7, 8-dihydroxycoumarin

Group	Time after injury (week)	
	1	2
7, 8-dihydroxycoumarin		
High dose	19.96±0.32[ab]	41.43±0.57[ab]
Medium dose	18.73±0.31[ab]	34.22±0.72[a]
Low dose	12.88±0.19	31.98±0.89[a]
Injury	10.41±0.18	26.71±0.56

Group	Time after injury (week)	
	4	8
7, 8-dihydroxycoumarin		
High dose	65.31±1.58[ab]	64.11±1.42[ab]
Medium dose	54.39±1.13[ab]	59.29±0.63[ab]
Low dose	51.07±0.63	52.86±0.29[a]
Injury	48.22±0.29	44.18±2.44

[a]$P < 0.05$, *vs*. injury group; [b]$P < 0.05$, *vs*. low dose 7, 8- dihydroxycoumarin group. The results are expressed as mean ± SD of 10 mice in each group. Intergroup differences were compared using one way analysis of variances and two-sample *t*-tests.

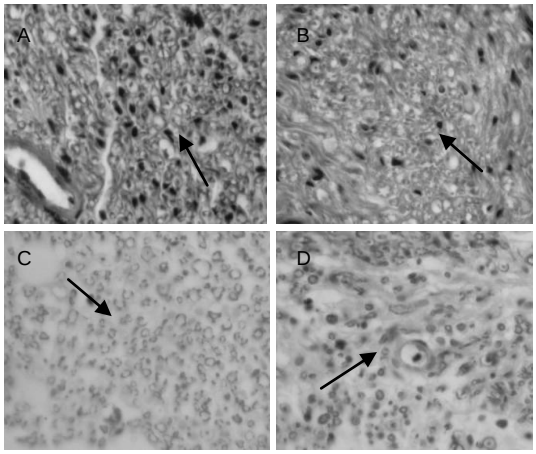


Figure 3 The morphology of sciatic nerve samples in the injured side of mice in high (A), medium (B), low (C) dose 7, 8-dihydroxycoumarin, and injury groups (D) at 8 weeks after injury (Luxol fast blue staining, × 40).

The number of myelinated nerves differed in each group, which corresponded to a variety of color intensities. Arrows represented myelin sheath, and the axons did not stain.

At 8 weeks after injury, the number and diameter of myelinated nerve fibers in the injured region of sciatic nerve was greater in the high and medium dose 7, 8-dihydroxycoumarin groups compared with the low dose 7, 8-dihydroxycoumarin and injury groups ($P < 0.05$; Table 4).

Table 4 The number and diameter of myelinated nerve fibers in the injured region of the sciatic nerve of each group

Group	Number of myelinated nerve fiber (n/mm^2)	Diameter of myelinated nerve fiber (μm)
7, 8-dihydroxycoumarin		
High dose	75±3[ab]	2.47±0.31[ab]
Medium dose	71±1[ab]	2.31±0.16[ab]
Low dose	56±4[a]	1.99±0.32[a]
Injury	50±2	1.57±0.26

[a]$P < 0.05$, *vs*. injury group; [b]$P < 0.05$, *vs*. low dose 7, 8- dihydroxycoumarin group. The results are expressed as the mean ± SD of 10 mice in each group. Intergroup differences were compared using two-sample *t*-tests.

DISCUSSION

Natural drugs exhibit particular advantages in neural regeneration. In previous studies, 7, 8-dihydroxycoumarin has been used for brain nerve and nerve recovery after cerebral ischemia/reperfusion injury[16-18]. Western blot and real-time PCR in the present study showed that high and medium dose 7, 8-dihydroxycoumarin significantly increased GAP-43 expression compared with the low dose 7, 8-dihydroxycoumarin and injury groups, and the increased GAP-43 expression was maintained for up to 2 weeks. GAP-43 protein and mRNA expressions increased in the L_{4-6} segments, Luxol fast blue staining of the myelin sheath demonstrated improvements and the electrophysiological index exhibited consistent change tendency with 7, 8-dihydroxycoumarin treatment. We conclude that 7, 8-dihydroxycoumarin promotes GAP-43 activation in the spinal cord anterior horn to regulate functional recovery after peripheral nerve injury[14-15]. GAP-43 protein expression is a sensitive and specific biochemical marker of central lesions. GAP-43 expression in a certain range can promote nerve recovery and regeneration, and overexpression can inhibit inflammation and scar formation[19-29]. In the present study, high and medium dose 7, 8-dihydroxycoumarin significantly increased GAP-43 expression. Furthermore Luxol fast blue staining of the myelin sheath and electrophysiological examination indicate high and medium dose 7, 8-dihydroxycoumarin promoted nerve recovery and regeneration. These results suggest high and medium dose 7, 8-dihydroxycoumarin promote GAP-43 expression in an appropriate range[7-10] and regulate peripheral nerve generation.

In conclusion, 7, 8-dihydroxycoumarin can improve functional recovery of mice with sciatic nerve injury.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiment was performed at the National Key Laboratory of China-Japan Union Hospital, Jilin University, China from November 2010 to May 2011.

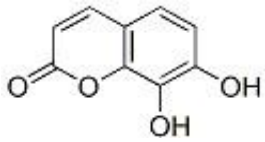
Materials

Animals

A total of 160 healthy, adult, male BALB/c mice, aged 8 weeks, weighing 20 ± 2 g, were provided by the Laboratory Animal Center of the College of Basic Medicine, Jilin University (No. SCXK(Ji)2007-0001). Experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals* issued by the Ministry of Science and Technology of China[30].

Drugs

Daphnetin (95.5% 7, 8-dihydroxycoumarin) was provided by Jilin Xidian Pharmaceutical Technology Development, China. Daphnetin samples were identified using high performance liquid chromatography[31]. The relative molecular mass was 178.141 5 and the chemical structural formula was as follows:



The daphnetin powder was weighed on an analytical balance (Shanghai Keda Instrument Factory) and dissolved in normal saline. The 7, 8-dihydroxycoumarin solution was filtered through a 0.02 mm micropore membrane and stored at −20°C. The 7, 8-dihydroxycoumarin solution prepared and stored in this manner is stable for 1 month.

Methods

Establishment of the sciatic nerve injury model

The mice were anesthetized by intraperitoneal injection of 1% penthiobarbital sodium (100 mg/kg) and fixed in the prone position. A 2 cm longitudinal incision was made at the posterior femur of the hind limb at the injured side, and the sciatic nerve was exposed. The sciatic nerve trunk and surrounding tissues were isolated. The sciatic nerve at 0.5 cm below ischial tuberosity was completely excised. The sciatic nerve was anastomosed using a 11/0 micro suture under a 12 × magnification microscope (Zhenjiang Surgical Microinstrument Factory, Jiangsu, China), and the muscle and skin were sutured layer by layer[29] (Figure 4). Successful models were demonstrated by nerve end-to-end anastomosis and smooth anastomotic stoma.

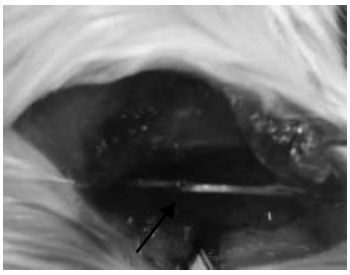


Figure 4 The establishment of the sciatic nerve injury model. The arrow represents the end-to-end anastomosis of excised nerves.

Intervention

The clinically used dose of 7, 8-dihydroxycoumarin was converted into a comparable dose for intraperitoneal injection in mice[32], and was labeled as the medium dose of 7, 8-dihydroxycoumarin in the present study. The high, medium and low dose 7, 8-dihydroxycoumarin and injury groups were respectively intraperitoneally injected with 10, 5 or 2.5 mg/kg reconstituted in 1 mL aliquots per day 7, 8-dihydroxycoumarin[32] or 1 mL per day normal saline, immediately after injury. The administration was not terminated until sampling.

Sampling

At 1, 2, 4 and 8 weeks after injury, 10 mice were selected from each group and anesthetized by intraperitoneal injection of 1% pentobarbital sodium (100 mg/kg). The vertebral canal was opened using gouge forceps through the median incision at the posterior vertebral column to expose and dissociate the L_{4-6} segments connected with the injured sciatic nerve (Figure 5). The spinal cord at the L_{4-6} segments of the injured side was harvested, placed in a marked frozen tube, and immersed in liquid nitrogen for Western blot and real-time PCR. In addition, the nerve trunk, 0.5 cm distal to the anastomotic stoma (including the stoma) was harvested, fixed in 10% neutral formalin for over 72 hours, dehydrated with gradient alcohol, paraffin embedded, and sectioned (2 μm thick).

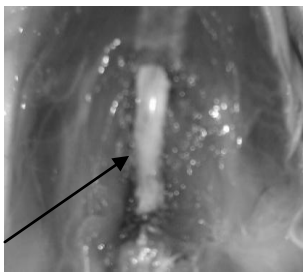


Figure 5 The samples of the L_{4-6} segments of the spinal cord. The arrow represents the lumbar intumescentia of L_{4-6}.

Western blot for GAP-43 protein expression in the L_{4-6} segments of the injured side in mice with sciatic nerve injury

The spinal cord tissues were rapidly harvested from

liquid nitrogen and ground by a mortar. These tissue fragments were mixed with radio-immunoprecipitation assay lysis buffer to extract the protein, then loading buffer was added, followed by boiling for 15 minutes. After centrifugation, the supernatant was harvested for 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein was transferred to a polyvinylidene difluoride membrane. The membrane was soaked in a rabbit anti-mouse GAP-43 monoclonal antibody (1: 1 000; Roche, Nutley, NJ, USA) overnight at 4°C and washed with 0.01 M PBS for 5 minutes 4 times. The membrane was then immersed in a goat anti-rabbit IgG antibody (1: 10 000; Roche) at room temperature for 1 hour and then washed with 0.01 M PBS for 5 minutes 4 times, followed by enhanced chemiluminescence (Roche). The bands were scanned and analyzed. A gel imaging system (Alpha-Innotech, San Leandro, CA, USA) was used for absorbance analysis, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as an internal reference. The absorbance ratio of the target band to GAPDH was measured. A high ratio indicates strong protein expression.

Real-time PCR for GAP-43 mRNA expression in L_{4-6} segments of the injured side in mice with sciatic nerve injury

Primers were designed using Beacon designer 7 software (Premier Biosoft, California, USA).

Primer	Sequence (5'–3')	Product size (bp)
GAP-43	Upstream: GCC TAA ACA AGC CGA TGT GC Downstream: TTC GTC TAC AGC GTC TTT CTC C Probe: TGC TGC TGT CAC TGA TGC TGC TGC	276
GAPDH	Upstream: AAT GTG TCC GTC GTG GAT CTG Downstream: CAA CCT GGT CCT CAG TGT AGC Probe: CGT GCC GCC TGG AGA AAC CTG CC	462

Specificity was verified using the Blast test (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Spinal cord tissues of L_{4-6} segments at each time point were harvested. Total RNA was extracted using the TRIzol reagent (Shanghai Sangon, Shanghai, China). RNA was reverse-transcribed into cDNA. With cDNA as template, GAP-43 at L_{4-6} segments was amplified by PCR. One pair of GAPDH primers was added in every reaction system and used as an internal reference. The amplification conditions were 40 cycles of 95°C for 30 seconds, 58°C for 60 seconds and 72°C for 60 seconds. The threshold cycle value of the target gene and the internal reference gene was determined and used to quantify the target gene and plot histogram (SigmaPlot 8.0, Bio-Rad, Hercules, CA, USA).

Myelin sheath luxol fast blue staining for neural regeneration after sciatic nerve injury

The nerve trunk, 0.5 cm distal to the anastomotic stoma (including the stoma) was harvested. Then the nerve trunk was fixed in 10% neutral formalin for over 72 hours, dehydrated with gradient alcohol, paraffin embedded, and sectioned for hematoxylin-eosin staining to observe structures, cell proliferation, and inflammatory cell infiltration. After dewaxing, the sections were immersed in luxol fast blue solution at 60°C for 12 hours, mixed with 95% alcohol for 5 minutes, treated with 0.05% lithium carbonate for 15 seconds, washed with 70% alcohol and distilled water, dehydrated, cleared and mounted. The sections were observed by light microscopy (Olympus, Tokyo, Japan) and the number of axons and the diameter stained by lithium carbonate were determined by an image scanner (40 ×, Olympus).

Nerve action potential conduction velocity of the injured side in mice with sciatic nerve injury

The injured sciatic nerve was detected at 1, 2, 4, and 8 weeks after injury using a Medtronic Keypoint myoelectricity/evoked potential system (Medtronic, Minneapolis, Minnesota, USA). Briefly, the room temperature was maintained at 24°C. The mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium, sterilized, and placed in a prone position. The sciatic nerve was exposed. A concentric needle electrode was punctured into the soleus muscle as the recording electrode (M point), and the ground electrode was placed in the tail. Parallel stimulating electrodes (with a 2 mm gap) were respectively placed at the ischial tuberosity proximal to the anastomotic stoma of the injured nerve (P point) and the bifurcation of the distal sciatic nerve (D point). The mice were stimulated with a 10 mA current. The interelectrode distance of stimulating electrodes was measured using a Vernier caliper and motor nerve conduction velocity was calculated as tge: interelectrode distance/difference in action potential latency (duration that the nerve impulse passed through two points, *i.e*. the motor conduction velocity between two points). The amplitude of the action potential was detected using a Vernier caliper.

Statistical analysis

The data were expressed as the mean ± SD and analyzed using the SPSS 17.0 software (SPSS, Chicago, IL, USA). The differences in the mean value between groups were compared using a one way analysis of variance; paired comparisons of the mean value between groups were performed using a two-sample *t*-test.

Author contributions: Qing Zhao was in charge of funds, provided and integrated the data, and conceived and designed the study. Jianshi Du integrated the data and revised the manuscript. Yingli Zhang analyzed the data. Qing Zhao and Jianshi Du drafted the manuscript and guided the study. Yu Wang contributed to statistical analysis. Ming Ma provided technical and data support.

Conflicts of interest: None declared.
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Ethical approval: This study received permission from the Animal Ethics Committee of Jilin University, China.

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