Comparison and Evaluation of 2,4-Dinitrofluorobenzene Induced Allergic Contact Dermatitis Model in Mice

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Research

Keywords: Allergic contact dermatiti,; 2,4-dinitrofluorobenzene, itch, animal model

Posted Date: October 27th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-988811/v1

Abstract

Objectives

2,4-Dinitrofluorobenzene (DNFB) induced allergic contact dermatitis model is widely used in the research of inflammation, immune response and pruritus. However, there are several modeling cycles of DNFB model in many literatures, which brings some confusion for choice in the experiments. Here, we established two-weeks and five-weeks models of DNFB and aimed to compare and evaluate them.

Methods

Two-week and five-week DNFB models were established in male SPF C57BL/6 mice.

Results

The results showed that the scratching bouts of the ACD mice in two groups both significantly increased. Following the modeling process, the skin of the neck and back of the modeling site of the ACD mice in two-weeks group gradually appeared erythema, edema, ulceration, scabbing, scaling and dryness. The skin surface features of ACD mice in five-weeks group obviously showed erythema, edema, ulceration, scabbing, scaling, re-scabbing, re-scaling, harden and dryness.The whole modeling process are both accompanied by severe scratching reaction after the skin repeated exposure to DNFB. Moreover, the results of hematoxylin-eosin (HE) staining showed that the two modeling methods both had thickened epidermis ($p < 0.001$) and hyperkeratosis. The toluidine blue (TB) staining revealed obvious inflammatory cell infiltration ($p < 0.0001$) in two groups.

Conclusion

In our opinion, the two modeling methods both are feasible. The two-weeks ACD model had a shorter modeling period, and while the 5-weeks model could have enough time to observe the effect of drug. Our results provided further experimental basis and evidence for animal models of skin pruritus and inflammation.

Introduction

Allergic contact dermatitis (ACD) is known as a delayed hypersensitivity inflammatory reaction [1, 2], that occurs when the skin or mucous membrane is exposed to certain external irritants or allergens [3, 4]. This reaction is mediated by a hapten-specific T cell-dependent reaction. After the sensitized body is attacked by antigen, it is mainly characterized by the infiltration of inflammatory cells and the release of inflammatory factors. The clinical symptoms are mainly allergic contact reactions such as erythema, erosion, scab, edema and itching in the sensitized skin [5–7]. The T cells in the delayed hypersensitivity

reaction play a key role in transplant rejection, graft-versus-host disease, autoimmunity and tumor immunity [8]. Therefore, studying the effect of drugs on the induction and regulation of ACD is very important for finding new drugs and elucidating the mechanism of action.

A good experimental animal disease model is helpful for people to understand the occurrence and development laws of human diseases more conveniently and effectively, and to study preventive measures. The allergic contact dermatitis model induced by 2,4-Dinitrofluorobenzene (DNFB) is one of the most commonly used animal models in the research of inflammation or chronic pruritus [9–14]. A good animal model must have the characteristics of accurate replication and similarity with clinical pathogenesis, which plays a key role in the research of disease pathogenesis and efficacy. DNFB is a hapten that activates skin keratinocytes and produces cytokines after the first contact with the body [15]. Cytokines combine with antigen-presenting cells to transfer to local lymph nodes, activate antigen-specific T cells, and make them produce memory T cells [16]. This process is called the sensitization stage [3]. After 4-7 days of sensitization, apply the hapten DNFB to the skin of the ear or the neck and back to supplement and activate memory T cells. Cytokines are produced by helper T cells 1 (Th1 cells) and helper T cells 2 (Th2 cells) to cause local delayed allergic reactions [4, 14, 17, 18]. This reaction generally occurs about 18 hours after exposure to the antigen and reaches a peak in 24-48 hours. There are literatures using the ACD two-week model to study the role of Toll-like receptors [19] and monoclonal antibody NaV1.7 channels in chronic pruritus [10, 20–22], while studying the development of chronic pruritus in sensory neurons requires the use of ACD in the BRAF signaling pathway for five weeks Model [13]. In order to clarify a more suitable animal model during the actual operation, this study compared and evaluated the two modeling methods of DNFB-induced contact allergic reaction in mice.

Material And Methods

Animals

Male SPF C57BL/6 mice, weighing approximately 20-25 g, are provided by Changzhou Cavens Laboratory Animal Co., Ltd., free to eat and water during the feeding and experiment period. The ambient temperature is 20-25°C, and the relative humidity is 40 %-60%. All experimental protocols described in this study were followed the protocol outlined in the Guide for the Care and Use of Laboratory Animals of Anhui Medical University Animal Care Committee, which comply with the National Institutes of Health Guidelines for the Care and Use of laboratory Animals in ARRIVE guidelines.

Chemicals and reagents

DNFB was purchased from sigma, batch number: BCCB1276, ready for use. Acetone (Hefei Xinle Co., Ltd.), batch number: 190131. Olive oil (Aladdin), batch number: K1922143. Paraformaldehyde (Aladdin) batch number: HJ201403. physiological saline (Shandong Qidu Pharmaceutical Co., Ltd.) batch number: 2B20012105.

Two weeks ACD model established by DNFB

ACD model was induced by repeated exposure mice skin to DNFB-acetone solution [4, 22–24]. DNFB was dissolved in a mixture of acetone:olive oil (4:1) [15]. C57BL/6 male mice were randomly divided into normal saline control, solvent control (acetone: olive oil 4:1) and DNFB model, with 5 mice in each group. After a week of adaptive feeding, the rostral area of the neck and the abdomen of all mice were shaved and the shaving area were about 2cm×2cm. Two days later after shaving, 0.5% DNFB-acetone solution 50μL was dripped on the shaving area of the abdominal skin, and evenly applied for initial sensitization [25]. Repeat the operation at the next day for intensive sensitization. After 5 days, 50μL of 0.15% DNFB was dripped into the shaved part of the neck and back of mice in DNFB model, and evenly smeared. The saline control mice and the solvent control mice were dripped with 50μL saline and 50μL acetone olive oil (4: 1) respectively. And the challenge with 50μL 0.15% DNFB-acetone solution was painting on first, third, fifth and seventh day [9, 10]. The specific timeline and sequence could be seen in Figure 1A. The video of itching behavior of mice was recorded for 60 minutes after each application 24 hours.

Five weeks ACD model established by DNFB

The intervention measures are similar as the two-week ACD model. C57BL/6 male mice were randomly divided into saline control, solvent control (acetone: olive oil 4:1) [17, 26] and DNFB model, with 5 mice in each group. Mice were shaved before the experiment. 0.15% DNFB 100μL were dripped on the shaving area of mouse abdomen, and evenly smeared for sensitization. After sensitization for one week, 0.15% DNFB 50μL was applied to the shaving area of the neck and back of model mice twice a week for four weeks, nine times in total [14, 27]. The control mice were coated with saline and acetone olive oil (4:1) respectively. The specific treatment and intervention measures were shown in Figure 1B. After each treatment for 24 hours, itch behavior video was recorded continuously for 60 minutes.

Scratching behavior test

Behavioral tests were videotaped using SONY digital video camcorders [13, 28, 29]. On the test day, Mice were placed in a plastic arena (10×10×13 cm) and given at least 10 minutes to get accustomed to recording conditions prior to recordings. Then mice were recorded for 60 minutes for spontaneous scratches. Videos were played back on a computer for assessments by observers [28–31]. Hind limb scratching behavior towards the painted area was observed for 60 minutes. A scratch is defined as a bout of scratching that occurs after the mouse lifts its hind paw to the moment the hind paw is returned to the ground or mouth [32, 33].

Skin morphology studies and histological observation

The skin which was challenged were fixed by 4% paraformaldehyde, and then the paraffin sections were made to be stained with hematoxylin-eosin (HE) solutions and toluidine blue (TB) staining [13]. And the staining images were visualized under a microscope, and images were taken. Finally, Image J software was used to measure the relative thickness of the epidermis from HE staining images and count the number of mast cells from TB staining images [13, 31].

Statistical analysis

Results are expressed as mean ± SEM of all independent experiments. Statistical analysis was conducted using GraphPad Prism 8.0 statistical software. For multiple-group comparisons, a one-way variance analysis (ANOVA) with Bonferroni's multiple comparison test were used. When $p < 0.05$, the difference is significant.

Results

Scratching behavioral changes in two-week and five-week ACD mice model induced by DNFB

The experimental results were shown in Figure 2. The number of scratches in the DNFB two-week ACD model increased significantly and showed an upward trend. The number of scratching in the saline control group and the vehicle control group did not increase significantly (Figure 2A). In the five-week ACD model group of DNFB, the scratches increased obviously. And the scratching times of hind limbs of ACD model mice in saline control group and solvent control group had no obvious increasing trend (Figure 2B). At the end of the two-week model building cycle, the number of scratches in the model group was significantly increased compared with the two-week model's normal saline control group and solvent control group, and the difference was statistically significant (Figure 2C, **** $p < 0.0001$). At the end of the five-week model construction period, the number of scratches in the model group was significantly higher than that in the saline control group and the solvent control group and was statistically significant (Figure 2D, **** $p < 0.0001$).

Skin gross morphology changes in two-week and five-week ACD mice model induced by DNFB

The experimental results are shown in Figure 3. In the two modeling methods, the skin surface of the neck and back of the mice in the saline control group and the vehicle control group has no obvious change, and the mice had better mobility. With the progress of the modeling process, the skin of the two-week model mice was ulcerated on the fifth day after applying DNFB on the neck and back. On the seventh day after applying DNFB on the neck and back, the skin in the ulceration area became crusted and hardened. At the end of modeling of two weeks, the skin was scabbed (Figure 3C). On the eighth day after applying DNFB on the neck and back of five-week model mice, the skin at the neck and back modeling site ulcerated. On the eleventh day, the skin had scab before administration. In the fifteenth day of administration, the scab of the skin had faded, and the skin under the scab was exposed and was dry. Before administration on the eighteenth day, the skin ulceration at the modeling site was aggravated. Before administration on the twenty-second day, the skin began to scab, and the skin around the administration area began to turn up. Before the administration on the twenty-fifth day, the turned skin around the administration area of the mice began to fade and the skin ulcerated. At the end of the five-week model, the scab on the neck and back fell off and exposed the skin (Figure 3F).

Skin histopathological changes in two-week and five-week ACD mice model induced by DNFB

As shown in Figure 4, HE staining results showed that the skin epidermis thickness of mice in the two-week model saline control group and solvent control group had no obvious thickening and the edge was clear (Figure 4A, 4B). Compared with saline control group and solvent control group, the model group mice obviously thickened the skin epidermis at the modeling site (Figure 4C), and the difference was significant (****$p < 0.0001$, Figure 4G). The skin at the modeling site was hyperkeratosis, spongy degeneration and dermal capillary dilatation. There was no obvious thickening of skin epidermis thickness at the modeling site of saline control group and solvent control group in the five-week model (Figure 4D, 4E). Compared with normal saline control group and solvent control group, the skin epidermis in the model group is obviously thickened (Figure 4F), and the difference is statistically significant (**** $p < 0.0001$, Figure 4G), dermal capillaries dilate, skin hyperkeratosis and spongy degeneration.

Inflammatory mast cell changes in two-week and five-week ACD mice model induced by DNFB

The experimental results are shown in Figure 5. Compared with the mice in the control group and the vehicle control group, the skin of the two-week ACD model group has obvious infiltration of mast cells (Figure 5C), and the difference is statistically significant (Figure 5G, **** $p < 0.0001$). Compared with the mice in the saline control group and the vehicle control group, the skin of the five-week ACD model group showed obvious infiltration of mast cells (Figure 5F), and the difference of mast cell count was significant (Figure 5G, **** $p < 0.0001$).

Discussion

ACD is a chronic skin disease with recurrence, pruritus and inflammation [34]. It is seriously affecting human health and quality of life [35–37]. ACD is caused by delayed allergy, and patients are often accompanied by itching, so itching is the most prominent clinical symptom of the disease [38]. Studies have shown that local sensitization by exogenous allergens on the basis of the destruction of the skin barrier is an important basis for inducing ACD [7], and the immune inflammatory response is mainly based on the secretion of IL-4 by Th2 cells [39, 40].

ACD is a common inflammatory skin disease caused by the skin or skin mucous membrane contacting with external allergens. It usually needs to be connected by proteins in the epidermis to form a new antigenic determinant, causing immune system activation [41, 42]. Primary irritant contact dermatitis is a strong irritation of the contact material to the skin, and dermatitis occurs after contact. The form, scope and severity of dermatitis are different due to the different contact modes and individual reactions. In severe cases, the erythema is swollen and there are many papules and blisters. When the inflammation is severe, the blisters may rupture, erosion, exudate crusting and peel off. The ACD model established by

DNFB is one of the most commonly used models [4, 10]. DNFB is a hapten of small molecular substance, which is easy to combine with skin tissue protein without significantly changing the internal structure of tissue protein. The DNFB-induced ACD model, as a model of immunity and itch, plays an important role in the research of T cell inflammation-related and itch [3].

In this study, the two-week model [9, 10] and the five-week model of ACD [13, 14] in mice were established by using the methods of DNFB abdominal sensitization and neck back excitation. The modeling methods of ACD are compared in order to provide sufficient experimental and theoretical basis for the selection of appropriate animal models for research in related fields. According to the experimental data, the number of scratches in the ACD model mice induced by the two methods was significantly increased ($p < 0.0001$), which indicated that there were significant differences in itch behavior: the itch behavior results of ACD two-week model and ACD five-week model mice increased significantly and showed an upward trend, while the itch behavior results of normal saline control group and solvent control group had no obvious upward trend. In addition, the HE staining results of the skin where the mice were modeled by the two modeling methods showed that the epidermis was thickened ($p < 0.0001$), hyperkeratosis, and telangiectasia. The results of toluidine blue staining showed the infiltration of mast cells in the model group obviously ($p < 0.0001$). Therefore, the results of this study show that the two methods of DNFB-induced ACD can successfully produce inflammation in the skin of the mouse model, and there are significant differences in itch behavior. However, in this experiment, the two modeling methods caused different changes in the appearance of the skin of the mouse model. The two-week model modeling cycle was relatively short. After DNFB was administered on the back and neck, the skin at the administration site of mice festered and hardened. The shape, scope and degree of dermatitis in the neck and back of the five-week model mice were more serious than those of the two-week model. After the administration of DNFB, the skin was ulcerated, crusted, scabed, re-crusted, and then dropped. Therefore, we believe that in the experimental research, we can choose the appropriate modeling time in combination with our research field and research direction. For the study of pharmacokinetics, the five-week model may be more suitable, and there is enough time to observe the effects of drugs.

Abbreviations

ACD allergic contact dermatitis

DNFB 2,4-Dinitrofluorobenzene

HE hematoxylin-eosin

TB toluidine blue

Th1 helper T cells 1

Th2 helper T cells 2

BRAF serine/threonine kinase

IL-4 interleukin-4

Declarations

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to being interim data from a trial.

Acknowledgements

Thanks to all participants for their help in the experiment, data processing and English editing.

Funding

This work was supported by the Basic Research Project (Natural Science Foundation) of Shenzhen Science and Technology Innovation Committee (JCYJ20190809110403601) awarded to Prof. Lanlan Zhou, Department of Medical College, Shenzhen Polytechnic.

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L.L Zhou and X.X Zhu developed the concept and designed the experiment. X.X Zhu performed the experiments. Y.C Zhao analyzed all the data; F. Bian performed the behavior test. W.Z Li provided helpful suggestions. X.X Zhu and L.L Zhou wrote the manuscript. All authors approved the final version of the manuscript.

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Ethics approval and consent to participate

Not applicable

Consent for publication

All authors understand the requirements of the journal and agree to publish.

Interest competition

All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

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Figures

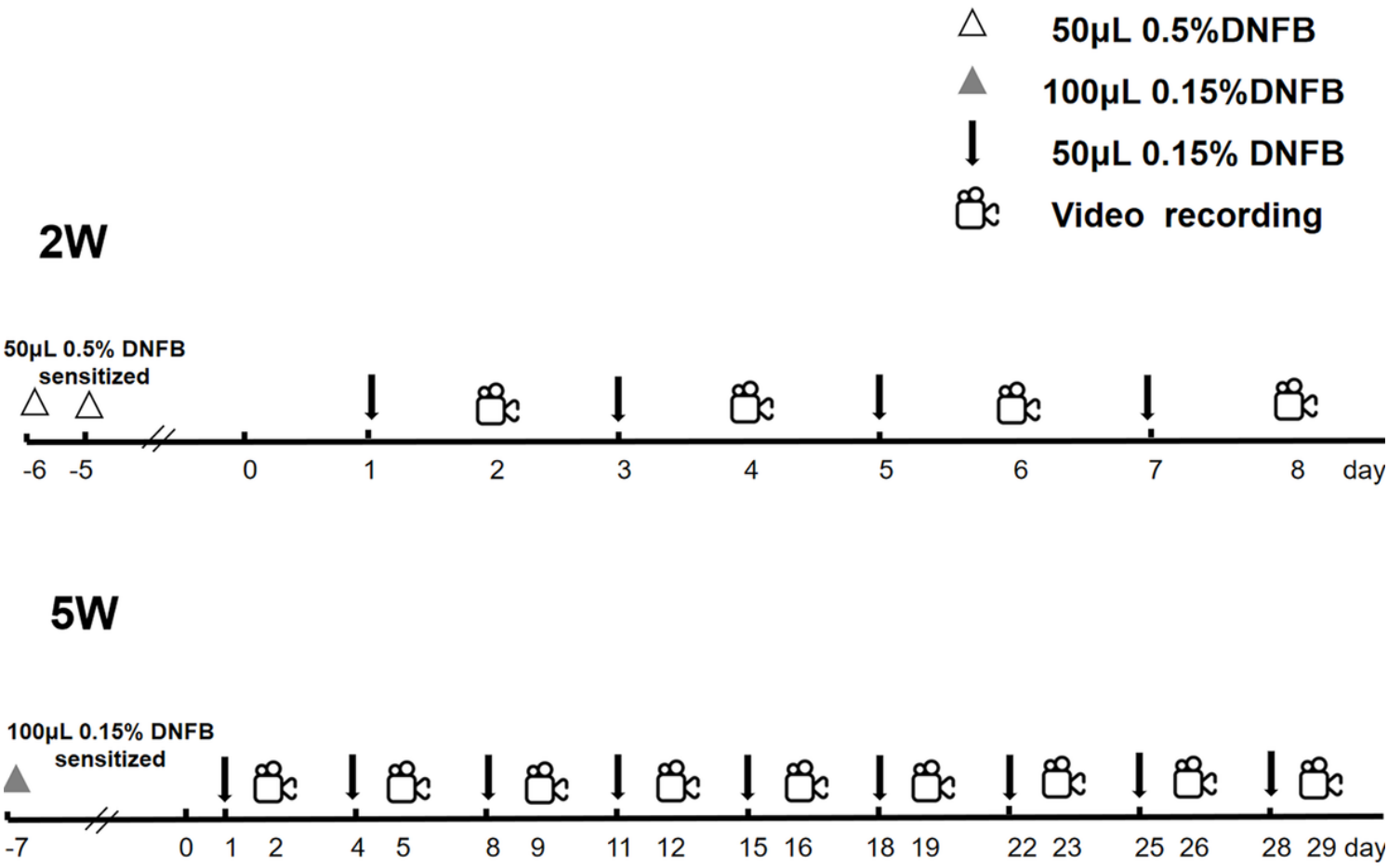

Figure 1

Protocol for two-week and five-week ACD model induction in mice. Two-week ACD model building. DNFB was dissolved in a mixture of acetone: olive oil (4:1). Mice were sensitized with 50 μL 0.5% DNFB solution by topical application of shaved abdomen skin. Repeat the operation on the next day. 5 days later, mice were challenged with 50μL 0.15% DNFB solution by painting the nape of neck, then on day 1, 3, 5, and 7. Scratching behaviors were video-recorded on 2nd, 4th, 6th, 8th for 60 min. Five-week ACD model building. Mice were painted with 100 μL 0.15% DNFB acetone solution onto the clipped abdominal skin for sensitization. Then 50 μL 0.15% DNFB challenge was repeated twice a week for 4 weeks, 9 times in total, from 7 days after the sensitization. The video of itching behavior was recorded for 60 minutes after 24 hours of each challenged on the nape of neck (n = 5).

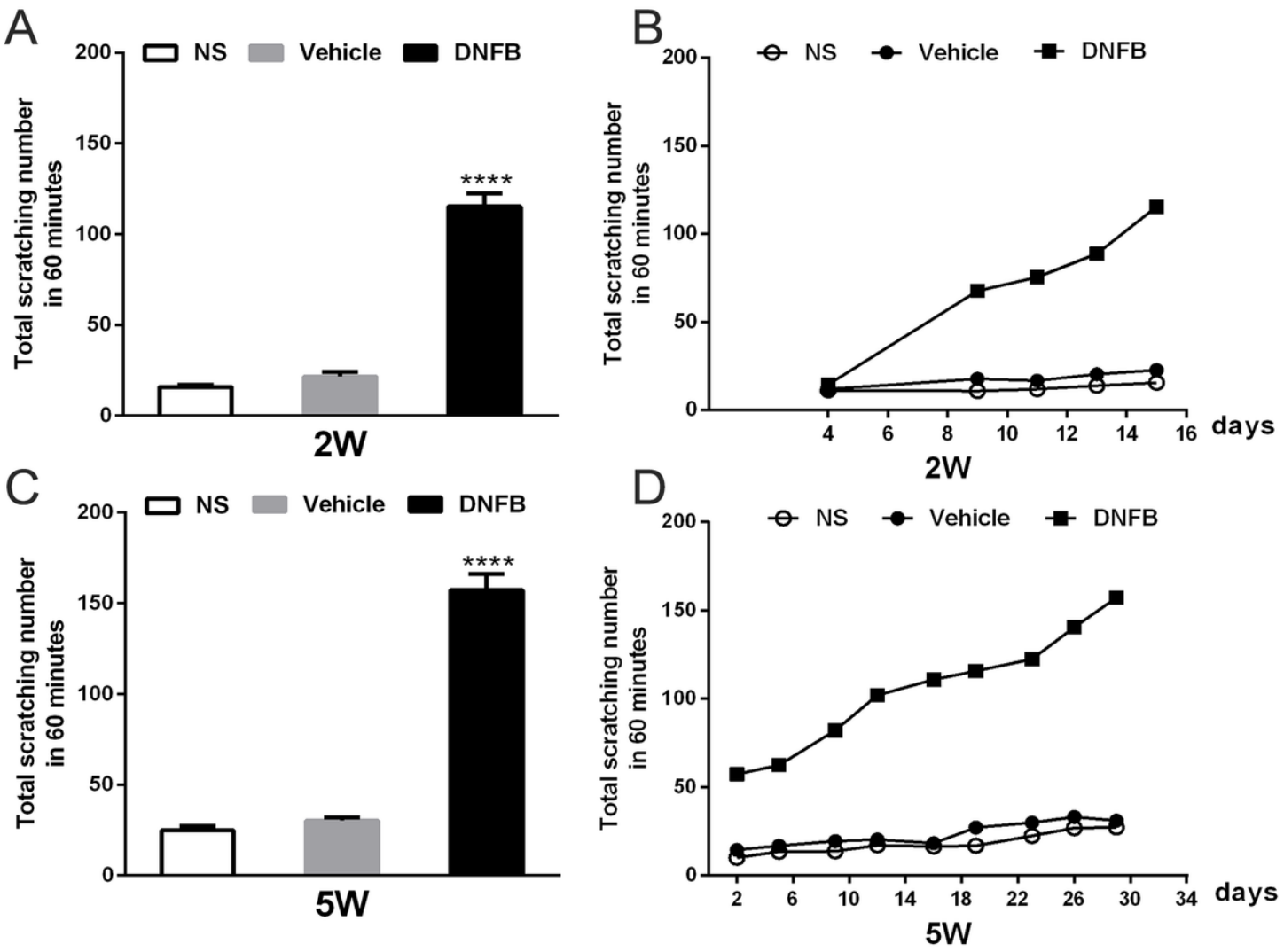

Figure 2

Comparison of the scratching behavior changes in two ACD models. (A) Total scratching number in 60 minutes during the two-week model building cycle. With the progress of model building, there is an upward trend. (B) The total number of scratches for 60 minutes in the five-week molding cycle. And the number of scratches increased significantly as the mold was made. (C) The scratches number at the end of the two-week model building. Analysis is statistically significant, **** $p < 0.0001$. (D) The number of scratches at the end of the five-week model making. And the difference was statistically significant, **** $p < 0.0001$. All data are expressed as the mean ± SEM (n=5). **** $p < 0.0001$ compared with control groups.

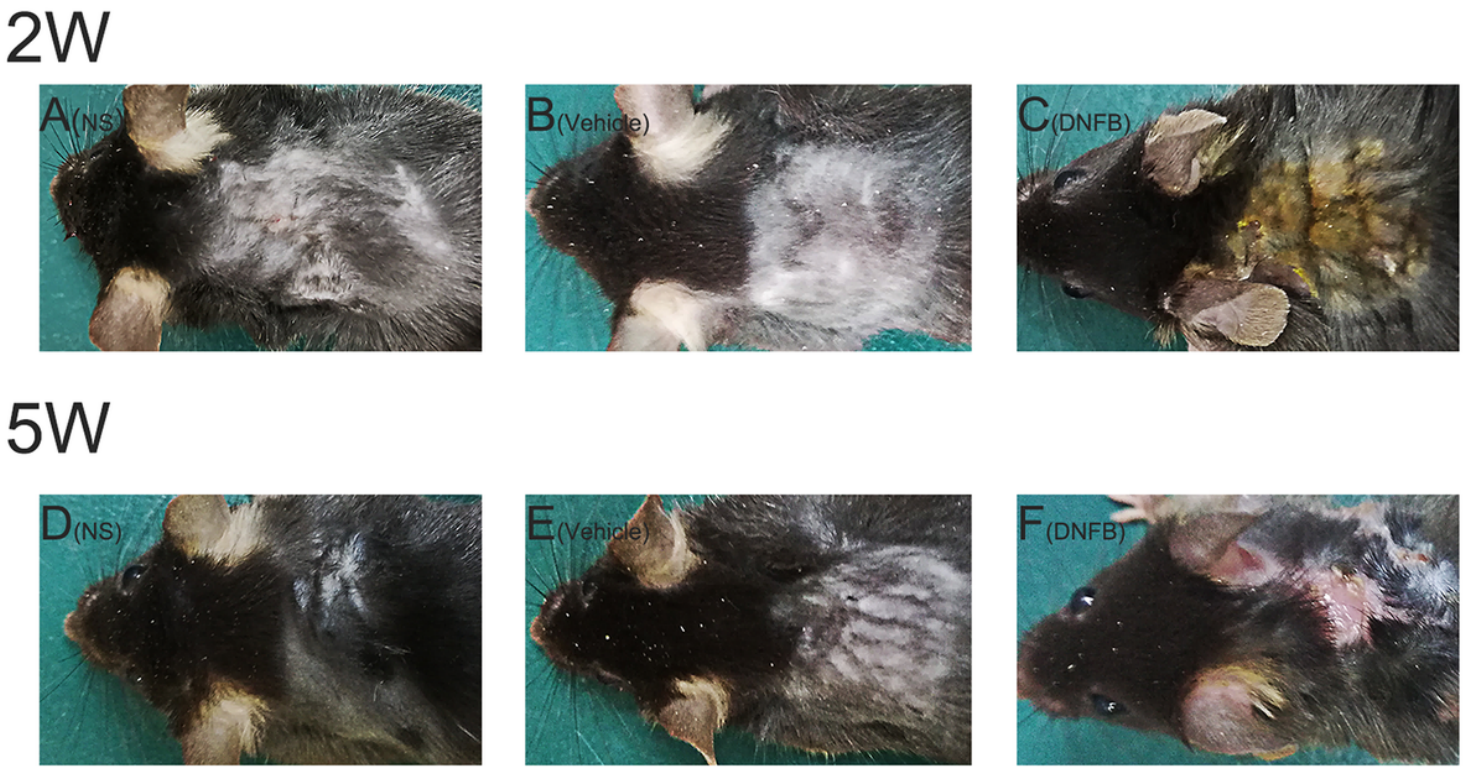

Figure 3

Comparison of the skin appearance changes in two ACD models. (A-C) Representative image of the appearance of the skin of each group at the end of the two-week ACD model on day 8. (A) Saline control group. (B) Solvent control group. (C) DNFB model group: mice in the model group developed inflammation, and the skin at the challenged became scabs and hardened at the end of the two-week ACD model (n = 5). (D-F) Representative image of skin appearance of each group at the end of the five-week ACD model building on day 29. (D) Saline control group. (E) Solvent control group. (F) DNFB model group: At the end of the five-week DNFB-induced ACD model, the skin of the model group mice was severely damaged and ulcerated (n = 5).

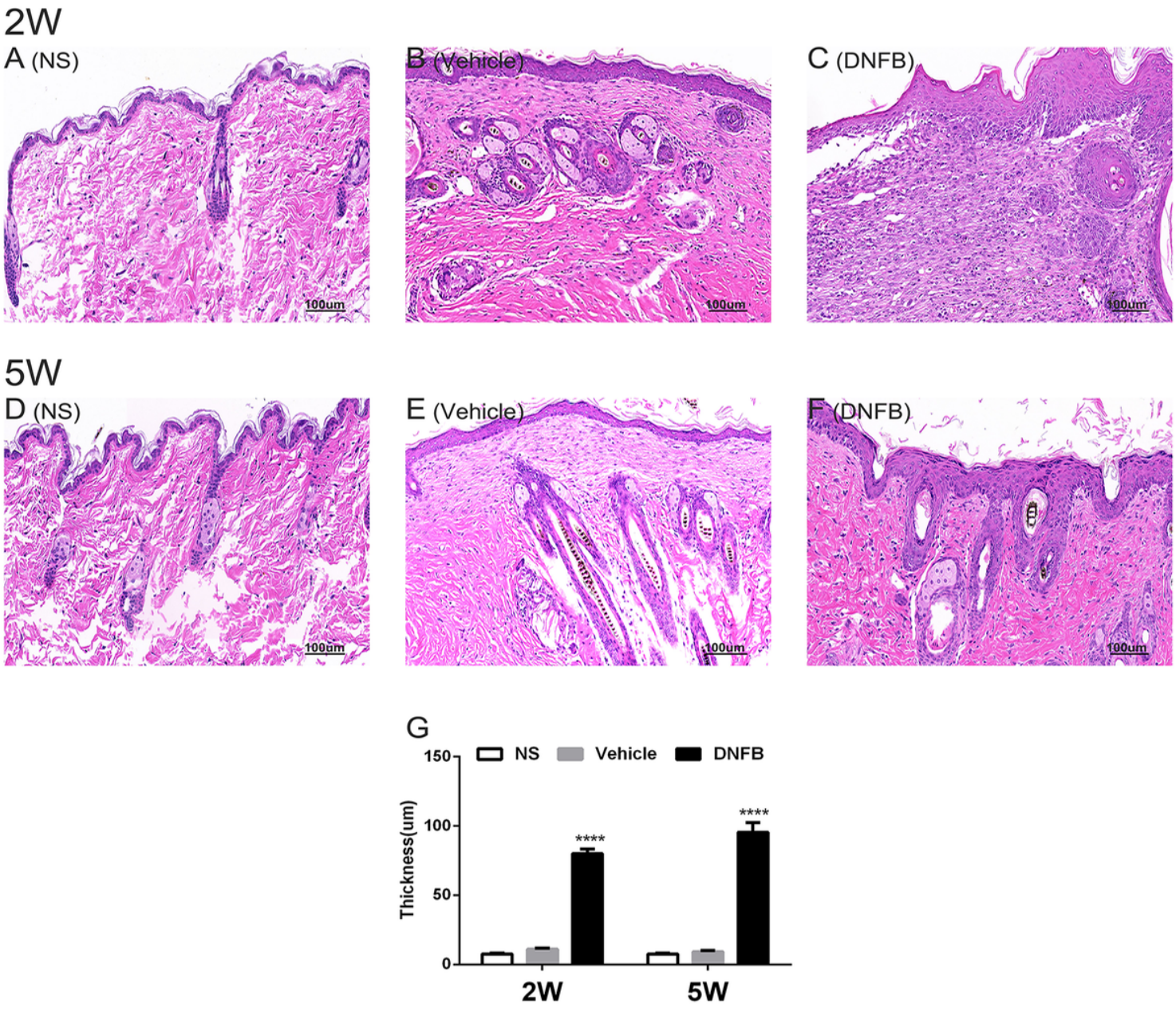

Figure 4

Comparison of the skin histopathological changes and epidermal thickness variation in two ACD models. (A-C) HE stained (100 μm) representative images of skin epidermal thickness changes in each group after completing the DNFB two-week ACD model modeling. (A) Saline control group. (B) Solvent control group. (C) DNFB model group: Telangiectasias and epidermal thickening are obvious. (D-F) Representative image of HE staining of skin thickness at the end of the five-week ACD model. (D) Saline control group. (E) Solvent control group. (F) DNFB model group: Dermal capillaries are dilated, and the epidermis is significantly thickened. (G) Statistical analysis of the degree of skin epidermal thickening at the two ACD models: The two-week ACD model group has obvious epidermal thickening and the difference is statistically significant, **** $p < 0.0001$. The thickening of the epidermis in the model group is significant

in the five-week ACD model and has analytical significance, **** p < 0.0001. All data are expressed as the mean ± SEM (n=5). **** p < 0.0001 compared with control groups.

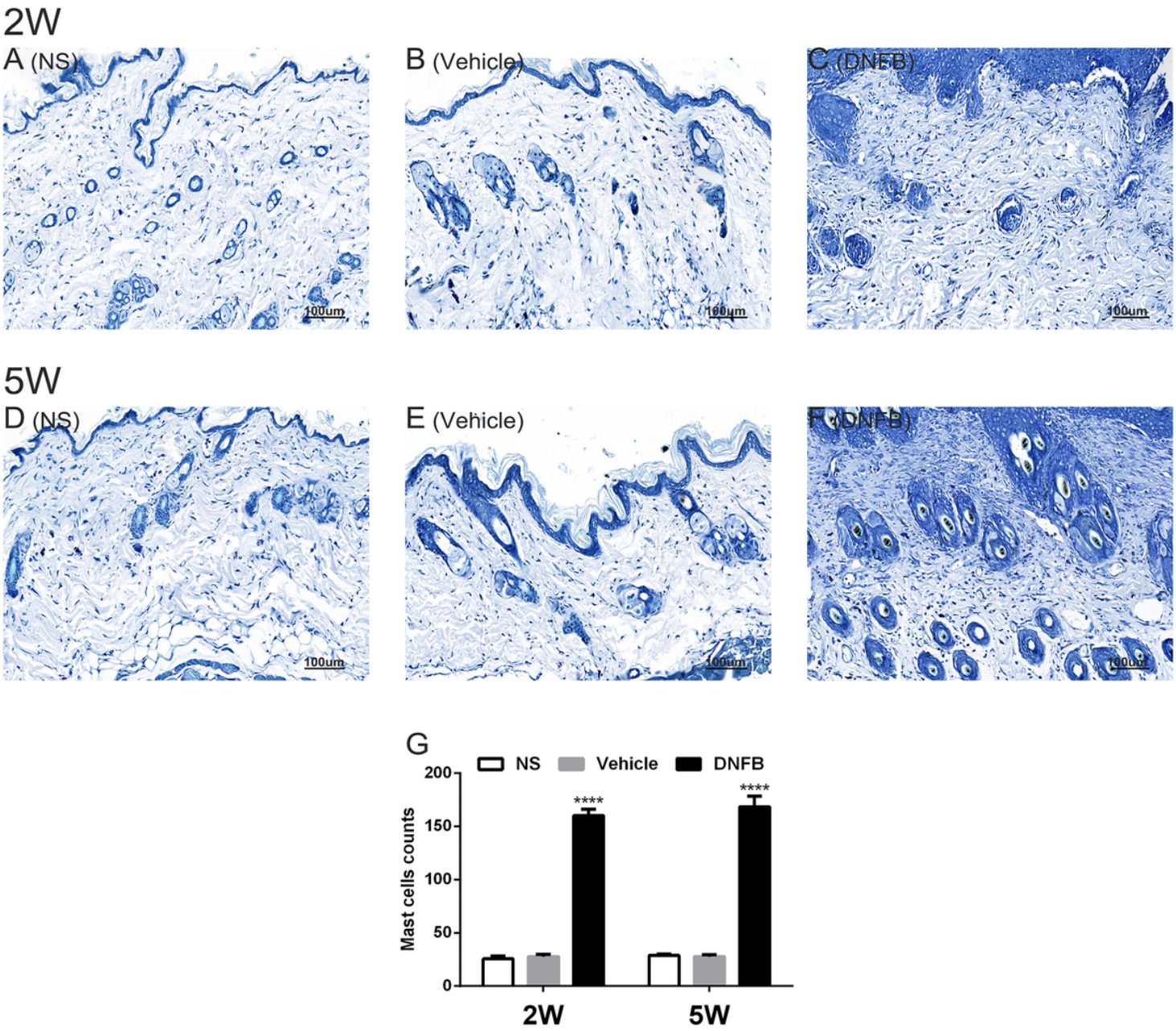

Figure 5

Comparison of the inflammatory mast cell changes in two ACD models. (A-C) Representative images of mast cell counts by TB staining (100 μm) in each group after the completion of the DNFB two-week ACD model modeling. (A) Saline control group. (B) Solvent control group. (C) DNFB model group: Mast cell infiltration is obvious. (D-F) The representative image of TB staining and mast cell counts at the end of the five-week ACD model. (D) Saline control group. (E) Solvent control group. (F) DNFB model group: Mast cell infiltration. (G) Statistical analysis of the mast cell counts results after the completion of the two ACD models. In two-week ACD model, mast cell infiltration is obvious and the difference is significant, **** p < 0.0001. The difference in the counts of mast cells with toluidine blue stained after

five-week ACD model was statistically significant, **** $p < 0.0001$. All data are expressed as the mean ± SEM (n=5). **** $p < 0.0001$ compared with control groups.