

Development of an animal model for rosacea-like skin lesions caused by *Demodex*

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Abstract. To develop an animal model of rosacea-like skin lesions caused by *Demodex* mites, a suspension of *Demodex* mites was injected into the skin of Japanese rabbits. The pathology of the skin lesion was assessed using H&E staining after 4 weeks of modeling. The skin lesions observed after 4 weeks were further treated with the recombinant bovine basic fibroblast growth factor (rbFGF) gel. Untreated lesions in the same rabbit were considered as the blank control. Erythema papules were observed in the model rabbit skin and could be observed most clearly in the 2nd week. Lumpy foreign bodies, telangiectasia and granuloma-like structure were observed in the model rabbit in the 1st, 2nd, and 3rd weeks, respectively. An organized granuloma-like structure was observed in the 4th week. The color of the skin lesions became lighter than that of the self-control after 4 weeks of rbFGF treatment. In conclusion, the model of *Demodex*-induced rosacea-like skin lesions can be developed through intradermal injection of suspension of *Demodex* mites into Japanese rabbits. The model can mimic the phenotype of skin lesions and histopathological manifestations in the *Demodex* mite-positive patient with rosacea.

Introduction

Rosacea is chronic inflammatory facial dermatosis, manifesting as transient or persistent erythema. Other symptoms

include papules, pustules, and telangiectasia in the central area of the face (1,2). The etiology of rosacea is complex, involving genetics, impaired skin barrier, neural and vascular dysfunction, immune system disruption and susceptibility factors (microorganism infection, ultraviolet radiation, and mental stress) (3). *Demodex* has recently been recognized as an important etiology in the pathogenesis of rosacea (4-6).

Rosacea is typically treated with topical drugs (such as ivermectin and metronidazole), oral medications (such as antibiotics) and physical modalities (such as lasers and intense pulsed light therapy). Patient education and proper skin care are also advised (1). It is generally recommended that patients with multiple rosacea symptoms should be treated with a combination of treatments.

Despite these therapies, however, only <50% patients with rosacea are satisfied with the current treatment regime, probably due to the lack of a specific treatment, particularly for those caused by infection with *Demodex* mites (6). *Demodex* may cause rosacea by impairing the skin barrier, thereby inducing a host immune response. Excessive *Demodex* mites fill and block the openings of hair follicles, leading to abnormal expansion of the sebaceous gland cavity of the follicles. Moreover, *Demodex* mites secrete proteolytic enzymes that destroy the intercellular junction, and their chelicera destroy the epithelial cells of the hair follicle, damaging the skin barrier (7). In addition, after they die, their ruptured bodies and fragments are released into the superficial dermis through damaged hair follicles that induce an immune response, forming granulomas (8). However, the exact underlying mechanism through which *Demodex* induces rosacea remains unclear. At present, continuous biopsy to observe dynamic histopathological changes cannot be performed in the human tissues affected by rosacea. In addition, *Demodex* mites do not survive for long *in vitro*. Both these factors have limited further research on the topic to understand the mechanism through which *Demodex* mites induce rosacea. Therefore, an animal model may be an alternative to study the underlying mechanism. The currently available animal models of rosacea only consider inflammatory cytokines during the development of rosacea (9). Therefore, an animal model of *Demodex* mite-induced rosacea is urgently needed.

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Abbreviations: rbFGF, recombinant bovine basic fibroblast growth factor; H&E, hematoxylin-eosin; PBS, phosphate buffer saline

Key words: rosacea, *Demodex* mites, animal model, granuloma, rbFGF

In the present study, live *Demodex* mites were extracted from the face of a patient and were used to make a suspension containing *Demodex* bodies and their fragments. This suspension was injected into the skin of Japanese rabbits to establish a model for rosacea-like skin lesions caused by *Demodex* mites to mimic the phenotype of chronic inflammatory skin lesions and their histopathological state. In a previous preliminary clinical study, it was revealed that the recombinant bovine basic fibroblast growth factor (rbFGF) gel could relieve the chronic inflammatory skin damage caused by *Demodex* mites (10). Therefore, the therapeutic efficacy of rbFGF gel in this animal model was also investigated.

Materials and methods

Ethics approval. The present study was approved (approval no. 2019KYLL006 for both human and animal studies) by the Ethics Committee of the 940th Hospital of Joint Logistics Support force of Chinese People's Liberation Army (Lanzhou, China). Written informed consent for participation in the study was obtained from the patient.

Animals. A total of 28 female Japanese rabbits (12-weeks-old, weighing 2.0-2.5 kg) were obtained from Chengdu Dossy Experimental Animals Co., Ltd. Our preliminary experiment showed that female Japanese rabbits had a higher success rate of skin lesions than that of males. Only female Japanese rabbits were used to avoid gender differences in modeling. Rabbits were held in a simulating natural growth condition with a 12/12 light/dark cycle under a temperature of $23\pm 2^{\circ}\text{C}$ and humidity of $54\pm 10\%$ with access to food and water *ad libitum*.

Modeling

Preparation of the suspension of *Demodex* mites and control suspension. A total of 39 patients with rosacea caused by facial *Demodex* mites (number of *Demodex* mites $>5/\text{cm}^2$ skin) (Fig. 1) were enrolled. The baseline characteristics of the patients are listed in Table I. Each patient first washed his/her face with warm water and compressed it with a hot sterilized towel for 2 min. Then, at $20-30^{\circ}\text{C}$, the doctor quickly squeezed the nasal groove, nose, or facial skin lesions of the patient with a sterilized mite extractor to obtain the thread-like milky white or light-yellow sebum (the color and texture slightly differed depending on the facial condition of the donor). Next, 2-3 drops of 10% KOH solution were added and mixed well with the sebum. The mixture was then transferred into a 5-ml glass tube and mixed with 2-ml phosphate buffer saline (PBS) solution. After standing for 1 h, the supernatant was discarded and the pellet was retreated with KOH and PBS. After performing the treatment for 2-3 times, the pellet was finally transferred to a 1.5-ml EP tube for further application. The suspension of *Demodex* mites in each 1.5-ml EP tube was collected from 3 patients. The final suspension was observed under a light microscope (BX53; Olympus Corporation). The suspension was considered qualified when the fragments or bodies of *Demodex* mites were observed.

The control suspension was prepared from 36 healthy donors without facial *Demodex* mites in the same way as the suspension of *Demodex* mites was prepared. The control suspension showed no fragments or bodies of *Demodex* mites under the microscope.

Table I. Baseline characteristics of included patients.

Clinicopathological characteristic	Patients (n=39)
Age, years	
40-50	18
50-60	14
60-70	7
Sex	
Male	11
Female	28
Type of rosacea	Papulopustular rosacea
Duration of disease	
<6 months	9
6-12 months	18
12-60 months	12
Genetic background	All patients had no family history of rosacea

Development of the animal model of rosacea-like skin lesions caused by *Demodex* mites. After 1 week of accommodation, the rabbits were randomly divided into three groups: the Model group (n=13), the Control group (n=12), and the Blank group (n=3). Rabbits in the Model group were further divided into Model A (n=4), Model B (n=3), Model C (n=3) and Model D (n=3) groups. The hair on the back of the rabbits was first removed to expose the area for injection. Rabbits in Models A, B, C, and D groups were then intradermally injected with the suspension of *Demodex* mites at 0, 1st, 2nd and 3rd week, respectively. Rabbits in the Control group were further divided into Control A (n=3), Control B (n=3), Control C (n=3), and Control D (n=3) groups corresponding to Models A, B, C, and D groups, respectively. Rabbits in the Control A, B, C, and D groups were intradermally injected with the control suspension at 0, 1st, 2nd and 3rd week, respectively. Each rabbit was injected at 6-8 sites with 0.2 ml of suspension at each site. The rabbits of the Blank group received no additional intervention except regular care. Skin lesion tissues were collected from 3 rabbits in each group at the 4th week and subjected to hematoxylin-eosin (H&E) staining. One rabbit remaining in the Model A group was used for the rbFGF treatment. The flowchart of the study is shown in Fig. 2. The health and behavior of rabbits were monitored once in a day and no rabbit succumbed during the experiment.

Assessment of lesion changes after modeling. Changes in skin lesions, including erythema, pustules, and papules, were observed and images were captured every week until the 4th week.

H&E staining. At the 4th week, rabbits were locally anesthetized with 2% lidocaine hydrochloride. The skin lesion tissues were collected and fixed in 10% formaldehyde at room

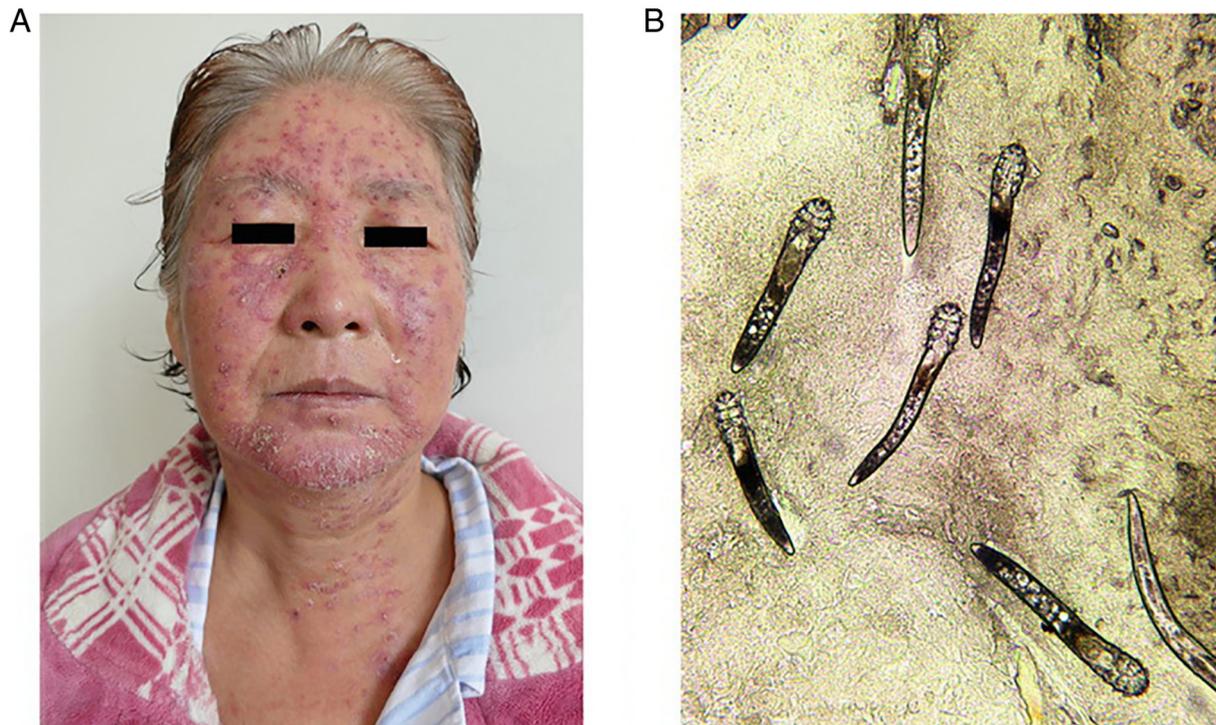


Figure 1. Clinical facial manifestations of *Demodex* mite donors and appearance of *Demodex* mites under microscope. (A) Lesions were scattered on the nose, cheek, forehead, and mandible, presenting as symmetric erythema, papules, and nodules with diameters of 0.3-1 cm. (B) *Demodex* mites (red arrow) in the facial sebaceous glands in a rosacea patient with *Demodex* mite infection (magnification, x100).

temperature for 6 h. Then, the samples were dehydrated in alcohol, embedded in paraffin, and cut into 4- μ m sections. Subsequently, the sections were deparaffinized with xylene and rehydrated with descending ethanol series before H&E staining following the standard protocols at room temperature. The sections were first stained with hematoxylin for 8 min, and then stained with eosin for 2 min. Images were captured under a BX53 light microscope. The pathological changes in each group were observed. After skin samples were collected, rabbits were euthanized by injecting sodium pentobarbital (100 mg/kg) through the ear vein. Death was confirmed by cessation of breathing and heartbeat, mydriasis and loss of nerve reflexes.

Treatment of model rabbit. One rabbit in Model A was used for treatment in the 4th week. The skin lesions were divided into two groups: treatment and self-control. The lesions of the treatment group were treated with the rbFGF gel (0.2 g/cm², 2 times/day; Zhuhai Yisheng Biopharmaceutical Co., Ltd.) for 4 weeks, while those in the self-control group did not receive any treatment. Changes in skin lesions were observed after treatment for 4 weeks. Then the rabbit was euthanized with 100 mg/kg sodium pentobarbital injected intravenously.

Results

Skin appearance of model rabbits. The appearance of the skin of rabbits in the Model A, B, C, and D groups (representing 4, 3, 2, and 1 weeks of the injection of suspension of *Demodex* mites, respectively) at the 2nd week showed several erythema and even purple-red papules on the back skin of

the rabbit (Fig. 3, panel Ab). The erythema was still obvious at the 4th week when the observation was completed (Fig. 3, panel Ad). Reddish papules were observed after the control suspension was injected in the rabbits of the Control group at the 1st week (Fig. 4, panel Aa); lesions in these rabbits recovered with a few pigmentations at the 2nd and 3rd weeks and subsided at the 4th week (Fig. 4, panels Ab-Ad). The rabbits of the Blank group had normal skin appearance without any abnormalities (Fig. 5A).

Pathological changes of skin lesions. Lumpy foreign bodies, telangiectasia, and granuloma-like structures were observed in the model rabbits in the 1st, 2nd and 3rd weeks, respectively (Fig. 3, panels Ba-Bc). An organized granulomatous-like structure was observed in the 4th week (Fig. 3, panel Bd). In the Control group, no angiotelectasis and granulomatous-like structure were observed (Fig. 4, panels Ba-Bd and Ca-Cd). In the Blank group, the skin structure was normal with no granuloma-like structure and inflammatory cell infiltration (Fig. 5B and C).

Treatment outcome. After treatment with the rbFGF gel for 4 weeks, the skin color of the lesions in the treatment group became significantly lighter than that in the self-control group (Fig. 6).

Discussion

An animal model of *Demodex* mite-induced rosacea-like skin lesions was developed by injecting the suspension of *Demodex* mites prepared using the lesions from clinical patients into

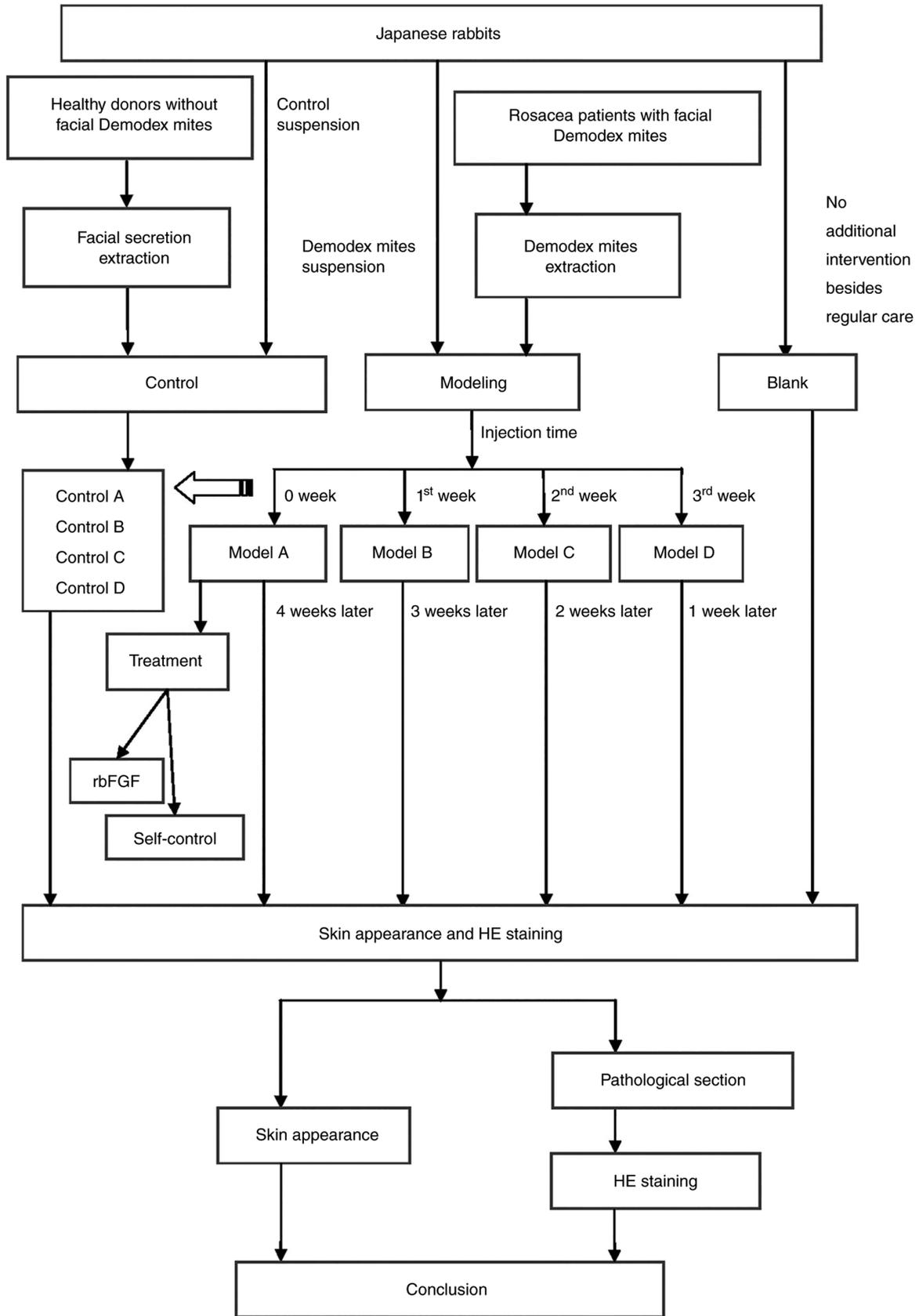


Figure 2. Flowchart of the present study.

the skin of Japanese rabbits. The model rabbits developed erythema and papules on their skin, which are typical symptoms of rosacea. In addition, telangiectasia and granuloma-like

structure were observed in the skin lesions, which is highly consistent with the pathological manifestations of chronic inflammatory skin lesions caused by *Demodex* mites.

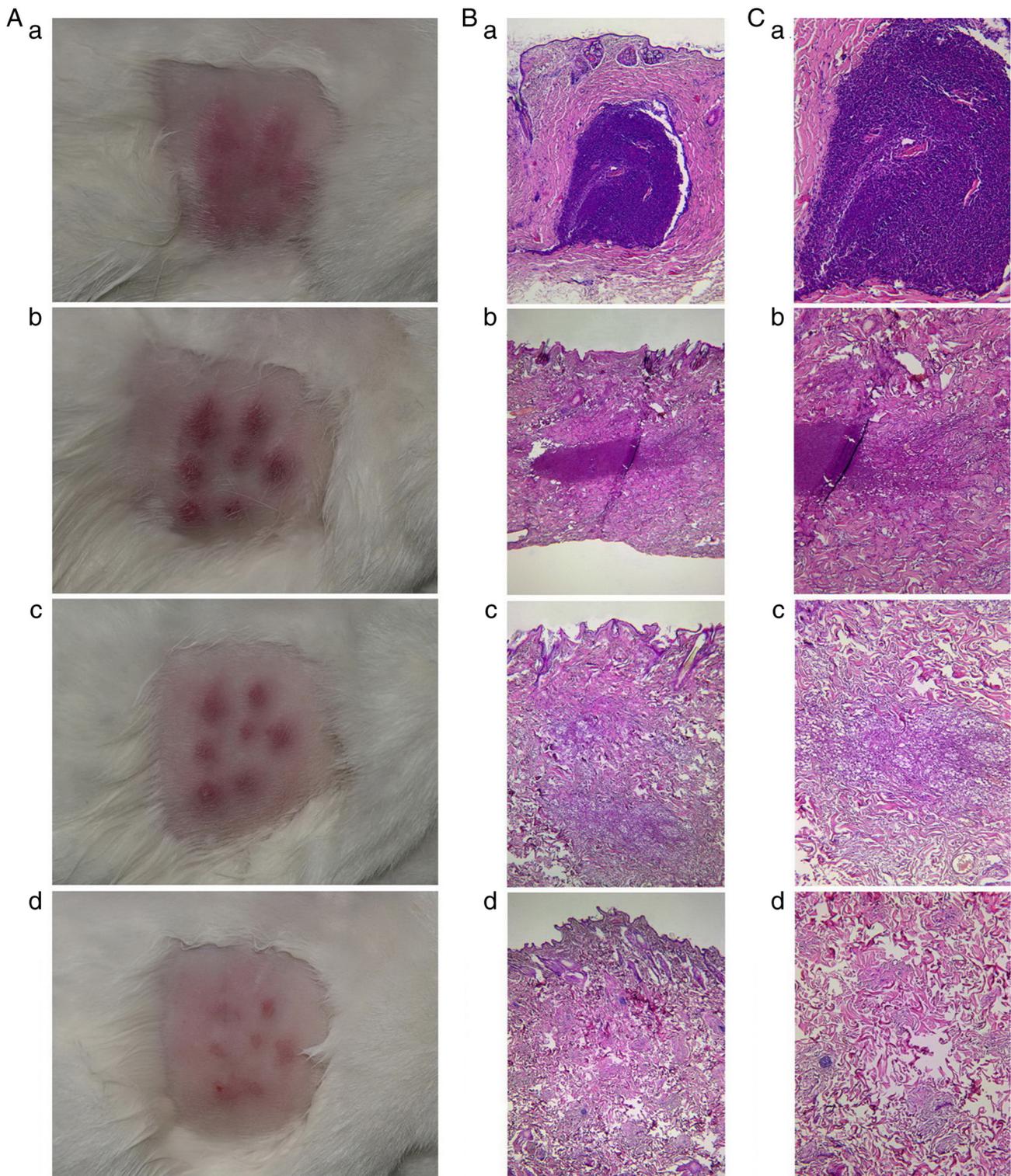


Figure 3. Appearance and H&E staining of skin lesions at the 4th week of modeling. (Aa-Ad) Skin appearance of the rabbits of Models D, C, B, and A respectively. (Aa) Skin appearance of the rabbits of Model D showing mild erythema and papule at the 1st week of modeling. (Ab) Skin appearance of the rabbits of Model C showing severe erythema and papule at the 2nd week of modeling. (Ac) Skin appearance of the rabbits of Model B showing less erythema at the 3rd week of modeling; however, a raised papule was still observed. (Ad) Skin appearance of the rabbits of Model A showing faded erythema at the 4th week of modeling; however, a raised papule was still observed, although it was less than that in Model B. (Ba and Ca) Skin of the rabbit of Model D showed lumpy foreign bodies. (Bb and Cb) Skin of the rabbit of Model C showed telangiectasia and some aggregated lesions, with numerous inflammatory cells surrounding. (Bc and Cc) Skin of the rabbit in Model B showed a granuloma-like structure. (Bd and Cd) Skin of the rabbit of Model A exhibited some organized granuloma-like structures. Magnification, (Ba-Bd) x40 and (Ca-Cd) x100.

Demodex is one of the most common ectoparasites of the human skin and is possibly involved in the pathogenesis of rosacea. Large population of *Demodex* mites can destroy the

skin barrier and induce skin inflammation (6,11-13). *Demodex* mites-induced rosacea may be a continuous process (14). Numerous factors may contribute to the proliferation of

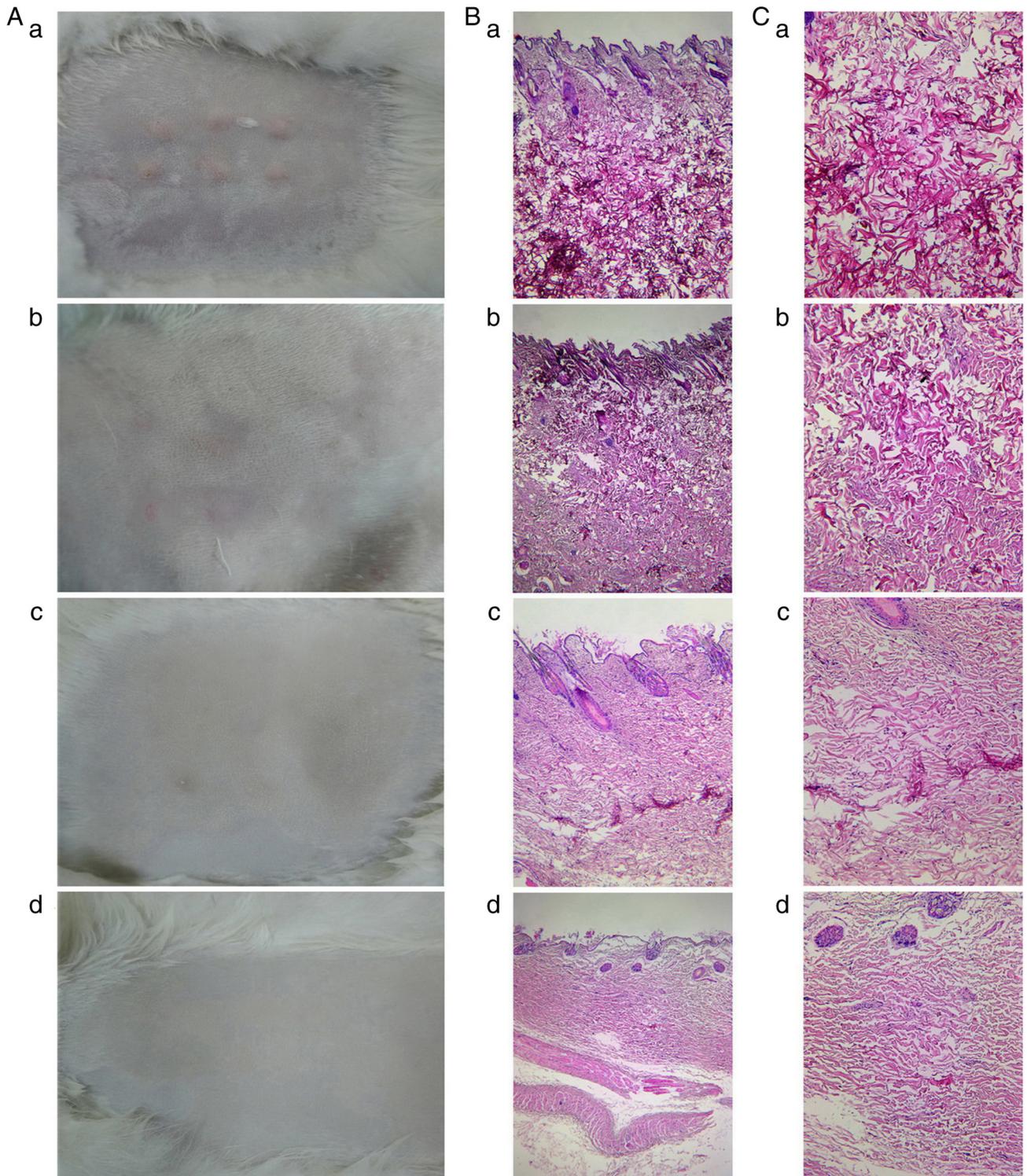


Figure 4. Appearance and H&E staining of the skin at the 4th week in Control groups. (Aa) Skin appearance of the rabbits of Control D showing reddish papules without obvious erythema. (Ab) Skin appearance of the rabbits of group Control C showing little pigmentation. (Ac) Skin appearance of the rabbits of Control B showing faded pigmentation. (Ad) Skin appearance of the rabbits of Control A showing subsided lesions. (Ba-Bd and Ca-Cd) Pathological examination showing similar to normal skin appearance. Magnification, (Ba-Bd) x40 and (Ca-Cd) x100.

Demodex mites in patients, including a susceptibility gene and immunosuppression (15-17), diabetes (18,19), abnormal expansion of skin vessels (20,21), or sebaceous gland hyperplasia (22). Subclinical proliferation of *Demodex* mites can be observed under numerous skin conditions (6). When *Demodex* mites proliferate, the patient presents with facial diffuse

erythema. Since *Demodex* mites do not have an anus, the accumulation of food residue crystals will eventually rupture their bodies, releasing their contents together with their skeleton into the host skin, which will further continuously induce the immune response of the host, aggravating the facial symptoms such as persistent erythema, papules, pustules and

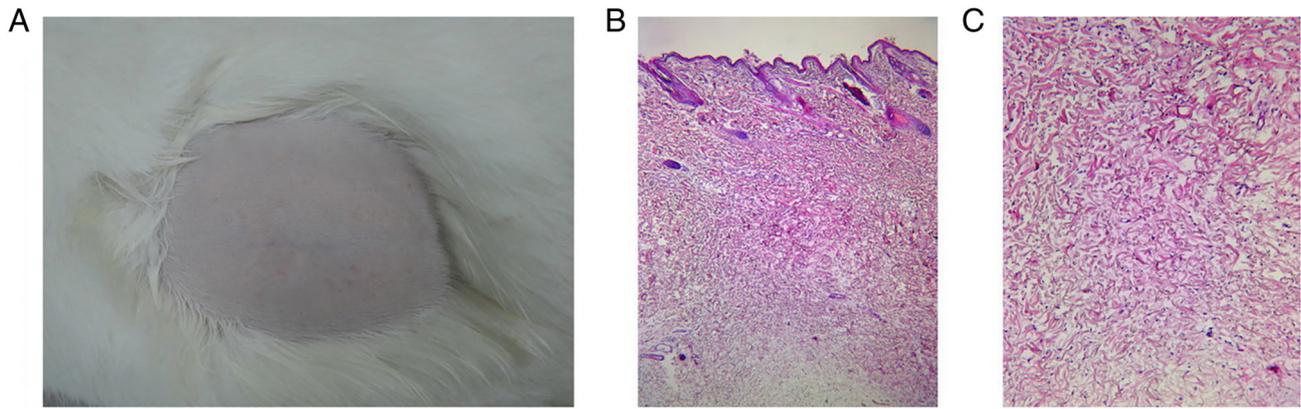


Figure 5. Appearance and H&E staining of skin at the 4th week of the Blank group. (A) Skin appearance of the rabbits of the Blank control showing normal skin appearance. (B and C) Skin structure of the rabbit of the Blank group showing normal skin appearance Magnification, (B) x40, and (C) x100.

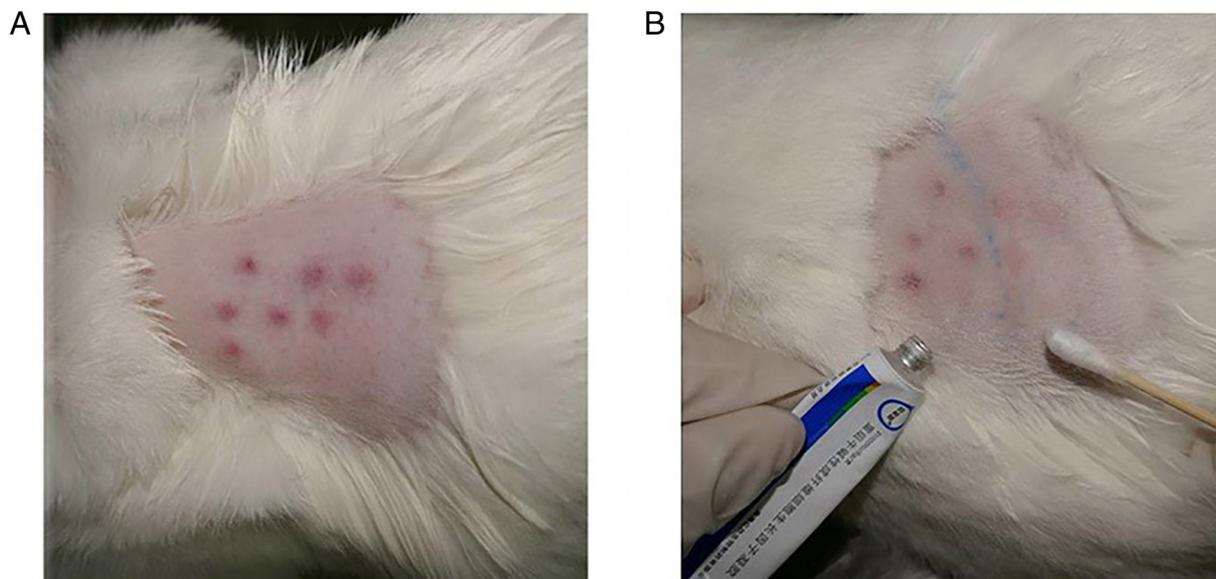


Figure 6. Skin appearance of the model rabbit before and after rbFGF treatment for 4 weeks. (A) Skin appearance of the model rabbit before rbFGF treatment for 4 weeks. (B) Lesions on the right side of the blue line were subjected to the rbFGF treatment, while those on the left side were used as self-control.

nasal hypertrophy (7,23). In the present study, the immune stimulation of *Demodex* mites to the host was simulated by intradermally injecting a suspension of *Demodex* mites into the skin of rabbits. In the 2nd week of the modeling, the skin of the model rabbits at the injection site exhibited obvious erythema and papules, similar to the clinical manifestations of rosacea.

Rosacea has no unique histological manifestation. It always presents as inflammation surrounding the hair follicles with a large number of aberrantly dilated vessels and sebaceous gland hyperplasia of the hair follicle (24,25). Dermal granuloma with lymphocyte infiltration can be observed in various subtypes of rosacea (26). Living *Demodex* mites can destroy the hair follicle wall and enter the dermis through mechanical stimulation. After death, the fragments of mites stimulate an inflammatory reaction, which possibly increases the possibility of granuloma formation in the skin lesions of rosacea (27). In the present study, pathological changes in the granuloma-like structure

were observed after the rabbits were injected with the suspension of *Demodex* mites. The fragments of *Demodex* mites in the dermis stimulate the host to produce T cells to mediate the host immune response. The tissue cells swallow the fragments of *Demodex* mites to form the granuloma-like structure (25). In a previous study, *Demodex* mites were observed at the center of the hair follicle in new facial skin lesions from patients with *Demodex* mites positive rosacea, with clearly visible mouthparts and body wall residues (28). However, in the present study, the body of *Demodex* mites was not found in the skin lesions under microscope even after 4 weeks of modeling, probably due to the fact that *Demodex* mites do not have an anus. Thus, when *Demodex* mites die and decompose, their intestinal contents are immediately released, which are phagocytosed by tissue cells to form a granuloma (29). The suspension of *Demodex* mites was injected into the skin of rabbits, which simulated the disease-causing process involving the death and decomposition of *Demodex* mites. Obvious skin erythema,

papules and granuloma-like structures were observed in the dermis under the microscope at the 2nd and 3rd weeks after the modeling. This observation is consistent with the skin appearance and pathological manifestations of skin lesions of the rosacea patients (10). H&E staining for rabbits in the control group showed no angiotelectasis and granuloma-like structure formation even after 4 weeks of observation.

The pathophysiology of rosacea is complex and unclear. It involves various factors apart from infection with *Demodex* mites. Animal models are always used to study the development of a disease. The most widely used animal model of rosacea at present has been developed by intradermal injection of LL-37 into animals, which can manifest typical rosacea symptoms (30). However, LL-37 is a common downstream inflammatory component induced by different pathogenic factors of rosacea. Therefore, this animal model cannot reflect the specific etiology of rosacea. By contrast, the animal model developed in the present study involved the specific pathogenic factor of rosacea-*Demodex* mites, which can be used to study the effects of *Demodex* mites during the pathophysiology of rosacea. Moreover, the method of preparing the suspension of *Demodex* mites is simple and convenient, which greatly reduces the cost of model building.

Recent studies revealed that the rbFGF gel can improve chronic inflammatory skin lesions caused by *Demodex* infection and reduce the granuloma (10,28). To verify whether the developed rabbit model can be used in basic research, the model lesions were treated with the rbFGF gel for 4 weeks. The result demonstrated that the rbFGF gel significantly improved the redness of the skin lesions, which was consistent with our clinical results (10), indicating that this animal model can be used for basic research on the topic.

It should be noted that since *Demodex* mites cannot survive or be cultured *in vitro* for a long time, the present animal model can only imitate the chronic inflammatory response of rosacea-like skin lesions after the death of *Demodex* mites; it cannot be used to model the mechanical irritation caused by living *Demodex* mites in the skin. Hence, an animal model of rosacea constructed with living *Demodex* mites should be developed, which can more comprehensively imitate the pathogenesis of *Demodex* mites during the development of rosacea.

In conclusion, an animal model for *Demodex* mite-induced rosacea-like skin lesions was successfully developed by injecting the suspension of *Demodex* mites into the skin of Japanese rabbits. The model rabbit showed skin lesions similar to those in rosacea patients infected with *Demodex* mites. A granuloma-like structure could also be observed in the lesions. This animal model provides a new platform for further exploring the underlying mechanism of and developing new drugs against *Demodex* mite-induced rosacea.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YL conceptualized the present study, provided methodology and conducted project administration. XL provided methodology, performed validation and formal analysis and wrote the original draft of the manuscript. NZ and ZJW provided resources and conducted data curation. LXW provided methodology, curated data and performed visualization. JHZ performed the experiment and provided resources. XLL wrote, reviewed and edited the manuscript and performed formal analysis. XL and NZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. 2019KYLL006) by the Ethics Committee of the 940th Hospital of the Joint Logistics Support force of the Chinese People's Liberation Army (Lanzhou, China). All participants provided written informed consent prior to enrollment.

Patient consent for publication

The patient with image presented in Figure 1 consent for publication of their images.

Competing interests

The authors declare that they have no competing interests.

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