MEDICAL SCIENCE MONITOR

ANIMAL STUDY

e-ISSN 1643-3750
© Med Sci Monit, 2018; 24: 105-113
DOI: 10.12659/MSM.905160

Received: 2017.05.04
Accepted: 2017.07.16
Published: 2018.01.06

Proprioceptive Changes in Bilateral Knee Joints Following Unilateral Anterior Cruciate Ligament Injury in Cynomolgus Monkeys

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Manuscript Preparation E
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Source of support: This work was supported by the National Natural Science Fund of China (grant number 81674095)

Background: The anterior cruciate ligament (ACL) is one of the most important structures maintaining stability of knee joints, and the proprioception of the ACL plays a key role in it. If the ACL is injured in the unilateral knee joint, it changes nerve electrophysiology, morphology, and quantity of the proprioceptors in the bilateral ACL. The aim of this study was to explore the proprioceptive changes in the bilateral knee joints following unilateral ACL injury, and to provide a theoretical foundation and ideas for clinical treatment.

Material/Methods: Nine normal cynomolgus monkeys were chosen and used to developed a model of unilateral ACL injury, and 3 monkeys without modeling were used as blank control. At the 4th, 8th, and 12th weeks, the changes in ACL nerves were inspected using electrophysiology [somatosensory evoked potentials (SEPs) and motor nerve conduction velocity (MCV)], and the changes of morphology and quantity of the proprioceptors in ACL were observed and measured under gold chloride staining.

Results: On the injured and contralateral knee joints, the incubations were extended and the amplitudes were decreased over time. In addition, with the extension of time, the total number of proprioceptors in the ACL decreased, and the variable number of proprioceptors in the ACL increased.

Conclusions: ACL injury leads to attenuation of proprioception on the injured side, and also leads to the attenuation of proprioception on the contralateral side, and there is a tendency could get worse over time.

MeSH Keywords: **Anterior Cruciate Ligament • Electrophysiology • *Macaca Fascicularis* • Proprioception**

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/905160

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Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Background

Proprioception refers to perception of position, motion, and vibration collected by the proprioceptors from muscles, tendons, and joints [1–3]. Trunk and limb proprioceptive pathways can be divided into consciousness and unconsciousness. The information between proprioception and the central nervous system mainly are transmitted by the proprioceptors from ligaments, muscles, and joints. Proprioception is affected by trauma, surgery, rehabilitation exercise, whole-body vibration exercise, and other factors [4–6].

As one of the important structures maintaining stability of knee joints, the anterior cruciate ligament (ACL) is one of the most studied structures [7]. It has been demonstrated that the ACL contains different types of proprioceptors, which include Ruffini corpuscle, pacinian corpuscle, Golgi tendon organs, and other free nerve endings. The proprioceptors can perceive the tension and strain of the ACL, and produce certain nerve reflexes leading to transmission of information to the central nervous system by the sensory pathway. Then, information is analyzed and integrated. Finally, these proprioceptors produce the motion and location of sense of the trunk and limbs [8–11].

ACL injury is clinically common, and is associated with high-energy injuries and sport trauma. Sometimes, surgical interventions of reconstructions are needed to repair ACL physical structure to stabilize the injured knee joints. Although ACL structure can be fundamentally reconstructed, there were some patients who feel knee-joint instability after surgery, and this is attribute to the effect of proprioception on the stability of knee joints [12–14]. When it occurs, ACL injury not only decreases the mechanical stability of knee joints directly, but also results in obstacles to proprioception at the same time [15–17].

It had been previously reported that both physical structure and proprioception of knee joints play important roles in maintaining the static and dynamic stability of knee joints [18,19]. The proprioception of knee joints is transmitted to ascending neurons by proprioceptors in the ACL. Once the ACL is injured, a series of feedback mechanisms of the knee joint are affected. Complicated injury of proprioceptors in the ACL can result in proprioception signal disorder. The information on position and movement of the knee joints cannot be quickly and accurately perceived by the central nervous system, followed by function disorder of related muscles and nerves. Eventually, knee stability decreases, causing a vicious cycle of repeated knee joint injuries [20]. Additionally, it has been confirmed that the whole process involves a number of links that interact with each other. Thus, in the treatment and rehabilitation of ACL injury, the recovery of proprioception is important as well. However, we have limited understanding of the relationship between proprioception and knee joint stability. It is still unclear whether ACL injury in the unilateral knee affects the contralateral proprioception and whether the impact is different, and the current understanding of the issue is insufficient [21–23].

With the current high level of concern about ACL injury, especially the proprioception function of knee joints, there have been many studies about related treatment. However, most studies focused on ACL repair and reconstruction, and there has been little research about the recovery of proprioception function, especially in conduction and tissue anatomy (morphology and quantity of proprioceptors).

Proprioception is important to balance for osteoarthritic knees, and functional improvement of injured knees [24–27]. Based on the information above, the aim of this study was to explore the effects of ACL injury on the proprioception in injured and contralateral knee joints. Firstly, when the unilateral ACL was injured, we explored the changes over time in proprioception on the injured side, and the change in the total and variable number of proprioceptors. We sought to determine whether the proprioception and the number of proprioceptors in the contralateral side (without injury) were affected, and if so, what was changed. Some studies have found that the unilateral ACL injury not only causes injury to the knee joint dysfunction, but also affects the contralateral knee joint activity, balance, and joint position sense [28–30]. However, studies related to contralateral proprioception and proprioceptors are scarce [31–33]. In the rehabilitation of ACL injury, the recovery of proprioception in the injured side has always been focused on, while the contralateral proprioception has received little attention, and appropriate rehabilitation of bilateral limbs is also affected clinically. Therefore, we hope that this study provides a foundation for further study, and also indicates treatment ideas and directions for clinical treatment.

Material and Methods

Ethics statement

All the procedures were approved by the Ethics Inspection Committee of Animal Experiments of Yunnan Yinmore Biological Technology Co. Ltd. (No. YBT1602). The welfare of animals was guaranteed by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and animal care was in accordance with the "Guide for the Care and Use of Laboratory Animals" (Office of Science and Health Reports CPRR/NIH 1996).

Animals

Twelve male specific pathogen-free (SPF) cynomolgus monkeys (weight range 6.0 to 7.0 kg) were provided by the Yunnan

Yinmore Biological Technology Co. Ltd. All monkeys were fed at the Laboratory Animals Breeding Center of Yunnan Yinmore Biological Technology Co. Ltd. In detail, the monkeys were fed in stable cages at the periods of sleeping, feeding, and rest, each cage measuring 1.5 m (H)×2 m (W)×1.5 m (D). The conditions were a 12: 12 h light: dark cycle, with a temperature of 22–24°C and relative humidity of 45–65%. The monkeys were fed daily with regular feed. Water was provided at all times. Additionally, all monkeys were moved out of cages to another spacious activity room, approximately measuring 4 m (H)×12.5 m (W)×8 m (D), for 6 to 8 h of free time per day. Sometimes, videos and music were played to relax the monkeys.

Reagents and consumables

We used gold chloride, dimethyl benzene, distilled water, anhydrous alcohol, natural lemon juice concentrated, formic acid, glycerin, paraffin wax, Zoletil 50 anesthetics, iodine volts, physiological saline, a sterile arthroscope instrument set, a tourniquet, a knife, syringes, sterile gauze, sutures, and drapes.

Instruments

We used arthroscopic instruments (Smith & Nephew), a photoelectric evoked potentiometer (MEB-9402C), an ordinary refrigerator, a super-clean operating table, an inverted microscope, an embedding machine, a paraffin section and a frozen section machine, and a roasting machine.

Grouping and modeling

Nine monkeys were randomly chosen for developing the model of unilateral ACL injury. All the knees were divided into 3 groups: the blank control group (3 normal knees were chosen from 3 normal cynomolgus monkeys, 1 knee per monkey); the model group included A and B, the model group A (contralateral knees in 9 monkeys that were developed as models), and the model group B (injured knees in 9 monkeys that were developed as models).

Cynomolgus monkeys in the model group underwent unilateral knee surgery for ACL injury by using arthroscopy. The arthroscopic instruments (Smith & Nephew, USA, 72200616) were prepared and the equipment was strictly sterilized by operators before the operation. Firstly, the monkeys were anesthetized by Zoletil 50 (Virbac, France, 5 mg/kg, IM) and were fixed in supine position with shaved skin on surgical area. Secondly, the operators marked the incision, and applied an ipsilateral lower-extremity proximal tourniquet. After all preparations, the anterior medial and anterior lateral approach of the knee joint were built with 0.5-cm in length for exploring the knee joints (Figure 1A, 1B). Exploration indicated that articular cartilage, ACL and posterior cruciate ligament, and meniscus were intact. Approximately 1/4 of the ACL was transversely cut (Figure 1C, 1D) under clear arthroscopy vision. The incision was closed with 3-0 absorbable sutures (Alcon, Alcon Laboratories, Inc., USA). The modeling operation was completed by the same group of experienced doctors. Three days after the operation, levofloxacin hydrochloride and sodium chloride injection (Heng Ao, China, 8 mg/kg, 1 time/12 h, IV) was used to prevent infection, with close observation of condition of incision, and tramadol hydrochloride for injection (QiMaiTe, China, 2 mg/kg, 1 time/day, IM) was used to relieve pain. A soft-padded bandage was placed and maintained on the operated limbs for 2 weeks. Animals were monitored daily.

Nerve electrophysiology and histopathology

At the 4th, 8th, and 12th weeks, 3 monkeys each were chosen from the model groups A and B, and then the changes in ACL nerves were assessed with the electrophysiology [somatosensory evoked potentials (SEPs) and motor nerve conduction velocity (MCV)]. SEPs and MCV are currently assessed by 2 methods of monitoring of peripheral nerve injury that mainly have 2 indicators: the incubations and amplitudes. If the incubations were extended and the amplitudes were decreased, it would indicate nerve injury. As control, the same electrophysiological inspections were performed on the monkeys in the blank control group after group division.

SEPs

Under Zoletil 50 (5 mg/kg, IM) anesthesia, the monkeys' heads and limbs were fixed. A recording electrode point was located by moving back 1 cm from the intersection of the attachments of bilateral tips of the ear and the root of the nose to the inion (lower limb cortex area), and the reference electrode was placed in the nasal root, the ground wire was connected beside the ear. SEPs was measured at 26–28°C, and bipolar surface electrodes were used to stimulate the surface areas of body at the ACL adherent place. Stimulation parameters were constant and electrical stimulations of a single square wave were used (the wave width was 0.1 ms, the frequency was 2 Hz, and the stimulation intensity ranged from 15 to 20 mA). Then, incubations and amplitudes of SEPs in cynomolgus monkeys were recorded by evoked potentiometer (MEB-9402C, Japan). Finally, the information was input to a microcomputer operation system, and we measured and analyzed the graphics, incubations, and amplitudes of SEPs (Figure 2A).

MCV

The stimulation electrode was placed at the popliteal space, the recording electrode was placed at the muscle belly of the hamstring, and the reference electrode was placed 2 cm from the recording electrode, and, at 26–28°C, bipolar surface electrodes

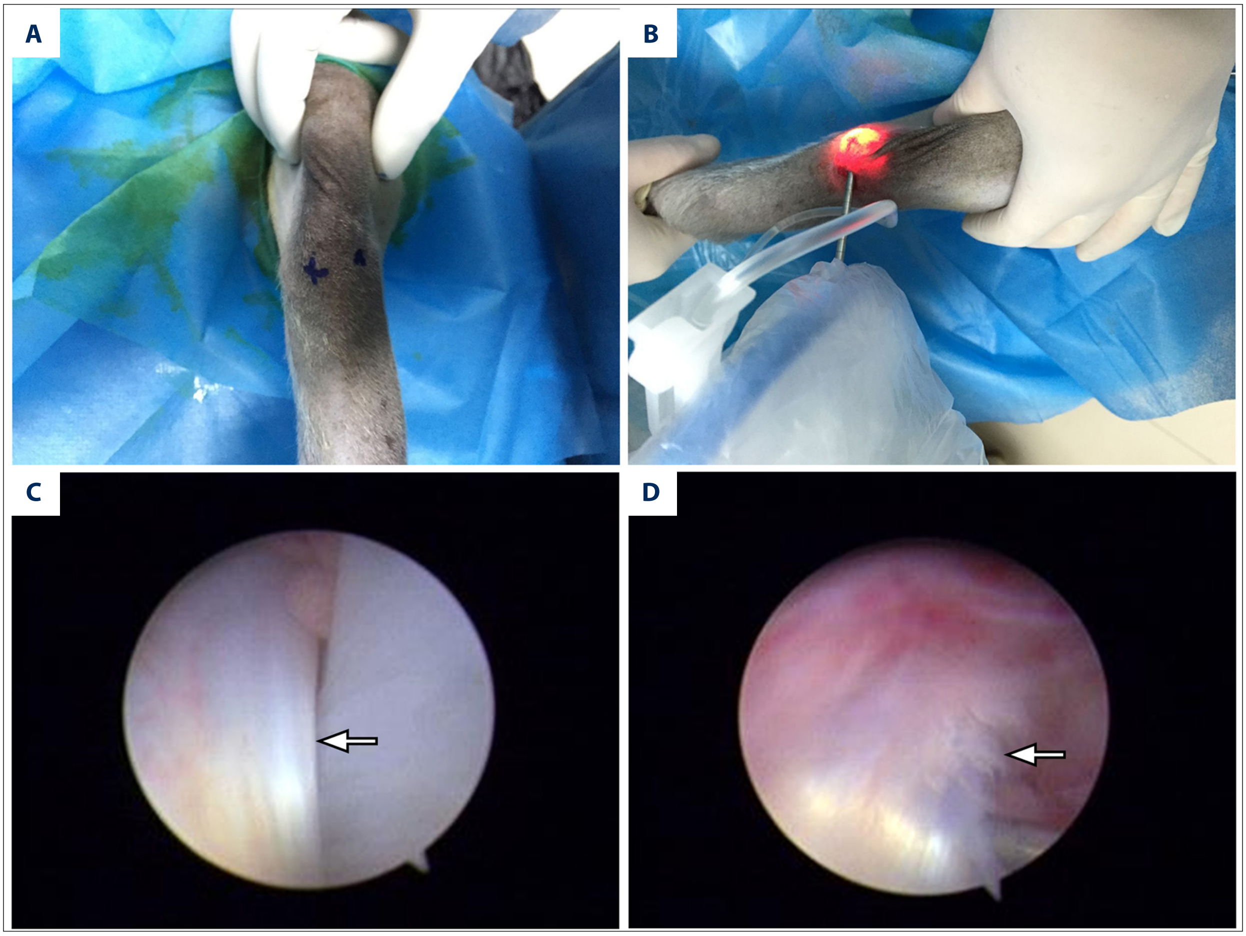

Figure 1. The development of anterior cruciate ligament injury models. (**A**) Arthroscopic approach. (**B**) Arthroscopy was performed in the cavity of knee joints. (**C**) Normal and smooth anterior cruciate ligament was observed in the arthroscopy. (**D**) 1/4 anterior cruciate ligament injury was performed by hook knife.

Figure 2. Inspection of nerve electrophysiology. (**A**) Somatosensory evoked potentials. (**B**) Motor nerve conduction velocity.

were used to stimulate the surface areas at the ACL adherent place. Stimulation parameters were constant, the wave width was 0.2 ms, the frequency was 1 Hz, and the stimulation intensity ranged from 25 to 30 mA. Then, incubations and amplitudes of MCV were recorded by the evoked potentiometer (MEB-9402C, Japan). Finally, the information was input to a microcomputer operation system, and we measured and analyzed the graphics, incubation, and amplitudes of MCV (Figure 2B).

Gold chloride staining

After the inspection of SEPs and MCV, under euthanasia, ACLs of all monkeys were obtained completely and put into the miscible liquids of lemon juice and 88% formic acid (lemon juice: 88% formic acid=3: 1) in preparation for staining. Firstly, all specimens were placed for 15 min in a dark room. Secondly, l% gold chloride solutions were added for 30 min and 25% formic acid solution for 15 h, washed by distilled water for 1 h, and the pure glycerin was added for 24 h, in that order. After staining, tissue blocks were dehydrated in an ascending series of ethanol, cleared in xylene, and embedded in paraffin. Then, 5-μm sections were prepared using a rotary microtome (Leica, CM3050S, Germany). Five sections were randomly chosen from each paraffin block of ACL, which would represent all parts of the ACL as far as possible. Finally, parts of the femur, tibia, and an intermediate location were marked by 3 operators, and each operator counted the number of proprioceptors in ACLs and hamstrings. Each operator utilized the surrounding tissues to avoid duplication.

Statistical analysis

All data are presented as the mean and standard error (SE). To determine the normal distribution, the Kolmogorov-Smirnov and Shapiro-Wilk tests were conducted. Then, repeated-measures ANOVA and Fisher's PLSD test were used to determine the effects of time duration after modeling on the level of plasma lipid. The differences among 3 groups in the level of plasma lipid were assessed by one-way ANOVA and Fisher's PLSD test. A value of $P<0.05$ was considered significant. Statistical analysis was performed using IBM SPSS version 20.0.

Results

Nerve electrophysiology

At the same time point, compared with the blank control group, the incubations of SEPs and MCV were extended in model groups A and B, while the amplitudes of SEPs and MCV were decreased, and the changes in model group B were more remarkable than in model group A ($P<0.05$). In model groups A and B, the incubations were extended and the amplitudes were decreased over time in SEPs and MCV ($P<0.05$) (Tables 1, 2 and Figure 3).

Histopathology

At the 4th week, the total and variable number of proprioceptors in model group A had no significant difference in comparison with the blank control group ($P>0.05$), but that in model group B had significant difference in comparison with the blank control group ($P<0.05$). At the 8th and the 12th weeks, compared with the blank control group, the total number of proprioceptors was decreased, but the variable number of proprioceptors was increased in model groups A and B, and the changes in model group B were more remarkable than in model group A ($P<0.05$). Additionally, in model groups A and B, the total number of proprioceptors was decreased and the variable number of proprioceptors was increased over time ($P<0.05$) (Table 3 and Figures 4, 5).

Discussion

Due to ethics and safety restrictions, it is difficult to perform research on the human body. Therefore, we performed experiment on an animal model. For many studies related to osteoarthritis, the main procedure is ACL injury in animal models [34,35]. Many animal models of ACL injury have been reported, such as rats, rabbits, and dogs, some of which were widely used in related research [36–38]. However, common animal models of ACL injury still have some differences from human ACL injury, especially in the physics of standing and walking. In contrast, primates show more similarities with humans in this aspect, and these physiological characteristics allow the knee joints of primates to better mimic the human model. Additionally, the instability of knee joints could be more obvious in the state of standing on hind limbs alone than on 4 limbs, which is also more similar to the clinical manifestations of real ACL injury in humans. Hence, the animal model of ACL of primates such as cynomolgus monkey is helpful to explore the proprioceptive effects as similar as possible to those of humans.

In this study, subjective evaluations were not effective, because the cynomolgus monkeys were selected as experimental objects. As a result, nerve injury was evaluated by nerve electrophysiological method and morphological changes. Moreover, the quantity of proprioceptors was observed by pathological staining. Finally, the result showed that the incubation was extended and the amplitude declined over time on SEPs and MCV in the injured side of knee joints, the same as in the contralateral knee joint. This phenomenon indicates that the function of afferent nerves that maintain proprioception of knee joints was decreased and the proprioception decreased over time. In addition, a statistically significant difference was noted between

Table 1. The comparison of the incubation and amplitude of somatosensory evoked potentials in three groups (mean ±SE).

	Model group A		Model group B		Blank control group	
	Incubation (ms)	Amplitude (µV)	Incubation (ms)	Amplitude (µV)	Incubation (ms)	Amplitude (µV)
4 weeks	$15.37 \pm 0.15^{\alpha\beta\gamma\delta}$	$4.10 \pm 0.10^{\alpha\beta\gamma\delta}$	$25.47 \pm 0.60^{\alpha\beta\delta}$	$2.23 \pm 0.15^{\alpha\beta\delta}$	11.97±0.21	7.57±0.21
8 weeks	$18.57 \pm 0.50^{\beta\gamma\delta}$	$3.47 \pm 0.06^{\beta\gamma\delta}$	$29.80 \pm 0.46^{\beta\delta}$	$1.37 \pm 0.06^{\beta\delta}$		
12 weeks	$21.27 \pm 0.32^{\gamma\delta}$	$2.90 \pm 0.10^{\gamma\delta}$	$34.07 \pm 0.29^{\delta}$	$0.93 \pm 0.12^{\delta}$		

$^{\alpha}$ P<0.05 *vs.* 8 weeks in the same group; $^{\beta}$ P<0.05 *vs.* 12 weeks in the same group; $^{\gamma}$ P<0.05 *vs.* Model group B at the same time; $^{\delta}$ P<0.05 *vs.* Blank control group.

Table 2. The comparison of the incubation and amplitude of motor nerve conduction velocity in three groups (mean ±SE).

	Model group A		Model group B		Blank control group	
	Incubation (ms)	Amplitude (mV)	Incubation (ms)	Amplitude (mV)	Incubation (ms)	Amplitude (mV)
4 weeks	$10.37 \pm 0.15^{\alpha\beta\gamma\delta}$	$2.40 \pm 0.10^{\alpha\beta\delta}$	$18.57 \pm 0.31^{\alpha\beta\delta}$	$1.30 \pm 0.17^{\alpha\beta\delta}$	3.12±0.03	9.53±0.42
8 weeks	$13.27 \pm 0.25^{\beta\gamma\delta}$	$1.97 \pm 0.15^{\beta\delta}$	$21.13 \pm 0.15^{\beta\delta}$	$0.78 \pm 0.02^{\beta\delta}$		
12 weeks	$24.70 \pm 0.26^{\gamma\delta}$	$0.47 \pm 0.02^{\gamma\delta}$	$16.30 \pm 0.27^{\delta}$	1.57 ± 0.06^{d}		

$^{\alpha}$ P<0.05 *vs.* 8 weeks in the same group; $^{\beta}$ P<0.05 *vs.* 12 weeks in the same group; $^{\gamma}$ P<0.05 *vs.* Model group B at the same time; $^{\delta}$ P<0.05 *vs.* Blank control group.

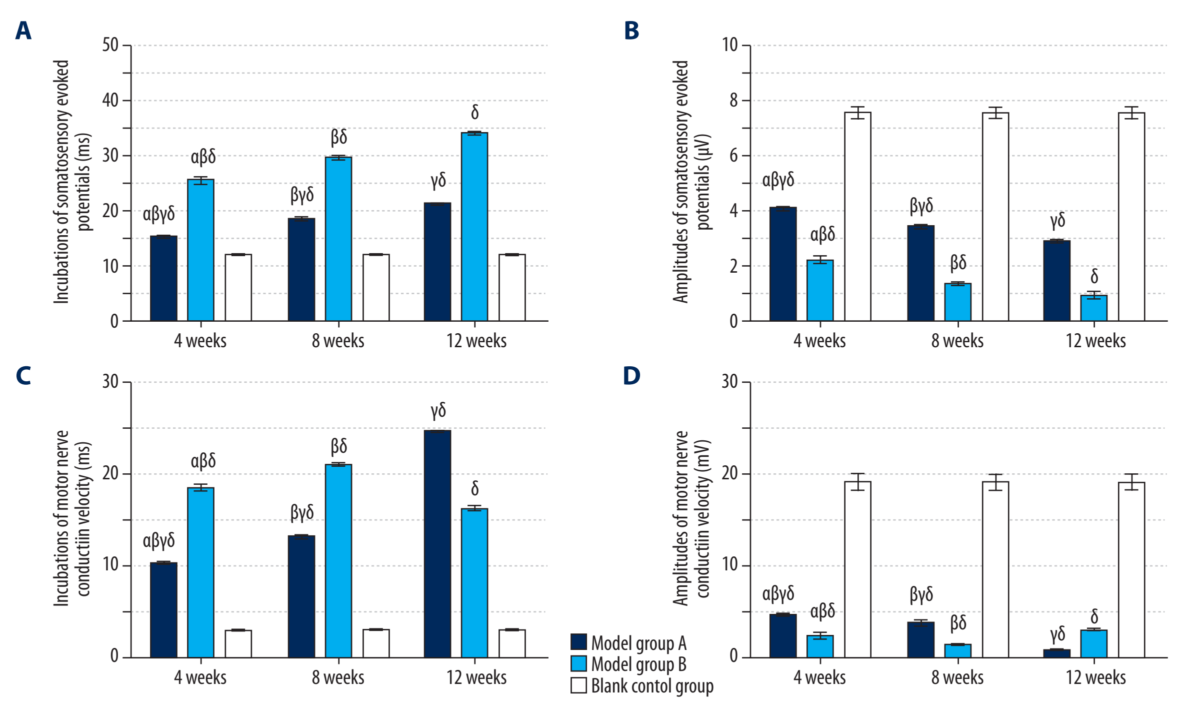

Figure 3. The comparison of nerve electrophysiology among 3 groups. (**A**) Differences in the incubation of somatosensory evoked potentials. (**B**) Differences in the amplitude of somatosensory evoked potentials. (**C**) Differences in the incubation of motor nerve conduction velocity. (**D**) Differences in the amplitude of motor nerve conduction velocity. α P<0.05 *vs.* 8 weeks in the same group; β P<0.05 *vs.* 12 weeks in the same group; γ P<0.05 *vs.* Model group B at the same time; δ P<0.05 *vs.* Blank control group.

Table 3. The comparison of total and variable number of proprioceptors in three groups (mean ±SE).

	Model group A		Model group B		Blank control group	
	Total (N)	**Variation (N)**	**Total (N)**	**Variation (N)**	**Total (N)**	**Variation (N)**
4 weeks	$975.67 \pm 12.01^{\alpha\beta\gamma}$	$0.00 \pm 0.00^{\alpha\beta\gamma}$	$578.00 \pm 2.65^{\alpha\beta\delta}$	$34.00 \pm 2.00^{\alpha\beta\delta}$	976.00±6.00	0.00±0.00
8 weeks	$878.00 \pm 2.00^{\beta\gamma\delta}$	$7.67 \pm 0.58^{\beta\gamma\delta}$	$459.67 \pm 6.81^{\beta\delta}$	$42.00 \pm 2.00^{\beta\delta}$		
12 weeks	$755.67 \pm 3.21^{\gamma\delta}$	$17.37 \pm 1.53^{\gamma\delta}$	$346.33 \pm 8.50^{\delta}$	$52.67 \pm 3.06^{\delta}$		

$^{\alpha}$ P<0.05 *vs.* 8 weeks in the same group; $^{\beta}$ P<0.05 *vs.* 12 weeks in the same group; $^{\gamma}$ P<0.05 *vs.* Model group B at the same time; $^{\delta}$ P<0.05 *vs.* Blank control group.

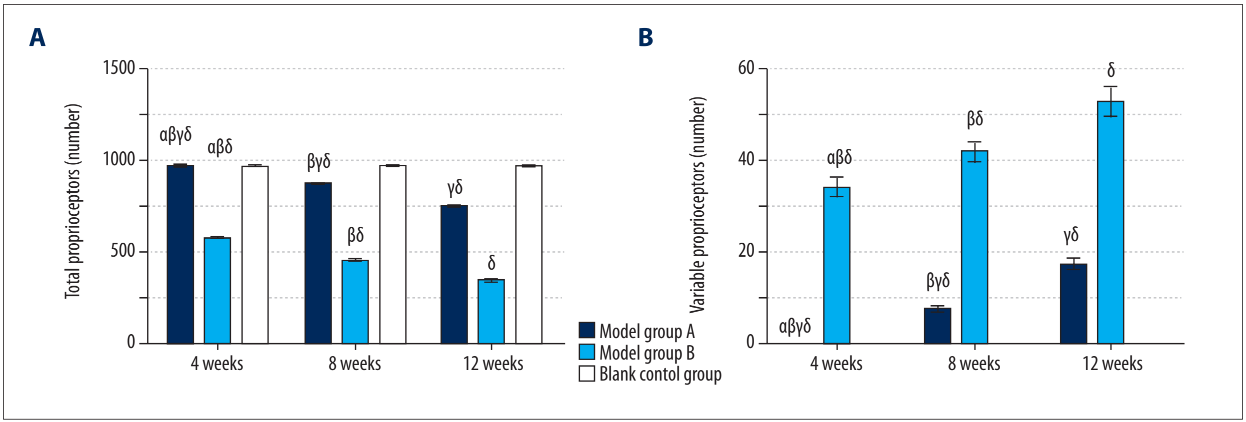

Figure 4. The comparison of the number of proprioceptors in ACL among 3 groups. (**A**) Differences in the number of total proprioceptors. (**B**) Differences in the number of variable proprioceptors. α P<0.05 *vs.* 8 weeks in the same group; β P<0.05 *vs.* 12 weeks in the same group; γ P<0.05 *vs.* Model group B at the same time; δ P<0.05 *vs.* Blank control group.

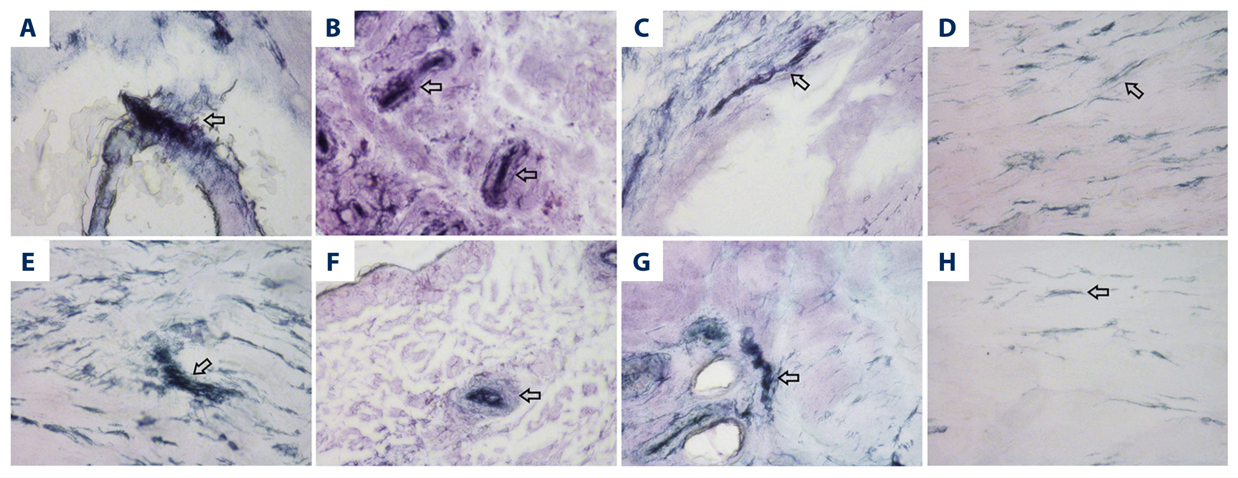

Figure 5. The pathological morphology of normal and variable proprioceptors (Magnification 40×). In the figure, the arrows point to places where the proprioceptors were located. (**A**) Ruffini corpuscles are shaped like a tree and there are normally branching structures; (**B**) Pacinian corpuscle was elliptic, and the outer layer usually had a layer of cyst; (**C**) Golgi tendon organs have a spiraling pattern: (**D**) Free nerve endings had no characteristic form; (**E**) Variable Ruffini corpuscle; (**F**) Variable pacinian corpuscle; (**G**) Variable Golgi tendon organs, which was dissolved, disordered, deformed, and even smaller; (**H**) Free nerve endings in the model group B, and there are fewer of them.

injured sides and contralateral sides of the knee joints, indicating that unilateral ACL injury affects bilateral proprioception of the knee joints.

On the other hand, the ACL proprioceptors were clearly observed by gold chloride staining, including Ruffini corpuscles, pacinian corpuscles, Golgi tendon organs, and other free nerve endings. At the 8th and 12th weeks, compared with the blank control group, the total number of the proprioceptors decreased and the variable number of proprioceptors increased in the model groups A and B. The changes in model group B were more remarkable than in model group A ($P<0.05$). This phenomenon could intrinsically explain the proprioceptive recession in morphology, where the mechanism should be related with decreased mechanical stimulation of proprioceptors in the ACL. This phenomenon also proves the bilateral interactive effect on ACL proprioception. At the 4th week, the total number of proprioceptors decreased and the variable number of proprioceptors increased in model group B, while all of that in the model group A had no significant difference compared with the blank control group ($P>0.05$). In conclusion, within a short time (4 weeks), ACL injury can lead to the contralateral proprioceptive recession. However, the recession only appeared in the SEPs and MCV, and the morphological characteristics and the number of proprioceptors change by 8 weeks later.

In addition, the unilaterally proprioceptive injury affects the contralateral proprioception in the knee joints, which can get worse over time. The result was similar to some extent. It has been proved that upward neurons of proprioception at different levels receive bilateral limb nerve conduction with the interactive connection [39–41]. Theoretically, because of the characteristics of nerve conduction, the unilaterally proprioceptive injury can affect the contralateral proprioception, but the details are unclear.

In recent years, the incidence of ACL injury has been increasing [42–45]. Rehabilitation therapy is important to the restoration of knee function and early re-exercise [46–49], and proprioception plays an important role in recovery of knee joint stability [50,51]. Most previous studies focused on clinical research, and we tested the changes of ACL proprioception after injury by nerve electrophysiology, especially observing the changes of proprioceptors in pathology. It is objectively proven that unilateral ACL injury can cause some effects on contralateral proprioception and our study may provide a basis for future treatment.

Our study has certain limitations. Firstly, due to objective reasons, the time of observation was limited to only 12 weeks, without a long-term follow-up. Secondly, the electrophysiological detection was performed under anesthesia, which might influence the experimental results to some extent. Thirdly, there was no recognized standard on the variable degree of proprioceptors, so we did not assess the degree of variation in pathomorphology. Additionally, because all of the experimental cynomolgus monkeys were males, the effects of sex on the final results were unclear.

Conclusions

Proprioception plays an important role in knee joint stability. ACL injury not only leads to unilaterally proprioceptive recession, but also affects the contralateral proprioception, and this phenomenon gets worse over time. Therefore, in the basic research and clinical treatment, we should not only focus on the unilaterally lack of proprioception caused by knee joint injury and recovery, but also on the contralateral side.

Conflict of interest

None.

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