

Original Article

Rhesus monkey is a new model of secondary lymphedema in the upper limb

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Abstract: Objective: This study is to establish the rhesus monkey model of lymphedema in the upper limbs, and assess the suitability of this model. Methods: An animal model of lymphedema was established by the combined irradiation and surgical techniques in the upper limbs of these rhesus monkeys. Physical examination, high-resolution MR lymphangiography, bioelectrical impedance analysis (BIA), and immunohistochemical staining were performed to determine the severity of the edema in the upper limbs of the animal model. Results: Our results from physical examination indicated that the rhesus monkey model present with typical appearance and features of lymphedema. MR lymphangiography further demonstrated pathologically modified lymphatic vessels in our rhesus monkey model. BIA revealed increased water content in the upper limb in these rhesus monkeys, which was in line with the pathology of lymphedema. Immunohistochemical staining showed the curvature of the lymphatic vessels in the rhesus monkey model, typical pathological changes in lymphedema. Conclusion: Rhesus monkey lymphedema model provides a more consistent background to elucidate the pathophysiology of the disease. This new model would help to increase our understanding of acquired upper limb lymphedema, and promote the development of new treatments for this intractable disorder.

Keywords: Secondary lymphedema, rhesus monkeys, upper extremity, breast cancer, animal model

Introduction

Lymphedema is a chronic, progressive disease resulting from the abnormality of the lymphatic system. It is mainly caused by the interstitial accumulation of lymph fluid, which leads to inflammation, adipose tissue hypertrophy and fibrosis [1]. Lymphedema can be categorized as primary and secondary concerning congenital and acquired etiologies. Generally, secondary lymphedema is more common, and has been associated with malignancy, particularly the breast cancer, and its treatment [2]. The pathological course induces significant swelling of the upper limbs after the lymph node dissection in the axilla. Although major advances have been made in the diagnosis and therapeutic treatments in recent years, lymphedema still represents one of the leading causes of physical and psychological morbidity in patients with breast cancer. Researchers are continuing with

the aim to improve the lifestyle and care of these patients.

An important aspect in researches on arm lymphedema after breast cancer treatment is about the animal model. So far, various animal models for clinical lymphedema have been described, including rodent and carnivore species. For examples, Liu et al. produced a practical model of secondary lymphedema in rats, and found that when treated with local intradermal VEGF-C transfection, a reduction of lymphedema would be observed in the therapy group [3]. Moreover, Kinjo and co-workers showed that lymphatic vessel-to-vein anastomosis would be an effective therapeutic method for the management of secondary lymphedema, in dog models [4]. Although encouraging results about the pathogenesis and therapeutic strategies of lymphedema have been found with these non-primate species, there are still disad-

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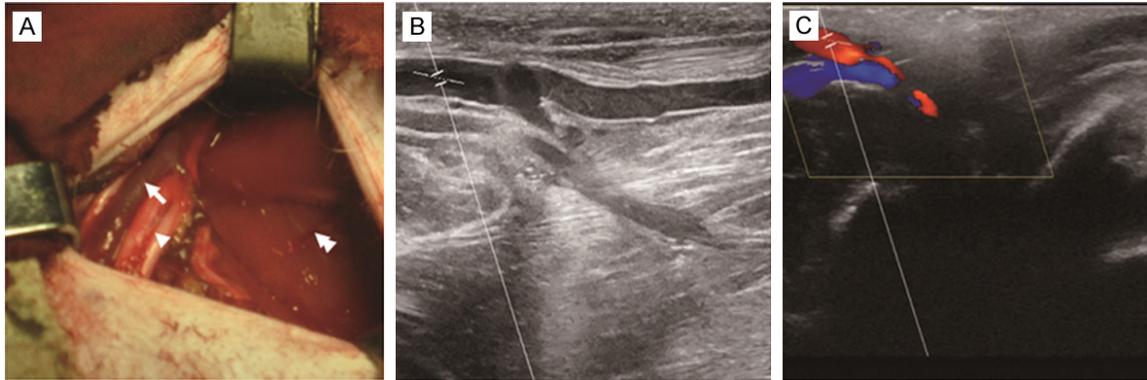


Figure 1. ALN dissection and ultrasound detection of axillary vein in rhesus monkeys. A: A transverse straight incision was made at the right axillary region and the subcutaneous fat and the deep lymphatic tissues around the axillary vessels were excised. Axillary blood vessels (arrow), nerves (arrowhead), and pectoralis major muscle (double arrowhead) were indicated. B, C: Ultrasound detection of axillary vein in rhesus monkeys after surgery. After wound healing, B-mode ultrasonic diagnostic equipment was used to confirm whether the blood flow in the axillary vein was pulsatile. B: Axillary vein; C: Ultrasound signal of axillary vein.

vantages in these models. The non-primate models of lymphedema are often difficult to create, with relatively low achievement ratio. In addition, significant differences exist in physiology and anatomy between the upper and the lower extremities in humans, which could not be reflected in these non-primate species. Due to the above reasons, pathophysiological processes in lymphedema in the upper limbs have not been easily replicated in these conventional animal models.

In the present study, a rhesus monkey model of lymphedema in the upper limbs was established, and high-resolution MR lymphangiography, bioelectrical impedance analysis (BIA), and immunohistochemistry were performed to assess the suitability of the model. Our results showed that the rhesus monkey model could achieve a similar pathophysiological environment to humans following the treatment of breast cancer, in physiology, neurobiology, susceptibility to infection, and metabolic diseases. This study provided a preferable model to study lymphedema in the upper extremities after the treatment of breast cancer.

Materials and methods

Animals

Female rhesus monkeys of 4-5 years old, approximately 5.5-6.2 kg, were obtained from The Central for New Drug Evaluation of Shandong University (CNDE; Jinan, Shandong, China). From 7 days before experiments, all animals started to be kept in standard laboratory

with an artificial 12-hour light/dark cycle. All animal experiments were conducted according to the ethical guidelines of The Institutional Animal Care and Use Committee of Shandong University (Protocol number 2012).

Animal model establishment

Animal model of lymphedema was established by the combined irradiation and surgical techniques in the upper limbs of rhesus monkeys. The right upper limbs were chosen for the intervention, and the left ones were used as control. Axillary fossa was irradiated two weeks before and four weeks after the surgery, with a single dose of 30 Gy emitted from an X-ray machine (MBR-1520R-3; Hitachi, Tokyo, Japan), at a dose rate of 4.12 Gy/min (150 kVp, 20 mA). Axillary lymph node (ALN) and fat dissection were performed to facilitate lymphedema, just duplicating the ALN dissection in humans. Before the surgery, the animals were anesthetized with ketamine (4 mg/kg i.m.; Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, Jiangsu, China) combined with diazepam (2 mg/kg s.c.; Jiangsu Hengrui Medicine Co., Ltd.). First, 5 ml of 2% patent methylene blue (Beijing Double-crane pharmaceutical Co., Ltd., Beijing, China) was injected into the subcutaneous tissue layer adjacent to the right mammary gland to identify drainage lymphatic vessels. ALNs were readily identified by blue staining within 5 min. A transverse straight incision was made at the right axillary region, and then the subcutaneous fat and the deep lymphatic tissues around the axillary vessels were excised to disrupt the superficial and deep lymphatic path-

Rhesus monkeys with secondary lymphedema



Figure 2. Physical examination of the upper limbs in rhesus monkeys at 12 m after the surgery. (A, B) The experimental right palm (A) and arm (B), and the control left palm and arm. (C) Right upper limb demonstrated obvious pitting edema in rhesus monkeys at 12 m after the surgery.

ways (**Figure 1A**). After wound healing, B-mode ultrasonic diagnostic equipment was used to confirm whether the blood flow in the axillary vein was pulsatile (**Figure 1B, 1C**). Postoperative analgesia was obtained by application of tramadol hydrochloride (2.5 mg/kg i.m.; Hexal GmbH, Holzkirchen, Germany) for three days after surgery.

Limb circumference assessment

For the circumference measurement of the extremity, water displacement method was used as previously described [5]. Tape rule was used to analyze the circumference of forearm, elbow, and upper arm.

Magnetic resonance imaging (MRI)

The animals were scanned by MRI with a 3.0-T scanner, Magnetom Symphony (Siemens Medical Systems, Erlangen, Germany) equipped with high-performance gradients. Four stations were examined: the palm, the forearm, the upper arm, and the shoulder region. The phased-array body coil was used to examine the upper extremity. Before MR lymphangiography, the extent and distribution of the lymphedema were evaluated using a heavily T2-weighted turbo spin-echo sequence (TR/TE, 2,400/705; matrix, 256 × 256; bandwidth, 247 Hz/pixel; slices, 66; FoV, 350 × 350; acquisition time, 5 min). To highlight the edema, maximum-intensity-projection (MIP) reconstructions were performed.

For HR MR lymphangiography, a spoiled gradient-echo sequence (volumetric interpolated breath-hold examination [VIBE]) was used, with the following parameters: TR/TE, 2400/724; matrix, 448 × 448; bandwidth, 490 Hz/pixel; slices, 79; FoV, 350 × 350; acquisition time, 5

min. Four doses of gadodiamide (0.1 mmol/kg) were injected cutaneously into the dorsal aspect of hand, at the region of the four interdigital webs, with a 25 G needle (BD Biosciences, Franklin Lakes, NJ, USA). After the injection, the injection sites were massaged for 60 s. The massage was repeated during data acquisition. The four stations were first imaged without gadodiamide, and subsequently repeated 5, 25, and 45 minutes after intracutaneous application of gadodiamide. To emphasize the gadolinium-containing structures, baseline images were subtracted, and 3D MIP reconstructions were performed.

Bioelectrical impedance analysis (BIA)

The extracellular fluid volume changes were detected by BIA with a multiple-frequency bioelectrical impedance analyzer (Inbody 3.0; Biospace, Seoul, Korea). During impedance measurements, the animals were seated at nonmetal desks, with their feet resting on the plate electrode of the equipment. The animals rested their arms with palms facing down on the cushion at shoulder level. The skin areas where electrodes were applied were first wiped with 75% alcohol. Then, lightly adhesive resting electrodes were placed on each hand at the dorsal surface of the wrist between the radial and ulnar bones, and at the dorsal surface of the hand, 1 cm proximal from the peak of the knuckle of the middle finger. The foot electrode was placed between the 2 bony processes on the ankle in the front of the foot. Color-coded alligator clips were placed on the targeted electrodes as previously described [6].

Histology and immunohistochemistry

Upper arm subcutaneous tissue was fixed in 4% formaldehyde in PBS overnight, and then

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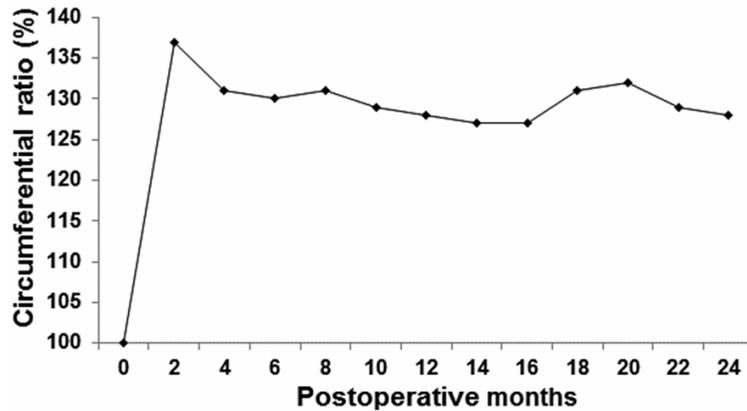


Figure 3. The circumferential ratios in rhesus monkeys after intervention.

decalcified in 10% formic acid in 10% formalin/PBS overnight. The tissues were embedded in paraffin after tissue processing of dehydration, clearance, and impregnation. Each tissue block was cut into 5 mm serial sections, and every fifth section was stained with rabbit anti-human vascular endothelial growth factor receptor-3/fms-related tyrosine kinase 4 (VEGFR-3/FLT-4) IgG polyclonal antibody (dilution 1:500; Santa Cruz Biotech, Santa Cruz, CA, USA) for immunohistochemical staining of lymphatic vessels. Slides were imaged with an upright Olympus BX-50 microscope equipped with a Moticam 2300 color camera (Cole-Parmer; Vernon Hills, IL, USA).

Statistical analysis

Data were expressed as mean \pm SD. SPSS 22.0 software was used to perform statistical analysis. Differences of the extracellular fluid volume in the upper limb of rhesus monkeys before and after operation were compared by paired t-test. $P < 0.05$ was considered as statistically significant.

Results

Macroscopic observation of the upper limb in rhesus monkeys

There are various diagnostic tests that could be counted to detect and assess lymphedema. In this study, physical examination, soft tissue imaging, bioelectrical impedance analysis (BIA), and immunohistochemical staining were performed to determine the severity of the edema in the upper limbs of the animal model. Firstly, macroscopic observation indicated that,

in the affected upper limbs, there was apparent swelling only 2 days after lymph node resection. As shown in **Figure 2**, at 12 m after the surgery, tissue texture, pitting edema, and larger skin folds were observed in the affected limb, which would be probably due to the accumulation of fluid and/or fat deposition in the extremity. On the other hand, the changes in the limb circumference were measured and recorded, from zero time-point to 24 months after the intervention. Our results

showed that the circumferential ratios of the affected limb to the contralateral control were between 100% and 137% over the 24 months after modeling (**Figure 3**), and the thickness of the palm increased about 15-40%. Accordingly, physical examination indicates that the rhesus monkey model present with typical appearance and features of lymphedema.

Magnetic resonance imaging (MRI) in the upper limb in rhesus monkeys

Soft tissue imaging, like MRIs and CTs, detects excess fluid in the tissues. Since lymphedema mainly results from the accumulation of interstitial fluid, these imaging technologies are usually used to assess lymphedema conditions. In this study, MR lymphography was performed, to evaluate the lymphedema in the upper limb in these rhesus monkeys. Our results showed significant changes in lymphatic vessels, at 3 m after the surgery. The major lymphatic trunks disappeared on the treated arm, compared with the normal side. The vessel structure was replaced by a bright, punctate fluorescence pattern against a foggy background (**Figure 4A, 4B**). In all animals, the lymphedema showed an epifascial distribution with high signal intensity on T2-weighted images. In frontal 3D spoiled gradient-echo high-resolution MRI and digital subtraction angiography (DSA) lymphangiography image, delayed lymphatic flow with reticular pattern of dilated lymphatic vessels was observed, indicating neovascularization associated with obstruction (**Figure 4C**). These MRI results further demonstrate the pathologically modified lymphatic vessels in our rhesus monkey model.

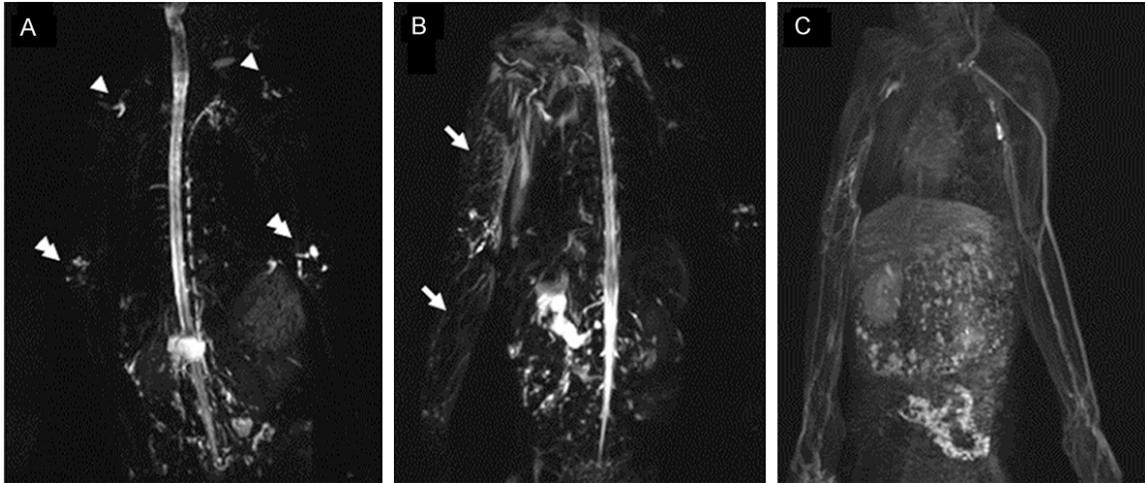


Figure 4. Magnetic resonance imaging (MRI) of lymphedema in the upper limb in rhesus monkeys. A: MR lymphography of bilateral upper limbs in rhesus monkeys at 1 m after surgery. Only elbow (arrowheads) and axillary lymph nodes (double arrowheads) were visible, without delayed lymphatic flow. B: MR lymphography of bilateral upper limbs in rhesus monkeys at 3 m after surgery. In the right limb, delayed lymphatic flow with reticular pattern of dilated lymphatic, and dermal backflow (arrows) was detected. C: Frontal 3D spoiled gradient-echo high-resolution MRI and digital subtraction angiography (DSA) lymphangiography image. In the right limb, delayed lymphatic flow with reticular pattern of dilated lymphatic vessels was observed.

Table 1. Extracellular fluid volume changes in the upper limb in rhesus monkeys

	Preoperative (L)	Postoperative (L)	P
Extracellular fluid volume (n)	0.326 ± 0.022 (5)	0.430 ± 0.047 (5)	0.0220

Bioelectrical impedance analysis (BIA) in the upper limb in rhesus monkeys

BIA detected water content in the tissue by inducing a small, unharmed electrical current through the limb, and the impedance to current flow was measured. Higher water content in tissues was linked with lower resistance. The lymphedema condition was assessed by comparing the resistance of electrical flow in the intracellular and extracellular fluid [7]. The rhesus monkey model of lymphedema was subjected to BIA to determine the extracellular fluid volume changes. As shown in **Table 1**, the extracellular fluid volume was significantly elevated after the operation, compared with the volume before the surgery. These results indicate increased water content in the upper limb in these rhesus monkeys, which is in line with the pathology of lymphedema.

Immunohistochemical staining of the subcutaneous tissue in the upper limb in rhesus monkeys

The lymphatic vessels in the upper limbs in the rhesus monkeys were next detected with immu-

nohistochemical staining. Our results showed that the lymphatic vessels were significantly increased and dilated, in the affected limb in this model (**Figure 5**).

These results suggest the curvature of the lymphatic vessels in the rhesus monkeys models, in accordance with the pathological changes in lymphedema.

Discussion

Nowadays, there are approximately 45 million people suffering from the secondary lymphedema of limbs worldwide. A recent meta-analysis has assessed the incidence of arm lymphoedema after breast cancer and explored the risk factors. The findings suggest that more than 20% women who survive breast cancer will develop arm lymphoedema, and it is very important to improve the understanding of risk factors and management strategies to reduce the individual and public health burden of this disorder [8].

Although breast cancer mortality rates have been significantly declined throughout recent years due to the adequate management and treatment, side-effects are still a significant issue, which have a severe impact on the quality of life [9]. Lymphedema is a long-term disorder, which lacks effective therapeutic strate-

Rhesus monkeys with secondary lymphedema

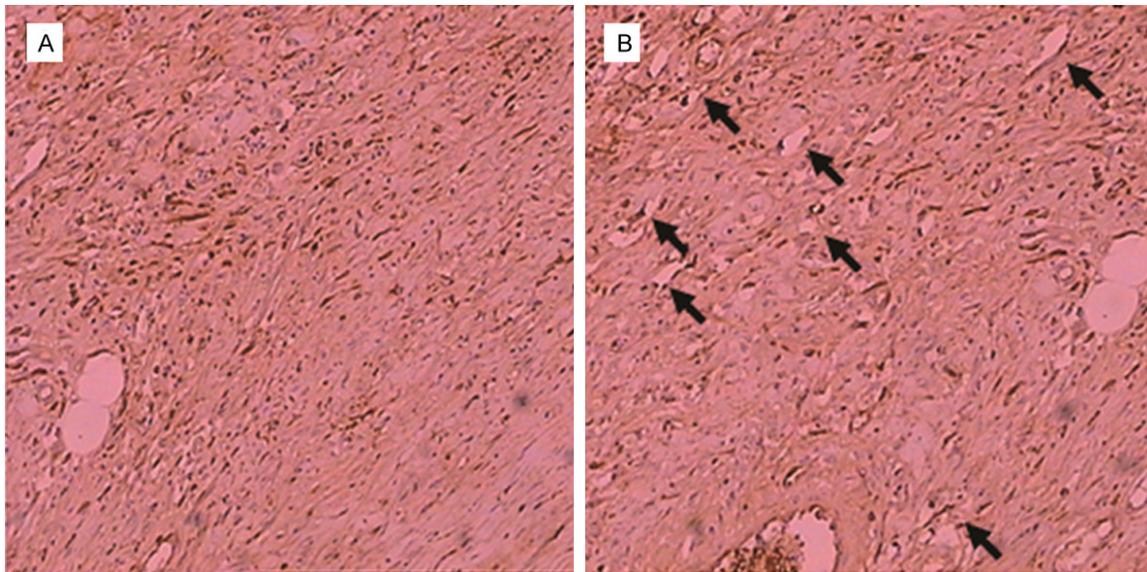


Figure 5. Immunohistochemical staining of the subcutaneous tissue in the upper limb in rhesus monkeys. Immunohistochemical staining with VEGFR3/FLT4 antibody were performed to identify the lymphatic vessels in the control (A) and the affected (B) limbs in rhesus monkeys. Dilated lymphatic vessels in the affected arm were indicated by arrows.

gies [10]. The disease causes functional impairment and psychological burden to the affected ones [11-17], and poses a complicated therapeutic challenge for the physicians. Traditional treatments, such as elevating the swollen limb, compression bandages, massage and remedial exercises, etc., can only promote the fluid circulation, reduce edema volume, and result in partial relief in the patients [10, 11, 18, 19]. Moreover, these conservative measures cannot guarantee the long-term freedom from pathophysiological alterations [10]. Based on these reasons, there has been substantial interest on the studies of disease treatments concerning stem cells and growth factor-mediated lymphangiogenesis. It has been suggested that the administration of growth factors and/or regulatory genes, and stem cell transplantation may provide new therapeutic options for lymphedema [20-26]. Cell biology and molecular biology approaches may provide a therapeutic method to reverse the pathophysiological changes in lymphatic insufficiency. Such approaches will require the documentation of appropriate treatment responses, and the assessment of safety, in suitable preclinical animal models [27].

Various animal models of lymphedema have been described, however their achievement ratios are relatively low, and the formation of

permanent lymphedema is inconsistent with humans. In addition, there are considerable differences in physiology and anatomy between the upper and lower extremities in humans and factors responsible for lymphatic transport in the upper extremity, including capillary filtration, muscular activity, breathing moments, body position or gravity, etc., are far more complex than those in the lower limb. Conventional animal models, like rodents and carnivore [1, 27-30] cannot fully replicate the pathophysiological conditions of lymphedema in the upper limb. Furthermore, there are few studies concerning the animal models with lymphedema associated with axillary lymph node dissection, and none of these studies have been conclusive [31]. Actually, an ideal animal model should closely simulate the biology and pathogenesis of the particular disease in human, including the natural history and the temporal patterns of the clinical expression of the disease. One might then expect to make valid predictions about the applicability of the therapy in humans, by extrapolating the observations from the animal models [27].

In this study, we performed the combined surgical and irradiation techniques in the upper limbs of rhesus monkeys. All animals survived, and developed progressive lymphedema as expected, demonstrating the reproducibility

and consistency of the models and methods. Compared with rodents, which are separated from humans by more than 70 million years [32, 33], monkeys are closely related to humans and share a last common ancestor from about 25 million years ago [32]. This species shares about 93% of the DNA sequence with humans [34]. In addition, compared with other previously described models, rhesus monkeys exhibit greater similarity to humans in terms of physiology, neurobiology, and susceptibility to infectious and metabolic diseases, and they have a greater range of research tools available such as antibodies and various databases. Therefore, our rhesus monkey model of upper limb lymphedema is more suitable for the comparison with humans. To the best of our knowledge, this is the first report of an experimental model with acquired lymphedema in the primate upper extremity to mimic the disease process in humans. However, due to the limited duration of the study, the late effects such as delayed reappearance of lymphedema could not be assessed.

In conclusion, we have successfully established a novel model of upper limb lymphedema in rhesus monkeys. Our model provides a more consistent background to elucidate the pathophysiology of the disease in humans. This new model would help to increase our understanding of acquired upper limb lymphedema, and promote the development of new treatments for this intractable disorder.

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Disclosure of conflict of interest

All authors declare no financial competing interests. All authors declare no non-financial competing interests.

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