



Research Article

Therapeutic Application of Glutinous Rice Ferment Extract for Atopic Eczema

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Abstract

Atopic eczema is a relapsing inflammatory skin condition which is characterized by severe itching, polymorphic skin lesions, repeated attacks and so on. Current research believes that there are two major links in the occurrence of eczema, including immune abnormalities of allergic inflammation and damage to the skin barrier. In this paper, the anti-eczema and antipruritic effects of glutinous rice ferment extract were explored through in vitro and animal experimental models. We found that glutinous rice ferment extract could not only reduce TSLP and reduce inflammation, but also repair damaged skin barrier by upregulating the expression of filaggrin. On animal models, the basic formula containing glutinous rice ferment extract could significantly increase the itching threshold and relieve itching. In summary, glutinous rice ferment extract has been shown to have a good anti-eczema effect, which provides a model for the treatment of eczema.

Keywords: Glutinous Rice Ferment Extract; Anti-Eczema; Skin Barrier; TSLP; Itching

Introduction

Studies believe that the main pathogenesis of eczema is related to the imbalance of Th2 immune response, and related inflammatory factors play a very important role [1-3]. TSLP (thymic stromal lymphopoietin) is a key “initiator” of the Th2 immune response, mainly expressed in epithelial cells and keratinocytes. On the one hand, after TSLP activates DC, it can promote the proliferation and differentiation of naïve CD4⁺ T cells into inflammatory Th2 cells [4, 5]. On the other hand, TSLP directly acts on natural immune cells, stimulating mast cells to produce a series of inflammatory factors to promote allergic reactions [6, 7]. Simpson, a dermatologist at Oregon Health and Science University, believes that TSLP is the hub of contact between epithelial cells and the immune system and participated

in a phase H trial of an anti-TSLP antibody called tezepelumab to treat eczema [8].

Numerous studies have shown that infantile eczema is closely related to skin barrier dysfunction [9-11], and the literature points out that it is mainly manifested as abnormal keratinocyte connections of the skin barrier caused by FLG gene mutations (FLG, filaggrin) [12, 13].

In addition, itching is a major manifestation of eczema, because the scratching caused by itching further aggravates the damage of the barrier, and the damage of the barrier further brings allergic inflammatory reactions, exacerbating eczema symptoms such as itching and dry redness, resulting in a vicious circle [14-16]. Therefore, effective itching relief may be an important entry point to solve the problem of eczema.

In recent years, fermentation broth, especially rice fermentation broth, has attracted extensive research work and

is widely used in skin care because it contains more beneficial substances such as polyphenols, flavonoids and polysaccharides [17, 18]. Using a unique solid-state layered brewing technology, we ferment the seed mash which contains diverse microorganisms and various enzyme systems by traditional inoculation method to obtain the preliminary ferment. And through 3 rounds of extraction, separation and molecular biology research, we finally obtained a kind of excellent rice fermentation broth, glutinous rice ferment extract (solid content is 1%, namely 10 mg/mL). Preliminary findings have found that rice ferment has skincare effects [19]. For example, Elena Babini et al. found that rice ferments displayed in vitro antioxidant, cell-based anti-inflammatory and anti-aging effects [20]. This inspired us to study the therapeutic effect of glutinous rice ferment extract on eczema, hoping to provide new ideas for the cure of eczema.

As mentioned, we used eczema-like 3D skin model, ELISA technology and qPCR technology to study the mechanism of glutinous rice ferment extract in the treatment of eczema, and through the animal itching model, the itching threshold was used as an indicator to investigate the antipruritic effect of glutinous rice ferment extract. The results showed that glutinous rice ferment extract had a good anti-eczema effect, which may be achieved by inhibiting the inflammatory cascade caused by TSLP while repairing the skin barrier and raising the itching threshold. In summary, this paper provides a theoretical basis and basis for the treatment of eczema with glutinous rice ferment extract, and also provides new ideas and perspectives for fighting eczema.

Materials and Methods

Experimental Model

The cells used in this test are human skin primary keratinocytes (EP200708). The skin model is the 3D epidermal skin model Epikutis® (ES210402). They are both produced and provided by Guangdong Boxi Biotechnology Co., Ltd. The animal model was guinea pigs (100 animals, 300 g each).

Main Reagents

Phosphate buffer solution (PBS, Solebo), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma), DMSO (Sigma), Histamine phosphor (Shanghai Yuanye Biotechnology Co., Ltd.), Clobetasol propionate cream (Jiangsu Yuanhuan Pharmaceutical), KC-Growth Culture Medium (Guangdong Boxi Biotechnology), Epi-Growth Culture Medium (Guangdong Boxi Biotechnology), Human TSLP ELISA Kit (Boxi Bio), Isopropanol (Sinopharm), PolyI:C (Sigma), Lipopolysaccharide (E.Coli. Sigma), RNAiso Plus (TaKaRa), ethanol (Sinopharm), DEPC water (Beyotime), reverse transcription kit (TaKaRa), fluorescent dye (TaKaRa).

The composition of matrix used in animal antipruritic experiments included the following ingredients: aqua, glycerin, petrolatum, cetearyl ethylhexanoate, isopropyl myristate, dicaprylyl ether, cetearyl alcohol, persea gratissima (avocado) oil, stearic acid, behenyl alcohol, glyceryl stearate, cetyl alcohol, palmitic acid, lecithin, myristyl alcohol, lauryl alcohol, dimethicone, dimethiconol, beeswax, cetyl palmitate, panthenol, hydroxyacetophenone, tocopheryl acetate, allantoin, xanthan gum, tromethamine, pentaerythrityl tetra-di-t-butyl hydroxyhydrocinnamate and acrylates/c10-30 alkyl acrylate crosspolymer.

Experimental Equipment

CO₂ incubator (Thermo, 150I), Biosafety cabinets (SW-CJ-1F), Inverted microscope (Olympus, CKX41), Microplate reader (BioTek, Epoch), High-speed refrigerated centrifuge (Changsha Xiangyi, H1850R), PCR instrument (BIO-RAD, TC-XP-G), Quantitative real-time PCR system (BIO-RAD, CFX96), digital display constant temperature water bath (Changzhou Guohua, HH-4A), stratum corneum moisture analyzer (Corneometer® CM825, Courage & Khazaka, Germany), closed skin trans-epidermal water loss meter (AquaFlux Model AF200, Biox Systems Ltd, UK), dermatomelanin and heme analyzer (Mexameter MX18, Courage & Khazaka, Germany).

Experimental Methods

The construction of eczema-like skin model

Firstly, the polyinosinic acid-polycytionate sodium salt and lipopolysaccharide (PolyI: C+LPS) induction working solution was prepared. Specifically, 1.44 mL of 5 mg/mL PolyI:C mother liquor and 3 mL of LPS mother liquor of 2 mg/mL were dissolved in the skin model culture medium for a total volume of 300 mL, so that the final concentration of PolyI:C is 24 µg/mL and the final concentration of LPS is 20 µg/mL.

Then, a working solution of the positive control group (0.01% dexamethasone) is prepared. 100 mg of dexamethasone was dissolved with 1 mL of DMSO to prepare a mother liquor at a concentration of 100 mg/mL. The above mother liquor was diluted 1000 times with skin model medium to obtain 0.01% dexamethasone and left for later use.

The group-labeled 6-well plates were prepared in advance and small molds were placed into the wells. The corresponding 3.7 mL of working solution was added into the prepared 6-well plates. Specifically, fresh culture solution was added into the BC group and NC group, and the corresponding working solution was added to the PC group and sample group. Then the skin model was transferred to the 6-well plate and incubated for 24 h in a CO₂ incubator (37°C, 5% CO₂).

Again, small molds and 3.7 ml of corresponding working solution were added to the prepared new 6-well plate, i.e., fresh normal culture solution was added to the BC group, and the induction working solution was added to the NC group, PC group, and sample group. After the dosing incubation is complete, the skin model is washed with sterile PBS and wiped with a sterile cotton swab. The skin model was then transferred to fresh, pre-prepared 6-well plates. Finally, the 6-well plates were incubated in a CO₂ incubator (37 °C, 5% CO₂) for 24 h.

Test of inflammatory factor TSLP

After 24 h of induction incubation, the model culture was collected into EP tubes. After that, TLSP assays were performed according to the protocol of the ELISA assay. If the TLSP detection could not be performed in time, the model should be stored at -80°C.

Real-time quantitative PCR experiment

The collected skin models were washed with PBS and cut off with a scalpel before loading into enzyme-free EP tubes. Each tube was added with 1mL RNAiso Plus and stored at -80°C for later use. According to the protocol, mRNA extraction and purification were performed sequentially with RNAiso Plus, chloroform, isopropanol and ethanol. The extracted mRNA was reverse-transcribed and the corresponding cDNA was obtained. After that, real-time fluorescence PCR quantification was performed on a real-time PCR instrument, and the relative gene expression between groups was calculated by 2^{-ΔΔCT} method.

$$\Delta C_T = C_T (\text{Target gene}) - C_T (\text{Reference gene})$$

$$\Delta\Delta C_T = \Delta C_T (\text{Experimental group}) - \Delta C_T (\text{Control group})$$

$RQ = 2^{-\Delta\Delta CT}$, RQ is the fold difference between the expression of the target gene in the control group and experimental group.

Establishment of an animal antipruritic model

This experimental protocol referred to the third edition of the Chinese medicine new drug research guide.

The day before the experiment, the sample was applied to the dorsum of the right hind foot of each group of guinea pigs. On the day of the experiment, the dorsal shaving area of the animal's right hind foot was scraped with coarse sandpaper, about 1 square centimeter, and then the sample was applied there once. Simultaneously, the blank control group was given the same amount of distilled water. 10 min later, 0.01% phosphor histamine was started to drop on the trauma surface, 0.05 mL for each. After that, the concentration was gradually increased by 0.01%, 0.02%, 0.03%, 0.04%..... every 3 minutes, and each time was 0.05 mL/piece. It was not until the guinea pig turned around and licked its right hind foot that the droplet addition of phosphorus histamine was stopped. The total amount of phosphorus histamine given when the guinea pig finally licks its right hind foot was the itching threshold. The itching thresholds were recorded and compared across groups.

Statistical Analysis

For multi-group comparisons, one-way ANOVA and post-hoc analysis were performed via Statistical Product and Service Solutions (SPSS). All data was graphed using GraphPad Prism 9 (GraphPad Software, CA, USA). Data is expressed as an average ± SD.

Results

Preparation of glutinous rice ferment extract

Glutinous rice ferment extract was prepared by multiple processes, as shown in Figure 1. Firstly, after the glutinous rice was crushed, steamed, and saccharified, yeast and wheat koji were added to ferment for the mash. Secondly, wheat bran and rice husk were added and stirred well. Traditional solid-state seed grains containing composite microbial strains were added to carry out solid-state layered fermentation to obtain the vinegar grains. After that, the vinegar brine that naturally oozed from the bottom of the vinegar grains was collected. Finally, the vinegar brine was purified by macro-porous resin to obtain the vinegar brine extract. After the vinegar brine extract was concentrated and dried, the glutinous rice ferment extract were obtained.

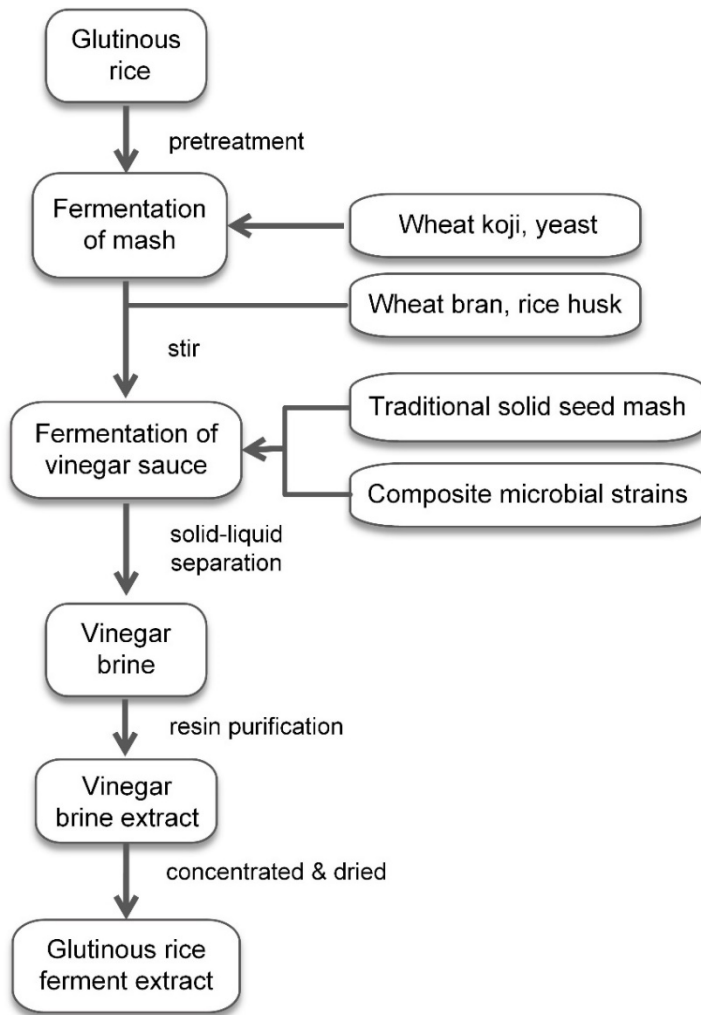


Figure 1: Flow chart for preparing the glutinous rice ferment extract.

Inhibition of TSLP

As mentioned above, the influencing factors of eczema are diverse, the most important among which are TSLP, skin barrier and itching. In this paper, we will conduct in-depth research and characterization of the skin care efficacy of glutinous rice ferment extract from three aspects: inhibiting TSLP, promoting the health of skin barrier and increasing itching threshold, so as to verify the application potential of glutinous rice ferment extract in the treatment of eczema.

A large number of studies have shown that the occurrence of eczema is accompanied by an increase in the secretion of inflammatory cytokines such as TSLP, which in turn can further expand the immune allergic response by acting on immune cells and aggravate eczema. Therefore, TSLP can be used not only as a

criterion for the occurrence of eczema, but also as a key target for eczema treatment research. We used a 3D epidermal skin model as the research model and Polyinosinate-polycytoglycate sodium salt and lipopolysaccharides (PolyI:C+LPS) as the inducer to establish an eczema-like model. We examined the TSLP content of the 3D skin model after induction and found that the TSLP content increased significantly, indicating that the eczema-like model was successfully established.

Next, we applied glutinous rice ferment extract to eczema-like skin model, and dexamethasone was used as a positive control to study the effect of glutinous rice ferment extract on the eczema-like skin model TSLP. The results of the TSLP test are shown in Figure 2. It can be seen from results that TSLP increased significantly after model induction, and the concentration of TSLP in the positive control group decreased significantly, which proved that the experimental results were scientific and effective. At the same time, we found that glutinous rice ferment extract (0.3%, V/V) can significantly reduce the expression of inflammatory factor TSLP by 32.06%, and the effect is better than that of the positive control 0.01% dexamethasone, indicating that glutinous rice ferment extract can reduce inflammatory factor TSLP.

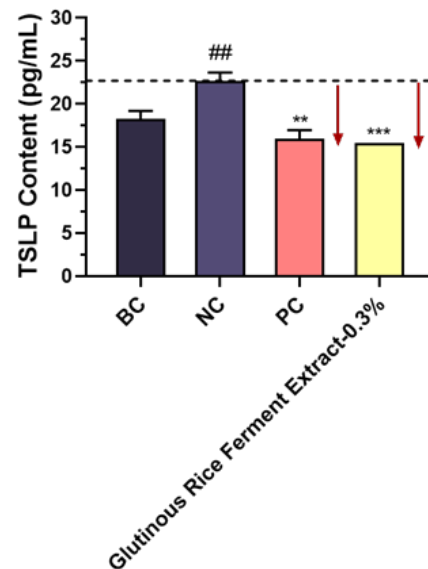


Figure 2: Glutinous rice ferment extract (0.3%) significantly downregulated the secretion of TSLP in eczema skin. NC, negative control, eczema skin. PC, positive control, dexamethasone (0.01%). TSLP, thymic stromal lymphopoietin. * $p < 0.05$. *** $p < 0.001$.

Improvement of the skin barrier

The strong correlation between the skin barrier and eczema suggests that eczema could be treated by improving the health of

skin barrier. FLG protein is one of the most important proteins to maintain skin barrier function. First, we applied glutinous rice ferment extract to keratinocytes, and detected the FLG gene expression of keratinocytes by PCR experiment, and the increase rate reached 46.25% (test concentration is 0.3%) and 51.86% (test concentration is 0.6%). Also, we studied the role of glutinous rice ferment extract on the AQP3 gene, which codes for aquaporins, is another important gene for skin moisturizing [21]. It can be seen from Figure 3 that glutinous rice ferment extract (0.3%) could significantly increase the expression of AQP3 by 48.55%. The promoting effect of glutinous rice ferment extract on FLG gene and AQP3 gene showed that glutinous rice ferment extract has great potential to improve the moisturizing barrier function of eczema skin.

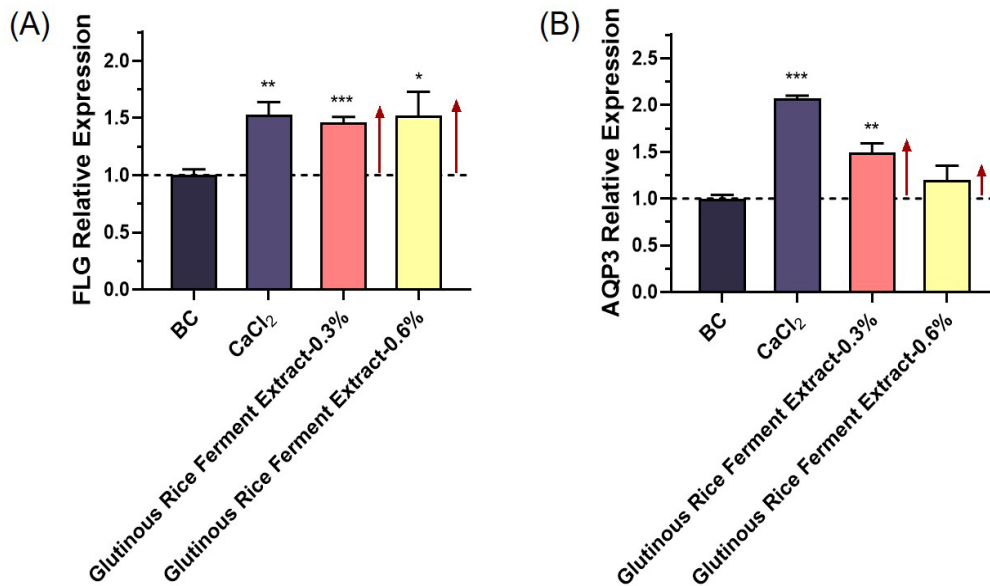


Figure 3: Glutinous rice ferment extract improves the moisturizing barrier function. (A) Glutinous rice ferment extract (0.3% and 0.6%) significantly increased the expression of FLG gene in keratinocytes. (B) Glutinous rice ferment extract (0.3%) significantly increased the expression of AQP3 gene in keratinocytes. * $p < 0.05$. *** $p < 0.001$. *** $p < 0.001$.

Furthermore, we established a 3D cutaneous eczema-like model, and used qPCR technology to detect the expression of FLG genes in the skin model after applying glutinous rice ferment extract to the eczema-like skin model. As can be seen from the Figure 4, the expression of FLG gene in the eczema model group decreased by 39% compared with the BC group, which confirmed the strong correlation between the skin barrier and eczema mentioned earlier. Surprisingly, we find that glutinous rice ferment extract (0.3%, V/V) could increase the expression of FLG gene in eczema-like skin models with an increase rate of 131.15%. We believe that glutinous rice ferment extract can increase the expression of FLG genes, thereby promoting the repair of the skin barrier and ultimately improving eczema.

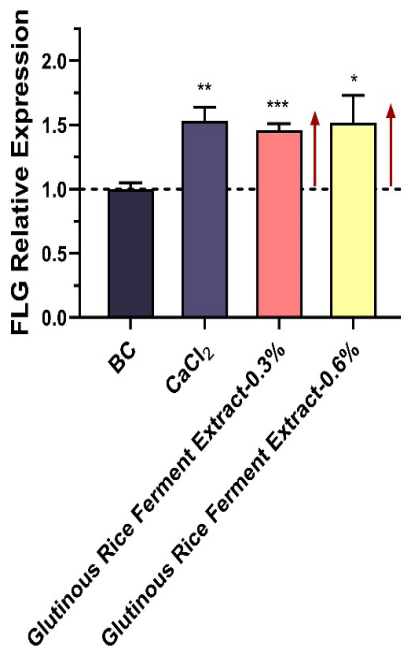


Figure 4: Glutinous rice ferment extract (0.3%) could significantly upregulate the expression of FLG gene in eczema skin. NC, eczema skin. PC, WY14643. * $p < 0.05$. *** $p < 0.001$. *** $p < 0.001$.

Reduction of the itching threshold

Itching, is a typical manifestation of eczema, but also make patients miserable. More serious, itching caused by scratching will further damage the skin. and the damage of the barrier further brings allergic inflammatory reaction, aggravates itching dry red and other eczema symptoms, which cause eczema repeatedly, into a vicious circle. Therefore, effective antipruritic may be an important breakthrough point to solve the problem of eczema. We established an animal antipruritic model using PolyI: C+LPS (the polyinosinic acid-polycytionate sodium salt and lipopolysaccharide) as an inducer. It is worth mentioning that in animal antipruritic experiments, glutinous rice ferment extract was added to the matrix and applied to take effect. The composition of the matrix can be found in the supplementary material.

Animal experiments showed that compared with blank, glutinous rice ferment extract had a certain effect on increasing the itching threshold of guinea pigs ($P < 0.01$), with an increase rate of about 37.03% as shown in Figure 5. From the application perspective, we added glutinous rice ferment extract to the matrix to simulate the anti-itching effect of skin care products containing glutinous rice ferment extract (in Figure 5). We found that the substrate containing 2.5% and 5% rice grain extract could increase the itching threshold of guinea pigs by 41.26% and 30.49%, respectively, thereby alleviating itching caused by eczema.

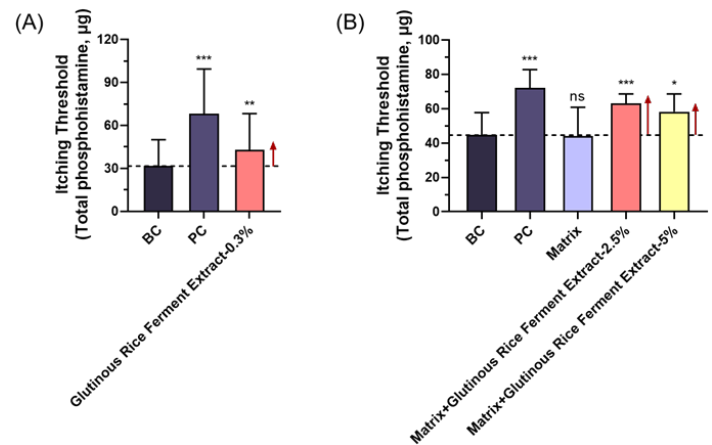


Figure 5: (A) Glutinous rice ferment extract (0.3%) significantly increased the itching threshold of guinea pigs. (B) The matrix containing glutinous rice ferment extract (2.5% and 5%) significantly increased the itching threshold of guinea pigs. PC, positive control, hormonal ointment. * $p < 0.05$. *** $p < 0.001$. *** $p < 0.001$.

Discussion

As a very common skin disease, eczema greatly affects the quality of life of patients, and new ideas are needed to combat eczema. This paper comprehensively considers three most important factors of eczema, namely TSLP, skin barrier and itching. Glutinous rice ferment extract obtained by fermentation can have a good regulatory effect on these three influencing factors, and the three effects are combined to fight eczema.

Compared with previous studies, glutinous rice ferment extract in this paper could target multiple eczema targets, which would make glutinous rice ferment extract more widely used. Combining multiple targets often results in better therapeutic outcomes. The disadvantage of this study is that no clinical trials have been conducted, and the anti-eczema effect of glutinous rice ferment extract has only been verified from in vitro experiments or artificial eczema models. In subsequent studies, the addition of human experiments will make the study more complete and more convincing for the anti-eczema efficacy of glutinous rice ferment extract.

In addition, we speculate that the reason why glutinous rice ferment extract has such excellent efficacy is inseparable from the way it is made. The fermentation process is able to break down the macromolecular substances or harmful substances in the extract into more active substances. However, what role the increased active substances play in anti-eczema needs to be studied in more depth. Linking specific actives with anti-eczema efficacy will definitely play a more important role in anti-eczema research.

Conclusions

Starting with TSLP, the skin barrier and itching, which are three important factors of eczema, we seek the treatment of eczema. Firstly, we found that glutinous rice ferment extract can significantly reduce Th2-related TSLP during the occurrence and development of eczema. Secondly, glutinous rice ferment extract can significantly help with the repair of skin barrier. Furthermore, glutinous rice ferment extract was able to significantly increase the itching threshold. Therefore, we believe glutinous rice ferment extract has great application potential in eczema treatment. Overall, we provide new ideas for the research of plant fermentation broth in the treatment of eczema.

Disclosure

Author Contributions: Conceptualization, Y.Z. and H.J.; methodology, X.S.W., M.C. and L.Z.; software, X.S.W.; validation, X.S.W.; formal analysis, X.S.W.; investigation, X.S.W., L.Z. and X.L.; resources, Y.Z., and H.J.; data curation, X.S.W. and L.Z.; writing-original draft preparation, X.S.W.; writing-review and editing, X.S.W., L.Z., W.C. and Y.Z.; supervision, Y.Z. and M.C.

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Conflicts of Interest: None.

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