Original Article
Pilose antler extracts protect against imiquimod-induced psoriasis-like inflammation

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Received November 3, 2016; Accepted February 7, 2017; Epub March 1, 2017; Published March 15, 2017

Abstract: Psoriasis is a chronic inflammatory skin disease characterized by excessive proliferation of keratinocytes. Current existing therapies only relieve symptoms but cannot cure disease. Pilose Antler (PA) is a well-known Traditional Chinese Medicine (TCM) used in the prescription, which has the effects of invigorating kidney Yang, replenishing essence and supplement the blood, strong bones and muscles based on Chinese traditional medicine theory. Although it is not traditionally used to treat psoriasis, but it is reported that PA and its extracts have immunomodulatory activities. The present study aimed to identify the curative effect and mechanism of Pilose Antler Extracts (PAEs) in imiquimod- (IMQ-) induced psoriasis animal model. Severity of inflammation of the dorsal skin was scored according to the clinical Psoriasis Area and Severity Index (PASI) and epidermal hyperplasia was measured by H&E staining. The mRNA expression of inflammatory factors in the epidermis was analyzed by Real-Time polymerase chain reaction (PCR). The results showed that PAEs can attenuate the IMQ-induced psoriasis-like inflammation, accompanied with increased epidermal hyperplasia. Real-time PCR analysis indicated that PAEs inhibited the levels of inflammatory factors, including interleukin (IL)-12, interferon-γ (INF-γ). In order to find out the active compounds in PAEs in the treatment of Psoriasis, the composition of proteins are profiled by LC/MS/MS, the results show that 216 different proteins were involved in PAEs, 10 of them had immune response function. Annexin A1 and Cathelicidin-1 were two typical proteins which may have positive effects on Psoriasis. In conclusion, we consider the PAEs as a potential treatment for Psoriasis, anti-inflammatory protein/peptide are the active ingredients of PAEs. Further research on the new drug development based on above research will be carried out for the next step.

Keywords: Pilose antler extracts, imiquimod-induced psoriasis, inflammation, interleukin-12, interferon-γ

Introduction
Psoriasis is an autoimmune disease and in some aspects an autoinflammatory disease of the skin [1], which is characterized by hyperproliferation of epidermal keratinocytes. The active leukocytes are involved in the immunopathology of the disease. It can be triggered by the topical biological response modifier imiquimod, mediated by the IL-23/IL-17 axis and activated dendritic cells (DCs).

Recent study has shown that severe psoriasis is associated with an increased risk of mortality as male and female patients in the study died 3.5 and 4.4 years younger respectively than those without psoriasis [2]. Therefore, psoriasis poses a major social and economic burden on society. Current existing therapies only relieve symptoms but cannot cure disease. Conventional therapies for psoriasis have not been fully satisfactory [3]. Dexamethasone, Prednisone, Betamethasone and other steroids have some short-term effect, but easy to induce diabetes, osteoporosis, ulcers, and other disease, if medication is discontinued, symptoms will be recurrent. Therefore, safe new drugs with longer term effect and less side effects are urgently needs for the treatment of psoriasis.

Pilose Antler has been used in China for over 2000 years in clinic as a traditional animal medicine. Based on TCM basic theory, it has the effects of invigorating kidney Yang, replenishing essence and supplement the blood, strong bones and muscles. Although it is not traditionally used to treat psoriasis, but it is reported that PA and its extracts have immunomodulatory activities. Orally administrated PA extracts increased monocytes in rat [4]. Intra-
peritoneal injection of Pantocrin could enhance phagocytosis and immunoglobulin levels in mice [5]. In addition, the PA extracts have been used in the prevention and treatment of certain immune-related diseases, such as rheumatoid arthritis. Some studies provide evidence that PA and its extracts exhibit anti-infective activity against pathogenic Staphylococcus aureus both in vitro and in vivo [6]. According to thousands of years of application and modern research, we try to develop a new potential precursor drug from PA for the treatment of Psoriasis. In our previous study, we have extracted proteins/peptides from PA with good biological activity [7]. This work is to investigate the effects and mechanism of PAEs on IMQ-induced psoriasis of animal model, it’s also the foundation of new drug development of psoriasis.

Material and methods

Pilose antler extracts preparation

Male sika deer, at the early, fast-growing stage of antler development, were obtained from a local deer farm (Jiyunluye Ltd., China). The antlers were cut into small sections (1×1 cm). Then the sections were sliced into 0.3 mm pieces using a WND-200 grinder (Weinengda Electric Co., Ltd. Zhejiang, China). The pieces of antler tissues were homogenized 8-12 hrs in a JM-L 50 colloid mill (Shanghai Nuoni Light Industrial Machinery Co., Ltd.) and then added a certain amount of acetic acid to maintain the pH 6.4. We used water to extract Pilose antler, because water was able to extract more lower molecular protein which can be absorbed well, so water was deemed to be a superior extract solution and more suitable for subsequent experiments. The mixture was centrifuged at 5,000× g for 10 min at 4°C, the supernatant was collected and then rotary evaporated in Rotavapor (Buchi, Switzerland) at 60°C. The concentrated supernatant was dialyzed with P1000D dialysis membrane (Solorbio, China) and then lyophilized in FD-1D-50 Freeze drying machine (Beijing Bo Kang laboratory instruments Medical Co., Ltd., China). The Pilose antler powder was stored at 4°C.

Gel electrophoresis

To prepare 4°C aqueous extract, 4 mg of Pilose antler powder was dissolved in 1 mL of de-ionized water (4 mg/mL). A bradford protein assay kit (Tiangen Biotech (Beijing) CO., Ltd., China) was used to measure protein concentration. Total protein of PAEs was then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). And stained by Coomassie brilliant blue R-250 stain (Bio-Rad Laboratories).

Protein identification by LC/MS/MS

The expressed band of antler was excised from the gel, transferred into sterile 2 mL microcentrifuge tubes. Then, all the bands were destained with 20 mM (NH₄)HCO₃ (containing 50% methanol) and dehydrated with 100% acetonitrile. The gel particles were reduced by 10 mM DTT for 45 min and alkylated with 50 mM iodoacetamide for 45 min at RT in 50 mM (NH₄)HCO₃ (pH 8.5) solution. Gel particles were washed with 20 mM (NH₄)HCO₃ (pH 8.5), dehydrated with acetonitrile, and air-dried. The proteins were digested by trypsin (Promega (Beijing) Biotech Co., Ltd, USA) using an enzyme-to-substrate ratio of 1:50 (w/w) for overnight at 37°C. Digested peptides were extracted with 1% fluoroacetic acid (FA) in 50% acetonitrile, and samples were dried with a SpeedVac and redissolved in 0.1% FA for LC/MS. Sample was separated on an analytical C18 column (Agilent Technologies; 4.6 mm×150 mm, 5 μm) connected inline to the mass spectrometer, at 200 ml/min using a linear acetonitrile gradient the 1 s survey scans were acquired over the mass range 350-1350 (m/z) and a maximum of 2 concurrent MS/MS acquisitions. The proteins were identified by searching the LC-MS/MS data of Swiss-Prot database using MaxQuant.

Ethics statement

Animal experiments were performed in accordance with Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, the Guide for the Care and Use of Laboratory Animals' protocol, published by the Ministry of the People's Republic of China (issued 3 June, 2004), and approved by the Institutional Animal Care and Use Committee of China Academy of Chinese Medical Sciences. The protocol was approved by Experimental Research Center, China Academy of Chinese Medical Sciences. All surgery was performed under 1% pentobarbital sodium anesthesia and all efforts were made to
minimize suffering. Healthy BALB/c male mice purchased from Vital River Laboratories (China) were housed in polypropylene cages and maintained under standard conditions of a 12 h light/dark cycle, a temperature of 25°C, and 35-60% humidity. Mice were fed standard diet without limitations until 24 h before sacrifice.

**Animal treatment**

Healthy BALB/c male mice purchased from Vital River Laboratories (China) were used in all experiments and fed standard mouse chow and water. Animals were housed in polypropylene cages and room temperature maintained at 25±1°C with a 12/12 h light/dark cycle. The 48 mice were randomly divided into the control and IMQ-induced groups. In all experiments, control groups received a daily topical dose of Vaseline cream (Hebei Lanlian Feitian Petrochemical CO., Ltd.) on the shaved back for 7 consecutive days. IMQ-induced groups were treated with 42 mg of commercially available 5% IMQ cream (Sichuan Mingxing Pharmaceutical Corporation, Ltd.) on the shaved back for 7 consecutive days. IMQ-induced groups were treated with 42 mg of commercially available 5% IMQ cream (Sichuan Mingxing Pharmaceutical Corporation, Ltd.). Those mice were divided into the following six groups (n = 8/group): Control group, (WT); Model group, (IMQ); Methotrexate group (1 mg/Kg/d, MTX), and low (0.13 g/kg/d), medium (0.26 g/kg/d), and high (0.52 g/kg/d) dose PAEs groups. All mice were treated orally for 7 days. At the end of the experiment period, all the mice were sacrificed by 1% pentobarbital sodium and their shaved skins were removed.

**Scoring severity of skin inflammation**

The severity of inflammation of the dorsal skin was scored using the clinical PASI. Erythema, scaling, and thickening were scored independently on a scale between 0 and 4: 0, none; 1, slight; 2, moderate; 3, marked and 4, highly marked. The cumulative scores (the amount of erythema, scaling and thickening) was used to measure severity of inflammation.

**Histology and polarization imaging**

Skin samples were fixed in 10% formaldehyde for 24 h at 4°C and then placed in 70%-100% ethanol. All samples were embedded in paraffin, cut into 10 μm sections, detected by Abrio™ Imaging System (CRI, USA) and stained with H&E. Epidermal thickness was accurately measured by ImagePro Plus software (Media Cybernetics).

**Real-time PCR**

Total RNAs were isolated from shaved back skin tissues using an ultrapure RNA extraction kit (Promega (Beijing) Biotech Co., Ltd, USA). Each sample was run in triplicate. Samples after evaluating RNA quality were further reversed transcription with the HiFi-MMLV cDNA first strand synthesis kit (Kang Biotechnology Co., Ltd., China). According to manufacturer’s protocols, real-time PCR for each transcript was executed with a fluorescent dye UltraSYBR Mixture chimeric fluorescence (Kang Biotechnology Co., Ltd., China). The cycle threshold (Ct) fluorescence values for amplification were recorded for each target gene and GAPDH gene. The differences between cycle thresholds for target genes and GAPDH in each of the samples were calculated (ΔCt), and the average fold change in expression between PAEs-treated and MTX-treated mice was calculated by the following formula: average fold difference = $2^{\Delta Ct_{\text{A}} - \Delta Ct_{\text{V}}}$.

**Statistical analysis**

Data were presented as mean ± S.D., Student’s t-test was used to compare two experiments and ANOVA was used to compare three and more groups. A value of $P<0.05$ was considered statistically significant.

**Result**

**Quality control of pilose antler extracts**

According to the ancient records of TCM, there has no clear active ingredient was discovered and no clear mechanism of activity was elucidated in all animal medicines, which greatly influenced the development and application of animal medicine. Many studies indicated that protein(s) is one of the most active components of Pilose velvet-related products [8]. In order to control the quality of the PA we investing the expression of proteins by SDS-PAGE. A water soluble fluffy white powder containing 36% protein had been obtained. **Figure 1** represents a typical SDS-PAGE pattern of the antler proteome visualized by coomassie blue. From this gel, the specific band was cut and used to in-gel
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LC/MS/MS approach

Because present public databases contain only a limited entry of deer sequences, we also searched sequences from other artiodactyla species to find matches. 216 different proteins were identified from the PAEs using a routine LC/MS/MS approach. According to gene ontology, the searching of the peptides led to the identification of 147 proteins of molecular function, 146 proteins of cellular component and 154 proteins of biological process. Psoriasis is an auto inflammatory and in some aspects autoimmune disease of the skin. We analysis all the proteins found that there were 10 proteins had immune response function. Annexin A1 and Cathelicidin-1 were two typical proteins which may have special distribution on psoriasis.

Annexin A1 plays important roles in the innate immune response as effector of glucocorticoid-mediated responses and regulator of the inflammatory process. Annexin A1 is a protein mainly located on basal keratinocytes of the basement membrane. In lesional psoriatic skin, annexin A1 appears only in the cell membrane, suggesting a translocation of the protein [1]. This transition may occur to promote the binding of annexin A1 to phospholipids, therefore reducing the production of inflammatory prostanooids [9]. Annexin A1 has anti-inflammatory activity, plays a role in glucocorticoid-mediated down-regulation of the early phase of the inflammatory response. As we know that annexin A1 contributes to the adaptive immune response by enhancing signaling cascades that are triggered by T-cell activation, regulates differentiation and proliferation of activated T-cells, promotes the differentiation of T-cells into Th1 cells and negatively regulates differentiation into Th2 cells [27].

Cathelicidin-1 is potent microbicidal activity and active against Staphylococcus aureus (S. aureus) and E.coli. S.aureus is a notorious pathogen causing a broad range of infections both in and outside hospitals [10, 11]. 50% psoriasis patients’ skin were injected with S.aureus, which increased skin lesions. The staphylococcal enterotoxins- the so-called superantigens- are extremely potent T cell activators, that promoting keratinocyte proliferation [12].

PAEs mitigates inflammation on IMQ-induced psoriasis in mice

Mice with IMQ-induced psoriasis were used to observe the anti-inflammation effects of PAEs. IMQ cream was applied on shaved back skin for 7 consecutive days. As a result, the skin of the mice in control group with Vaseline cream had no erythema or scaling and the hair was shiny. Three days following the application of IMQ onto the shaved back skin of the mice started to exhibit symptoms of erythema, scales and thickening. Signs of inflammation in IMQ group continually increased in severity until the end of the study. Both the PAEs and MTX treated groups show fewer symptoms of inflammation, as shown in Figure 2A. From the images revealed in Figure 2B, it is possible to detect and quantify the severity scores of the mice. The PAEs-H and PAEs-M groups, but not the PAEs-L.
Figure 2. PAEs mitigates skin inflammation on IMQ-induce psoriasis in mice. (A) Representative macroscopic views of mouse shoved back skin following continuous treatment for 7 days. (a) WT group, skin was smooth, rosy and thin; (b) IMQ group, hypertrophic lesions with heavy scales over, skin lesion was dark red; (c) MTX group, scattered scales, skin lesion was not so thick as model group, the color was less red; (d-f) low, medium, and high dose of PAEs groups, less scales, skin lesions was significantly thinner than model, the color was less red (e and f). (B) Epidermal thickness and erythema, scaling of the shoved back skin were respectively identified on 7 days indicated on a scale from 0-4. The cumulative score (erythema plus scaling plus thickness) is recorded.
group, exhibited anti-inflammatory activity. The PASI score shown that PAEs suppressed IMQ-induced psoriasis in a dose-dependent manner. Compared with the IMQ group (5.13±0.72), inflammation scores were significantly decreased in the other groups. The MTX and PAEs-L, PAEs-M, and PAEs-H experiment groups had inflammation scores of 5.3±0.48, 5.53±0.49, 4.55±0.26, and 4.08±0.48, respectively; there was a significant difference between the scores of the high dosage PAEs group and the low dosage PAEs group.

**PAEs suppressed proliferation of keratinocytes on IMQ-induced psoriasis in mice**

It has been previously revealed that IMQ-treated skin increased epidermal thickening, hyperproliferous keratinocytes, parakeratosis and altered differentiated epidermis symptomatic of psoriatic skin lesions [13]. In the present study, through the analysis of H&E-stained and Abrio sections from the IMQ-treated shaved back skin, we observed increasing epidermal thickness, stratum corneum, prickle cell layer

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**Figure 3.** Oral PAEs alters keratinocyte proliferation on IMQ-induced psoriasis in mice. (A) Abrio view (×200) of the shaved back skin of the six groups of mice treated by three types of drugs. a. WT group; b. IMQ group; c. MTX group; d-f. low, medium, and high dose of PAEs groups (B) Histological view (H&E staining of skin lesions). (C) PAEs reduces the thickness of epidermis cells, n = 4, **p<0.01 vs. the WT group.

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**Figure 4.** Real-time PCR confirmation of mRNA levels of IL12p40 and INF-γ in dorsal skin of PAEs-treated mice. IMQ treatment increased IL12p40 and INF-γ mRNA levels, while PAEs relieved those changes. **p<0.01 compared with control.
in the IMQ-treated group compared with the WT group (Figure 3A, 3B). The indication of inflammation of MTX group (2.72±0.6×10) and PAEs-H group (2.32±0.25×10) was evidently weaker than in the IMQ group (6.39±0.37×10) in each respect (Figure 3C) (P<0.001).

PAEs decreased the mRNA levels of interleukin 12p40 on IMQ-induced psoriasis in mice

IL12p40 were regulatory cell factor in psoriatic lesions, Changing the expression of IL12p40 could improve skin inflammation. The amounts of IL12p40 and INF-γ were significantly higher in the skin of mice treated with IMQ (Figure 4). PAEs significantly decreased the levels of IL12p40 in skin and having a concentration-dependent manner, comparable to that in untreated mice. MTX on the expression of IL-12p40 mRNA had no effect.

Discussion

Psoriasis, which is regarded as a T-cell-mediated inflammatory skin disease, is characterized by hyperproliferation and poor differentiation of epidermal keratinocytes [14]. It is defined as an immunological disease, that is coupled with prominently increased vascularization of the skin, fibroblast activation and leucocyte infiltration. The underlying pathogenic mechanisms of this condition have not been entirely clarified. Currently, numerous therapeutic reagents are available including topical treatments, phototherapy, and systemic agents [15]. However, the therapeutic efficacy of these treatments is limited. Many patients with psoriasis commonly do not respond to or develop tolerance to these therapies. Novel biologic agents such as anti-tumor necrosis factor (TNF) α, TNFα blocker, and anti-IL-12/IL-23, which inhibit autoimmunity and target specific molecular signals in the pathogenesis of psoriasis, are effective in the treatment of severe psoriasis. However, the safety of these therapeutic reagents is of concern.

Pilose antler have been traditionally used as anti-aging medicines. Chinese people used antlers of various deer species as a conventional therapy for anti-aging and rejuvenation. Based on the TCM theory, we believed it has the effects of invigorating kidney Yang, replenishing essence and supplement the blood, strong bones and muscles. Previous studies demonstrated that Pilose antler extracts possessed various bio-activities such as anti-amnesic [16], anti-inflammatory [17], antioxidant [18], or anti-aging activities [19]. Although there no report is available on the effect of Pilose antler extracts on psoriasis, it has immunomodulatory effects both on animal model and clinic, therefore, this research is made a primary exploration of PAEs on the future treatment of psoriasis.

The IMQ-induced mouse is utilized as a model of human psoriatic lesions, as it exhibits similar characteristics, including erythema, epidermal thickening, scaling, neoangiogenesis, and the inflammatory infiltrate of T cells, neutrophils and dendritic cells (DCs) [20, 21]. In the present study, the effect of PAEs on psoriasis-like lesions was investigated in an IMQ-induced model. The shaved back skin of the six groups of mice were treated with equal IMQ and taken PAEs or MTX by oral for 7 consecutive days. IMQ treatment resulted in hyperproliferative keratinocytes, parakeratosis, increase stratum corneum and stratum spinosum, as it may have been expected. However, in the PAEs group, these inflammatory effects were more mild than those in model group. Following this, we further determined the anti-inflammatory effect of PAEs on psoriasis-like lesions.

Recently, A new subset of Th17-cells expressing IL-12 and IL-23 appears to play a major role in Th17-cell-dependent chronic inflammation in psoriasis [22]. IL-12 is an important factor to promote cell differentiation into Th1 from Th0, which can induce secretion of IFN-γ. Besides, in situ IL-12 may also have a role in the induction of new psoriatic skin lesions [23]. The p40 protein of IL-12 associate with a p19 subunit to form IL-23, they are heterodimers that share a common p40 chain. Previous studies reported that an antibody to the human IL-12/p40 (anti-IL-12p40) have significant effect in psoriatic patients [24]. Some study of cytokines in the serum of patients with psoriasis over-expressed IL-12 and INF-γ significantly. There was a significant correlation between serum levels of INF-γ, IL-12, and severity of the disease [25]. In addition, the levels of IL-12, INFy and IL-23R gene expression are elevated in psoriasis skin lesions [26].

The results from our study have revealed that under the condition of IMQ-treatment, the
mRNA level of IL-12 and INF-γ increased. The IL-12p40 mRNA expression rise but did not reach statistical significance, the reason may be that the IMQ-induced mice psoriasis skin lesions is a temporary inflammation process. PAEs after oral absorption inhibits the expression of pro-inflammatory or inflammatory cytokines, including IL-12, INF-γ and reducing thickening, erythema and scales. These data indicate the potential beneficial effects of PAEs on IMQ-induced psoriasis. In the present study, the IMQ-induced model was selected to expound the molecular mechanisms underlying PAEs effects in psoriasis, the IL-12p40 is a critical therapeutic target of inflammation in psoriasis and PAEs are potential candidates for the prevention of hyperkeratosis and parakeratosis.

Proteins and peptides are attractive drug candidates because of their potent biological activities as well as high target specificities. In present study, according to the protein and peptide content of PAEs lyophilized powder and SDS-PAGE analysis, we detected 216 proteins of extracts from nature sources via LC-MS/MS, Annexin A1 and Cathelicidin-1, two of the proteins may have exhibited immune response. We established the psoriasis model by IMQ, and the protein/peptide showed the activity of preventing hyperkeratosis and parakeratosis. All of these confirm the truth that the anti-inflammatory protein/peptide is one of the active ingredients of PAEs. Therefore, we consider the PAEs as a potential treatment for psoriasis. It is warranted to make a thorough research on the sequence and mechanism of anti-inflammatory protein/peptide.

Acknowledgements

Thank all the workers and controls for their participation in this study. This work is supported by Beijing Key Laboratory of Traditional Chinese Medicine Basic Research on Prevention and Treatment for Major Diseases, Experimental Research Center, China Academy of Chinese Medical Sciences; Project supported by the National Natural Science Foundation of China No. 81273884, 81603285; Basic scientific research fund from Ministry of Finance of China No. ZZ2013005.

Disclosure of conflict of interest

None.

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