Original Article

Proprioceptive changes measured by histopathological and electrophysiological evaluations after NGF injection of anterior cruciate ligament reconstruction

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Background: Proprioceptive recovery has received an increased amount of attention after undergoing anterior cruciate ligament (ACL) reconstruction. However, ACL reconstruction without rehabilitation training could not significantly improve the reduction and function of the proprioceptor. This study aimed to explore whether nerve growth factor (NGF) could cause proprioceptive changes after ACL reconstruction through histopathological and electrophysiological evaluations.

Methods: A total 28 mature New Zealand white rabbits were used in the study, 24 to develop the model of ACL injury and ACL reconstruction. These included the experimental group (n=12; injected with NGF 20 μg/week at the second month after surgery) and the experimental control group (n=12), and 4 for blank control group. In the 4th, 8th, and 12th months, the changes in ACL nerves were measured by somatosensory evoked potentials (SEPs) and electromyogram (EMG). Furthermore, gold chloride staining was performed to detect the changes in morphology and quantity of the proprioceptors in ACL.

Results: Electrophysiological tests showed that the incubation of SEPs and EMG in both the experimental group and the experimental control group were prolonged, and the amplitude decreased when compared with the blank control group (all, $P<0.05$). Also, in comparison to the experimental control group, the experimental group injected with NGF had a shorter incubation and higher amplitude (all, $P<0.05$). Furthermore, histopathology analysis revealed that the number of proprioceptors in the experimental group injected with NGF was significantly increased, and the atypical structure was reduced (all, $P<0.05$).

Conclusions: The results showed the injection of NGF (injected with 20 μg/week in the second month after surgery) could improve joint function rehabilitation by promoting function and quantity of proprioception after ACL reconstruction.

Keywords: Nerve growth factor (NGF); rabbit; knee; anterior cruciate ligament (ACL); reconstruction, proprioception

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Introduction

An anterior cruciate ligament (ACL) is one of the major ligaments that, with resistance to anterior tibia deformation and rotational load (1), and its injury could result in balance disorder and proprioceptive dysfunction (2,3). Proprioception has been reported to be composed of incoming and outgoing pathways of somatosensory systems that control the reflexes and muscle tone of muscles contributing to regulating the preciseness of the articular angles of the knee joint (4). Consequently, proprioceptive dysfunction should also be assessed after ACL reconstruction surgery. Currently, there are few treatment methods, including reconstructing ACL and rehabilitation exercise to reconstruct the proprioception, applied to the reconstruction of ACL injury proprioception (1,5). However, several disadvantages have been presented, suggesting that ACL reconstruction or several months of rehabilitation might not result in improvement proprioception due to limitations such as the lack of ACL residue or uncertainty of efficacy along with long recovery time (6).

Nerve growth factor (NGF) is a neurotrophic factor released by mast cells, lymphocytes, and monocytes/macrophages in response to tissue inflammation and nociception (7). As a small secretory protein, NGF has been reported to play important roles in nerve cell proliferation and function (8). Moreover, NGF engages in the differentiation and survival of specific target neurons contributing to the maintenance and development of the sensory nervous system (9,10). According to the principle of osseoperception, it may be effective for promoting proprioception of the dental implant to induce peri-implant nerve regeneration, and He *et al.* (11) has revealed that NGF stimulates nerve regeneration and bone formation by its biological activities on both neuronal and non-neuronal cells. But until now, the effect of NGF on proprioceptive function after ACL reconstruction has not been reported.

In this study, we examined the role of NGF in the proprioceptive system following ACL injury rehabilitation, changes in the somatosensory evoked potentials (SEPs) and electromyograms (EMG), as well as the morphology and quantities of proprioceptors in white New Zealand rabbits after ACL injury injection.

Methods

Study design and research equipment

A total of 28 mature New Zealand white rabbits, weighing 1,500–2,500 g, were used in this study. The rabbits were randomized into 3 groups: the blank control group (n=4), the experimental group (n=12), and the experimental control group (n=12). The Laboratory Animal Center approved this study of the Gansu University of Chinese Medicine, and animal care was following the "Guide for the Care and Use of Laboratory Animals".

The research equipment were as following: rotary type slicer (RM2135 type, LEICA company), optical microscope (BH2, push around), induce voltmeter (photoelectric MEB2200 type, Japan), and muscle all (photoelectric MEB2200 type, Japan), electronic balance (AR1530/C, OHAUS companies in the United States), 81-2 type constant temperature magnetic stirrer (Shanghai music instrument factory), PHS-3 ph meter (Shanghai Yi instrument factory), portable pressure steam sterilizer (Jiangyin Binjiang medical equipment factory), automatic double distilled water machine (Shanghai Broadcom), electrothermal blowing (Chongqing Sida experimental apparatus), ultra-low temperature freezer (ULT1386-3 v, the GS), BH-type 2 biological microscope.

Surgical technique

The treatment of the three groups is briefly summarized in *Table 1*. Specifically, rabbits were anesthetized using 3% pentobarbital (1 mL/kg). After proper anesthesia, the rabbits were fixed in the supine position with shaved skin on the surgical area and sterilized. First, the operators marked the skin incision in the center of the knee and the iliac medial side and removed the fascia from the muscle. Next, it was washed with 30°C sterile normal saline, 3/4 of the ACL was cut off, leaving 1/4 of the ACL connecting to the femur and tibia. The wound was closed, disinfected, and covered with penicillin powder. Three days after the operation, 1×10^4 penicillin injection (1 time/24 h, IV) was used to prevent infection.

The anterior tibial tendon and periosteum of the medial tibia (length ~1.5 cm, diameter ~0.5 cm) were excised and used as the tendon graft. Next, the tendon was woven to 3 cm with the non-absorbable line 1, and the femoral head was 3 mm in diameter and 4 mm in the tibia. The graft was pre-tensioned manually with 20 N and was then fixed with the doornail and non-absorbable line 1 outside of the femoral tunnel and tibial tunnel. The wound was cleaned, sutured and pressed by the elastic bandage. All surgery was performed under general anesthesia, as well as all the operations were to minimize the suffering during

Table 1 The treatment of distinct groups

Groups	No. New Zealand white rabbits	Treatment	
		ACL injury and reconstruction	Injection of NGF
Blank control group	4	None	None
Experimental group	12	Yes	None
Experimental control group	12	Yes	20 μg/week

ACL, anterior cruciate ligament; NGF, nerve growth factor.

the operation. After the 2nd month of the operation, NGF (20 μg/week; N2513 Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was injected into the bilateral knee joint of the rabbits in the experimental group.

Histopathological evaluation

At the 2nd, 4th, and 6th months of ACL reconstruction, 4 rabbits were randomly obtained from the experimental group and control group, respectively. SEPs and EMG were performed for electro neurophysiological detection. Accordingly, the rabbits were executed by air embolism, and then the ligaments of the rabbits were removed and stained with gold chloride.

Electrophysiological evaluation

Under inhaling ether and local anesthesia in 1% lidocaine, the head and limbs of rabbits were fixed. After exposing the ACL, the electrodes were placed between the posterior margin of the two orbiters and the median sagittal line at 0.5 and 0.2 cm by the side, which was the sensory area of the lateral posterior extremity. Moreover, the reference electrode was placed under the subcutaneous of the nasal root, and the proximal end of the lower limb was grounded on the electrode. SEPs were measured at 26–28 ℃, and the stimulation was constant at an intensity of 18–20 V with a single square wave (the wave width was 0.1 ms). Next, SEPs were recorded using the photoelectric evoked potentiometer, and the information was input to the microcomputer operating system.

Biostatistical evaluation

For EMG examination, the electrode was placed in the hamstring muscle, and the reference electrode was placed in malleolus medialis. The bipolar surface electrode was placed at the ACL adherent at 26–28 ℃. The stimulation parameters were constant with the wave width at 0.1 ms and the frequency at 3 Hz. Next, EMG was monitored and recorded using the photoelectric electromyography, and the waveform and latency of EMG were analyzed.

Gold chloride staining

Firstly, the ACLs of all rabbits were obtained completely and soaked in fresh lemon juice and 88% acid mixture at room temperature for 15 min. Secondly, ACLs were placed in 1% gold chloride solutions for 30 min, and then 25% formic acid solution was added for 15 h. After washed by the distilled water for 1 h, the ACLs were put in the pure glycerin for 24 h in order. Next, tissue blocks were dehydrated with alcohol gradient, cleared in xylene, embedded in paraffin, and filleted at the thickness of 15 μm. For avoiding repetition, the femoral head, tibia end, and middle part of the specimens were marked and two operators respectively counted the number of proprioceptors.

Statistical analysis

All data were presented as mean ± SD, and the statistical analysis was performed by SPSS 13.0 software. One-way ANOVA was used to compare groups, and the normal test was performed using the Kolmogorov-Smirnov (K-S) test. A $P<0.05$ was statistically significant.

Results

Compared with the blank control group, the incubations of SEPs and EMG were both extended in the experimental group and the experimental control group at 2nd, 4th, and 6th months, respectively. Moreover, incubations of SEPs and EMG in the experimental group at 4th and 6th months were significantly decreased when compared to those in the experimental control group (all, $P<0.05$;

Table 2 The comparison among the three groups about the incubation and wave crest of SEPs in different detecting time

Index	Experimental group			Experimental control group			Blank control group
	2nd month	4th month	6th month	2nd month	4th month	6th month	
Incubation	22.41±2.96$^{\gamma}$	23.61±2.31$^{\gamma,\delta}$	20.19±1.95$^{\alpha,\beta,\gamma,\delta}$	21.36±3.32$^{\gamma}$	25.47±3.26$^{\alpha,\gamma}$	30.03±3.59$^{\alpha,\beta,\gamma}$	14.6±1.87
Amplitude	14.33±3.17$^{\gamma}$	13.27±2.02$^{\gamma,\delta}$	16.28±3.06$^{\alpha,\beta,\gamma}$	13.97±3.21$^{\gamma}$	6.35±1.27$^{\alpha,\gamma}$	6.13±1.54$^{\alpha,\gamma}$	21.09±3.05

$^{\alpha}$, P<0.05 versus 2nd month in the same group; $^{\beta}$, P<0.05 versus 4th month in the same group; $^{\gamma}$, P<0.05 versus the blank control group at the same time; $^{\delta}$, P<0.05 versus the experimental control group at the same time. SEP, somatosensory evoked potential.

Table 3 The comparison among the three groups about the incubation and amplitude of EMG in different detecting time

Index	Experimental group			Experimental control group			Blank control group
	2nd month	4th month	6th month	2nd month	4th month	6th month	
Incubation	12.79±2.21$^{\gamma}$	11.66±2.19$^{\gamma,\delta}$	10.39±1.65$^{\alpha,\beta,\gamma,\delta}$	12.36±2.09$^{\gamma}$	17.65±4.62$^{\alpha,\gamma}$	19.86±4.52$^{\alpha,\beta,\gamma}$	6.4±0.81
Amplitude	0.37±0.06$^{\gamma}$	0.39±0.19$^{\gamma,\delta}$	0.43±0.26$^{\alpha,\beta,\gamma,\delta}$	0.34±0.016$^{\gamma}$	0.23±0.21$^{\alpha,\gamma}$	0.20±0.04$^{\alpha,\beta,\gamma}$	0.49±0.03

$^{\alpha}$, P<0.05 versus 2nd month in the same group; $^{\beta}$, P<0.05 versus 4th month in the same group; $^{\gamma}$, P<0.05 versus the blank control group at the same time; $^{\delta}$, P<0.05 versus the experimental control group at the same time. EMG, electromyogram.

Tables 2,3 and *Figure 1A,B*), suggesting NGF could effectively shorten the incubation. However, the difference of incubation between the experimental group and the blank control group was still obvious at 6th month (P<0.05). On the contrary, the amplitudes of SEPs and EMG in the experimental group and the experimental control group were decreased compared with the blank control group at the same time point, respectively. And the amplitudes in experimental group were significantly higher than those in experimental control group at 4th and 6th months, respectively (all, P<0.05; *Tables 2,3* and *Figure 1C,D*), but the amplitudes of experimental group were still lower than those in the blank control group at 6th month (P<0.05). The results revealed that NGF could effectively put off the decline of proprioceptor function altogether.

To further prove the role of NGF on the proprioception function after ACL reconstruction surgery, the number of proprioceptors was recorded after gold chloride staining. As shown in *Figure 2*, the volume of the Ruffini corpuscle and Pacinian corpuscle in the experimental control group were reduced, the leaf shape gradually disappeared, as well as the thickness of the packaging plate was smaller over time. As for the experimental group, there was a leaf structure found in the Ruffini corpuscle, and the atypical structure was reduced over time.

Furthermore, the number of receptors in the experimental control group significantly decreased over time (P<0.05), but in the experimental group was remarkably up-regulated (P<0.05) (*Table 4* and *Figure 3*). More importantly, in comparison with the experimental control group, the number of receptors in the experimental group was increased at both 4th and 6th months, and the changes were significant (both P<0.05), suggesting the injection of NGF could promote the generation of proprioceptors. However, the difference of proprioceptors number between the experimental group and the blank control group was significant (P<0.05).

Discussion

The findings in the present study showed that ACL reconstruction without rehabilitation training was limited to the recovery of proprioceptor function, and NGF had a significant effect on proprioceptor function and quantity after ACL reconstruction.

During the past few years, the research on the treatment of proprioceptive rehabilitation is based on physical rehabilitation, but the curative effect is not satisfactory. Furthermore, instead of the biomechanical stability of the restorative joints, the research of proprioception in supporting the dynamic stability of the knee joint has received increased attention. Proprioception has been reported to play a significant role in the regulation of the nervous system and motor performance (12), and it has a significant correlation with the natural feeling of the knee joint (13,14). Since Freeman *et al.* (15) found the improved

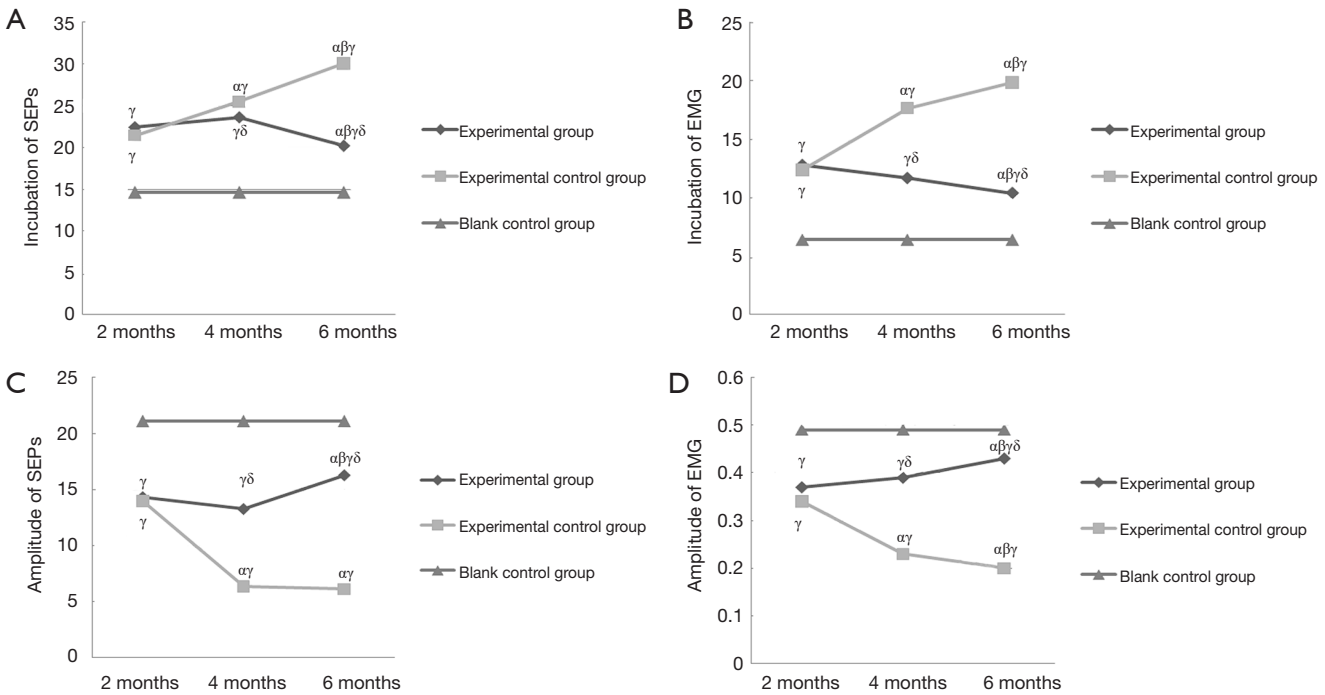

Figure 1 The comparison of nerve electrophysiology among 3 groups. (A) Differences in the incubation of SEPs; (B) differences in the incubation of EMG; (C) differences in the amplitude of SEPs; (D) differences in the amplitude of EMG. α, P<0.05 versus 2nd month in the same group; β, P<0.05 versus 4th month in the same group; γ, P<0.05 versus the blank control group at the same time; δ, P<0.05 versus the experimental control group at the same time. SEP, somatosensory evoked potential; EMG, electromyogram.

chlorinated gold staining method was more convenient, clear and stable for research on the distribution of nerve in 1967, proprioception has been defined as an important parameter for functional rehabilitation of knee joint because of its correlation with knee function (16,17).

Currently, SEPs and EMG have been recognized as the methods for examining the sensory state of the animal's knee. For example, Lu *et al.* (18) revealed that the incubation of hamstring contraction related to the functional instability of the knee joint is often significantly prolonged after ACL injury, which can be used as an indicator of proprioceptive loss after the ACL injury. In the present study with the rabbit model, the electrophysiological test showed that compared with the blank control group, the incubation of SEPs and EMG in both the experimental group and the experimental control group were prolonged, and the amplitude decreased. The result indicated the ACL reconstruction without rehabilitation training could not significantly improve the reduction and function of the proprioceptor, which was following the previous study (19-21). Moreover, in comparison to the experimental control group, the experimental group injected with NGF performed shorter incubation and higher amplitude, suggesting NGF could promote the improvement of proprioception function after ACL reconstruction. However, the statistically significant differences of incubation and amplitude were noted between the experimental group and blank control group at the 6th month, which was speculated to due to the dose and use time of NGF. Hence, further research to investigate whether NGF could help proprioception of ACL to return to the normal level is still needed.

As a potential neurotrophic factor that may be used to reduce lidocaine-induced neurotoxicity, NGF may promote the growth and regeneration of neurons (22). Svensson *et al.* (23) revealed that the masseter muscle injection of NGF is associated with a distinct and prolongs sensitization to mechanical stimuli. Moreover, an increasing number of studies suggest that gold chloride staining has played a significant role in the observation of the existence, morphology, and distribution of proprioceptors (24). In our study, the results showed that the number of receptors in the experimental group injected with NGF was significantly increased, and the

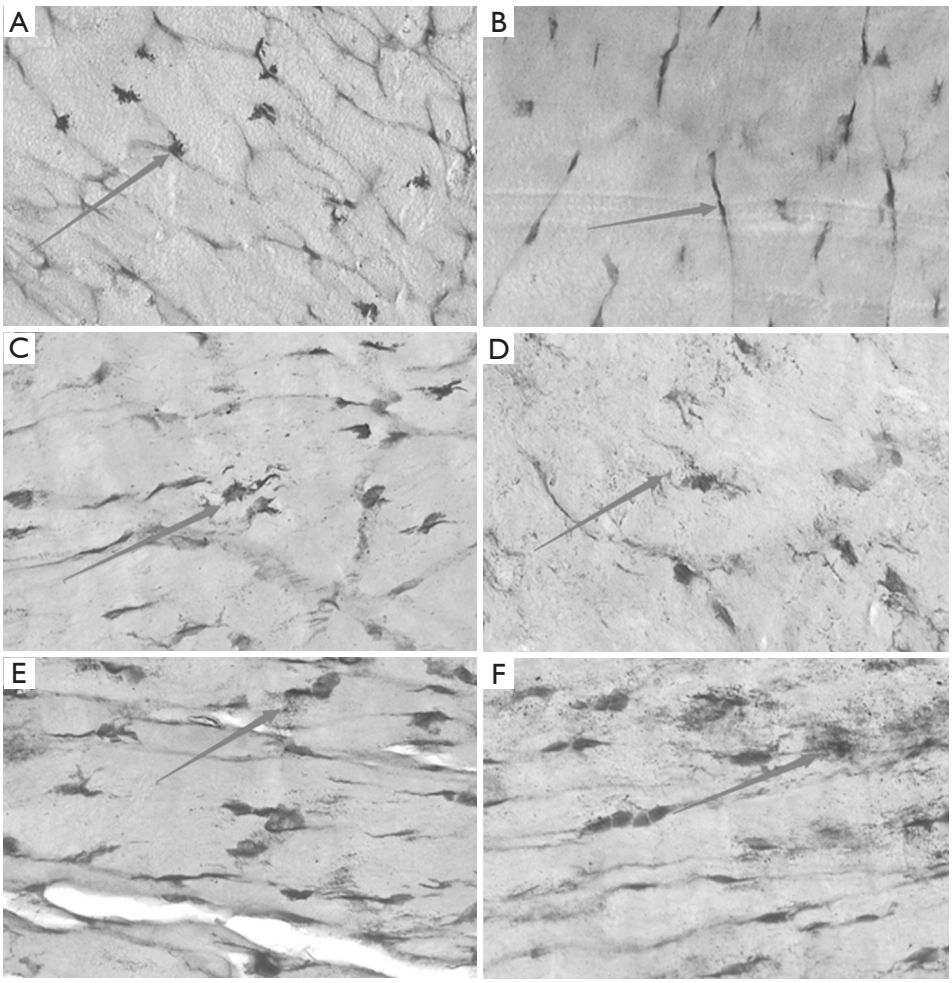

Figure 2 The pathological morphology of the proprioceptors in gold chloride staining result. Experimental group (A) and experimental control group (B) in 2nd month after surgery with gold chloride staining; the experimental group (C) and experimental control group (D) in 4th month after surgery with gold chloride staining; experimental group (E) and experimental control group (F) in 2nd month after surgery with gold chloride staining. The arrow in the figure refers to the notable change of the proprioceptors.

Table 4 The comparison among the three groups about the proprioceptive quantity in different detecting time

Index	Experimental group			Experimental control group			Blank control group
	2 months	4 months	6 months	2 months	4 months	6 months	
Proprioceptors number	$17.3\pm1.36^{\gamma}$	$18.74\pm1.54^{\alpha,\gamma,\delta}$	$20.35\pm1.90^{\alpha,\beta,\gamma,\delta}$	$17.26\pm1.22^{\gamma}$	$14.45\pm1.62^{\alpha,\gamma}$	$10.11\pm5.62^{\alpha,\beta,\gamma}$	28.39 ± 1.81

$^{\alpha}$, P<0.05 versus 2nd month in the same group; $^{\beta}$, P<0.05 versus 4th month in the same group; $^{\gamma}$, P<0.05 versus the blank control group at the same time; $^{\delta}$, P<0.05 versus the experimental control group at the same time.

atypical structure was reduced when compared with the experimental control group, suggesting NGF plays an important role in improving the proprioception function after ACL reconstruction. However, it was previously demonstrated that the numbers of mechanoreceptors significantly decreased, and their morphological changed appear with aging in rabbits (25); therefore, further investigation about the effect of NGF on the proprioception function after ACL reconstruction based on different age groups of rabbits still needed.

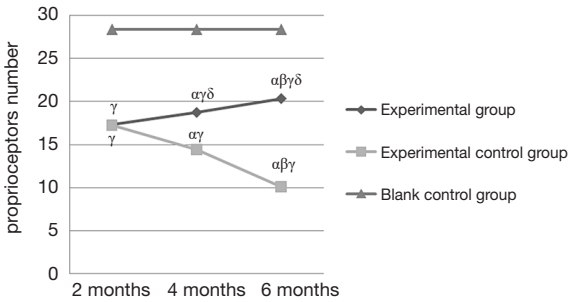

Figure 3 The comparison of the number of proprioceptors in ACL among 3 groups. Differences in the number of proprioceptors. α, P<0.05 versus 2nd month in the same group; β, P<0.05 versus 4th month in the same group; γ, P<0.05 versus the blank control group at the same time; δ, P<0.05 versus the experimental control group at the same time. ACL, anterior cruciate ligament.

Conclusions

In summary, our study has proved that the injection of NGF could improve joint function rehabilitation by promoting function and quantity of proprioception after ACL reconstruction. However, to investigate whether NGF could restore the function and quantity of the proprioceptor of ACL reconstruction to the normal state and further study still is needed.

Acknowledgments

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The Laboratory Animal Center approved this study of the Gansu University of Chinese Medicine, and animal care was following the "Guide for the Care and Use of Laboratory Animals".

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