Recombinant human thymosin beta-4 (rhTβ4) improved scalp condition and microbiome homeostasis in seborrheic dermatitis

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Summary

Seborrhoeic dermatitis (SD) is a recurrent common inflammatory skin disease that affects all ethnic groups in all regions worldwide. However, no specific treatment or preventive measure is yet available. Identifying effective treatments with acceptable safety and tolerability is desirable. In this study, scalp microbiota alterations were measured in SD, showing significantly greater abundance of Malassezia and Staphylococcus and diminished fungal and bacterial diversity compared with healthy controls. We investigated the benefit of a 4-week treatment with 0.5 mg ml⁻¹ recombinant human thymosin β4 (rhTβ4) gel or 2% ketoconazole lotion on the scalp condition of 71 patients with SD compared with 21 healthy individuals. Clinical assessment (Adherent Scalp Flaking Score, and the Maximum Erythema Area) and physiological conditions (transpidermal water loss, hydration, and sebum secretion) were evaluated. The rhTβ4 treatment provided significantly greater efficacy than ketoconazole and a sustained effect in the treatment of scalp SD. More importantly, rhTβ4 dramatically improved the microbiome homeostasis and prompted a shift of scalp microflora towards healthy composition, helping symptoms and ameliorating physiological conditions more effectively and durably than ketoconazole. Our research demonstrated the scalp microbe dysbiosis of SD and highlighted rhTβ4 as a promising therapeutic strategy in the prevention and treatment of SD.

Introduction

Seborrhoeic dermatitis (SD) is a recurring chronic inflammatory dermatosis. The main clinical symptoms are redness, itching and flaking of the skin, especially on the seborrhoeic areas, such as the scalp, ears, and chest. Globally, 1–3% of the general population and 34–83% of immunocompromised persons suffer from this disease while the prevalence of its non-inflammatory variant, dandruff, is probably closer to 50% (Tucker and Masood, 2020). The pathogenesis of SD results from the combined effect of multiple factors. Immunocompromised persons or patients with neurologic disease are more susceptible to SD and external factors such as sun exposure, stress, and dysbiosis of both fungi and bacteria may also contribute to SD (Piquero-Casals et al., 2019). Among the many causes, an inflammatory reaction against the dominant fungus Malassezia is considered basic to the etiology of seborrhoeic dermatitis (Kim, 2009; Zisova, 2009). The current mainstays of treatment for SD focus on eradicating the symptoms of dryness and flaking of the scalp, along with providing relief of associated pruritus, and include antifungals, keratolytics, antipruritics, and anti-inflammatory agents (topical corticosteroids and calcineurin inhibitors) (Tucker and Masood, 2020). For instance, 2% shampoo formulations of ketoconazole have been used as the first-line treatment for SD of the scalp and lead to improvement of lesions, accompanied by reduction in yeast count on the skin (Carr et al., 1987; Green et al., 1987; Ortonne et al., 1992; Ortonne et al., 2011). Although the antifungal and anti-inflammatory therapies tend to palliate the symptoms of SD disease, no permanent cure is yet available.

Thymosin beta-4 (Tβ4), a 43-amino acid peptide, is involved in multiple cellular responses and plays dual roles in antimicrobial and immune modulation (Park et al., 2021). Tβ4 has shown potential antimicrobial effects against various bacteria, including E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Staphylococcus epidermidis (Tang et al., 2002; Huang et al., 2007; Schillaci et al., 2016; Carion et al., 2020) and downregulates inflammation. For example, Tβ4...
could directly target the NF-κB RelA/p65 subunit (Qiu et al., 2011), thus suppressing TNF-α-mediated NF-κB activation and reducing the levels of a large number of downstream key proinflammatory cytokines such as IL-8 (Sosne et al., 2007). Besides, Tβ4 could promote wound repair and skin regeneration. It has been reported that Tβ4, as an angiogenic factor, enhanced wound healing specifically by stimulating endothelial cell migration (Malinda et al., 1999). Additionally, Tβ4 increased hair growth by activation of hair follicle stem cells (Dai et al., 2021). The anti-inflammatory ability of Tβ4 also helps to promote wound healing after skin and eye injuries (Qiu et al., 2011). Numerous studies to date have shown that Tβ4 is safe and well-tolerated (Crockford et al., 2010; Ruff et al., 2010), and it is currently being used for clinical trials in dry eye syndrome, neurotrophic keratopathy, cardiac damage post-acute myocardial infarction, epidermolysis bullosa, and venous stasis ulcers (Zhang et al., 2009; Smart et al., 2011; Xiong et al., 2011; Yu et al., 2018).

The recombinant human thymosin beta-4 (rhTβ4) used in this study was produced in E. coli with N-acetylation (Yu et al., 2018). Compared with chemical synthesis of Tβ4, biological synthesis as rhTβ4 has many advantages, such as controllable quality, unlimited scale of production, low production costs, and no environmental pollution. In light of these studies, we hypothesized that rhTβ4 could provide a therapeutic benefit for SD. To this end, we sought to confirm in this study that a disequilibrium in the proportion of major bacterial and fungal populations, especially decreased microbiome diversity along with increased proportions of Malassezia and Staphylococcus species, is associated with SD. Furthermore, we hypothesized that rhTβ4 could be a potential solution to lessen seborrheic dermatitis more effectively and in a more durable manner than ketoconazole, not only reducing symptoms but also improving and maintaining microbiome homeostasis.

Results

The dominant scalp skin microbiomes were generally similar on healthy and SD subjects

The information about phyla and genera of scalp fungi and bacteria are shown in Fig. 1, and different color blocks in each column represent different microorganisms and their constituent ratios. At the phylum level, Basidiomycota (Filobasidium spp.) and Ascomycota (Acremonium spp.) made up over 99% of the healthy and SD scalp fungi flora (Fig. 1A), and the most abundant genus level taxons were Malassezia, Mycosphaerella, Aspergillus, and Cladosporium, with prevalence up to 100%, 100%, 100%, and 96%, respectively (Fig. 1B). For bacteria, four phyla that dominated both healthy and SD scalps were Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes, which together contributed over 90% of the bacterial microbiome (Fig. 1C). Additionally, the most common genera that constituted over 50% of the total bacterial sequences were as follows: Staphylococcus, Corynebacterium, Deinococcus, Acinetobacter, Enhydrobacter, Paracoccus, Bacteroides, Sphingomonas, Streptococcus, Propionibacterium, and Blautia (Fig. 1D). According to our results, the species of the colonized microflora were quite similar in healthy and SD subjects.

Association of the scalp microbiome with scalp condition

Although the dominant fungal and bacterial communities were similar between healthy and SD groups, a decrease in relative abundance of microorganisms was exclusively observed in SD patients. The alpha diversity of scalp microbial community was examined using two metrics, PD_Whole_tree and Shannon index, to measure community richness and evenness of scalp microbial community (Fig. 2A and S1A). Overall, we found significantly decreased community diversity in SD scalps compared with healthy controls (P < 0.001). We further explored the relationship among microbial communities isolated from healthy and SD scalps by calculating beta diversity using the Principal coordinate analysis (PCoA) based on weighted or unweighted UniFrac distance. Our results showed that the composition of both fungi and bacteria was significantly altered in the SD group as compared with the healthy group (P = 0.001, Fig. 2B and S1B).

Next, we used the LEfSe Analysis to construct a correlation network of the microbiome resident on the scalp. On the basis of the results, we found that five fungal genera (Malassezia, Alternaria, Naganishia, Hanseniaspora, and Cladosiphialophora) and five bacterial genera (Staphylococcus, Blautia, Bifidobacterium, Xylanibacterium, Fusobacterium, and Lysobacter) were enriched in the SD patient group, whereas four fungal genera (Mycosphaerella, Cladosporium, Rhodotorula, and Debaryomyces) were enriched in healthy controls (Fig. 2C and S1C). Consistent with previous findings (Xu et al., 2016), our results indicate that alterations in scalp microbiome diversity are linked to SD disease states and that the increase in Malassezia and Staphylococcus abundance is exclusively associated with SD scalp skin, which is highly consistent with our previous studies (Lin et al., 2021).

Treatment with rhTβ4 significantly improved physiological conditions of SD scalp and relapse was reduced at up to 20 weeks

As shown in the patient flow diagram (Fig. 3A), 92 patients were screened for recruitment including 21
healthy patients receiving placebo (group A). The remaining 71 patients were randomly allocated into three groups to receive placebo (group B, \( n = 25 \)), 2% ketoconazole lotion (group C, \( n = 21 \)), or 0.5 mg ml\(^{-1}\) rhT\(\beta\)4 gel (group D, \( n = 25 \)), respectively. Then, the changes from baseline (T0) to Day 168 (T5) in host clinical assessment (i.e., ASFS and MEA) and physiological conditions (i.e., TEWL, Stratum Corneum Hydration [SCH], and sebum values) of each sample were measured (Table S2). The results showed that the clinical severity score improved significantly relative to baseline after rhT\(\beta\)4 treatment. The scores of ASFS and MEA

Fig. 1. Microbial distribution of scalp on healthy controls and SD patients.
A. Fungal flora composition at the phylum level.
B. Fungal flora composition at the genus level.
C. Bacterial flora composition at the phylum level.
D. Bacterial flora composition at the genus level.
Results are presented as the percentage (%) of total sequences.
(with the highest baseline values) in group D significantly decreased after 4 weeks of Tβ4 treatment (from T0 to T3) and sustained reductions during the subsequent 2-week follow-up phase without treatment (from T3 to T4). In the ketoconazole control group, the score of ASFS in group C showed a slight downward trend from T0 to T5 while MEA levels were not significantly different over time. With respect to the placebo control (group B), the changes from T0 to T5 were miniscule in ASFS or even not significant in MEA during the entire treatment (Fig. 4A and C). We then compared the degrees of change by calculating the baseline differences between different groups. In group D, the ΔASFS at every follow-up time point ($P < 0.001^{***}$ minimum) relative to baseline, along with ΔMEA at T4 ($P = 0.0103^*$) differed significantly compared with group B. Although the score of ΔASFS in group C showed a significant difference ($P < 0.001^{***}$ minimum, except at T5) compared with group B, no obvious improvement of lesional erythema area was observed (Fig. 4B and D). Although we noticed that the absolute values of ASFS and MEA increased after the cessation of intervention (from T4 to T5) in group D (Fig. 4A and C), which may be due to the more severe symptoms at baseline, the remaining larger decline in ΔASFS and ΔMEA at T5 (Fig. 4B and D) meant that rhTβ4 was more effective for eliminating dandruff and improving dermatitis than ketoconazole or control shampoo. We concluded that after a 4-week treatment period, 0.5 mg ml$^{-1}$ rhTβ4 gel was significantly superior to 2% ketoconazole lotion in the treatment of subjects with seborrheic dermatitis of the scalp.

Consistent with these results, the scalp skin barrier also recovered after treatment with rhTβ4 gel. In group D, TEWL (with comparable baseline values) decreased during the 4 weeks of rhTβ4 treatment (from T0 to T3) whereas it increased after the cessation of the

Fig. 2. Fungal diversity comparison between the normal and SD scalps.
A. PD_whole_tree and Shannon index and of fungi between the healthy controls and scalp SD patients.
B. Principal coordinate analysis (PCoA) of the fungal community structures based on unweighted UniFrac distance matrix. Each point on the PCoA plot represents a scalp microbiome sample (red = SD, and green = healthy).
C. LEfSe results showing significantly different fungal taxa between the healthy control and scalp SD patients.
intervention (from T3 to T5) (Fig. 4E). The SCH score (with lower baseline values) increased from T0 to T3 and slightly dropped from T3 to T5 (Fig. 4G). Sebum secretion deceased continuously during the experimental period except at T4 (Fig. 4I). These results showed that rhTβ4 significantly improved the scalp skin barrier. Adjusting for baseline measurements, mean change percentages of baseline versus T0 were calculated. Compared with placebo group B, ΔTEWL from T1 to T4 (P < 0.0001****), as well as ΔSCH from T1 to T5 (P < 0.01** minimum), differed significantly in group D. Simultaneously, ΔTEWL at T3 (P = 0.0278*) and T4 (P = 0.0034**), together with ΔSCH at T3 (P = 0.0071*), T4 (P = 0.0027**), and T5 (P = 0.0171*) from baseline differed significantly between group C and group D (Fig. 4F and H), which means that using ketoconazole improved the physiological conditions as well, but to a lesser extent compared with rhTβ4 treatment. Sebum secretion was compared among different groups but there was no significant difference in Δsebum (Fig. 4J), indicating that neither ketoconazole nor rhTβ4 could help to rebalance skin prone to oiliness. Overall, according to the results obtained, rhTβ4 successfully reduced relapse up to 20 weeks after an initial treatment phase and was more effective than ketoconazole in helping symptoms and ameliorating physiological conditions in the treatment of scalp SD.

rhTβ4 improved the microbiome homeostasis

To identify changes in scalp microbiota induced by treatment with ketoconazole or Tβ4, PD_ Whole_tree and PD_ Core were used to compare between groups (Fig. 4F and H). The microbiome composition in each group was found to be significantly different from that in the control group. The microbiota in the rhTβ4 group was more similar to that in the placebo group, indicating that rhTβ4 treatment improved the microbiome homeostasis.
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Shannon index were calculated and compared. Consistent with our previous results, the richness of scalp fungal flora in groups B, C, and D (scalp SD population) was significantly lower than that in the healthy population before the intervention. After the intervention, the richness of scalp fungal flora in groups C and D increased significantly, reaching a level similar to or even higher than that in the healthy population, and remained so for 20 weeks after the intervention (Fig. 5A and B). In the bacterial communities, the diversity of group D increased first at T1 and then tended to stabilize gradually to near the healthy control levels. However, the richness of scalp bacterial flora in groups B and C was still at a low level during and after the intervention period (except at T1 and T5) (Fig. 5C and D). At the same time, the variation trend of $\beta$-diversity of scalp microbiota flora at different intervention stages of each group was evaluated using PCoA analysis. The scalp fungal and bacterial flora of the healthy group A and SD placebo group B did not show significant changes throughout the whole period. For group C, the taxonomic composition of fungi approached the healthy control status in fluctuation, but the effect of ketoconazole on the taxonomic composition of bacteria was minuscule and significant differences to healthy control were maintained over time. For group D, a gradual shift of taxonomic composition towards the healthy state of both fungi and bacteria was detected, and the effect remained up to 20 weeks after the intervention (Fig. 6A and S2A). Therefore, rhTb4 played a decisive role in regulating host physiological conditions and altering the taxonomic composition to move the latter closer to a healthy state.

Discussion

Seborrheic dermatitis is a common and chronic inflammatory dermatosis that tends to flare and remit spontaneously (Clark et al., 2015). In this work, we were...
surprised to observe that there was no significant shift of bacteria in genus and fungi in species from SD scalps compared with those from healthy scalps. However, we observed that disequilibrium in the proportion of the major bacterial and fungal populations are associated with SD. Compared with a normal scalp, the SD scalp had increased Malassezia and Staphylococcus, accompanied by a dramatic decline of microbial diversity, suggesting that the balance of the topical microbiome ecosystem might be important for the severity of SD.

At present, topical corticosteroids and antifungal agents are widely used for the treatment of SD, but with severe adverse effects such as skin atrophy and telangiectasia with long-term use of topical corticosteroids and the ineffectiveness of antifungal drugs in some cases (Sobhan et al., 2019). In this context, we found that rhTβ4 treatment was more effective than ketoconazole – the first-line treatment for seborrheic dermatitis of the scalp – in improving symptoms and severity, ameliorating physiological conditions, and restoring microbiome homeostasis for a longer duration. To the best of our knowledge, this is the first report that states that rhTβ4 is a powerful and well-tolerated scalp SD remedy and that it is even more effective than ketoconazole. In our study, rhTβ4 not only eliminated dandruff and lessened the extent of lesions, but also improved the scalp skin barrier to resist further microbial invasion. Both ketoconazole and rhTβ4 reduced the inflammatory symptoms of SD. Besides, rhTβ4 possessed additional activities including the modulation on multiple cytokines and chemokines, the promotion of wound repair, tissue protection, and skin regeneration (Malinda et al., 1999; Sosne et al., 2007; Crockford et al., 2010). Specifically, rhTβ4 could attenuate apoptosis and enhance the proliferation and division of cells, especially the recruitment and differentiation of stem cells (Zhao et al., 2011), which would be of great help in rebuilding the barrier function of skin. Additionally, rhTβ4 could promote blood vessel formation (Dube and Smart, 2018), helping to restore good blood circulation in the capillaries of damaged skin. Furthermore, its protective effectiveness also benefited from the gel form, which not only helped rhTβ4 to efficiently penetrate skin, but also helped to rebuild a balanced and moist film for the skin and prevent dryness, cracking, and pruritis. Although preliminary, the results of the present study showed that topical rhTβ4 gel was superior to topical ketoconazole and can be considered as an alternative therapeutic modality in the treatment of scalp SD.

More importantly, the changes of scalp microflora after ketoconazole or rhTβ4 treatment also varied. We found that ketoconazole was only effective in inhibiting commensal Malassezia restricta growth at the early stage of intervention, but had no significant bacteriostatic activity against Staphylococcus (data not shown), which was consistent with previous studies showing that although the broad-spectrum antifungal activity of ketoconazole had a good effect on scalp SD, relapse often occurred after discontinuation (Gupta et al., 2014). By comparison, rhTβ4 significantly improved the diversity of both fungal and bacterial flora and was associated with a significant reduction of the relative abundance of two predominant Malassezia and Staphylococcus species. Besides, the effect of rhTβ4 was maintained for up to 20 weeks after discontinuation of the intervention. This may be because rhTβ4 corrected the imbalance of bacteria and fungi in the SD scalp population, making the microbiota structure similar to that of healthy people. Above all, the interactions among host factors and microorganisms imply that rhTβ4 may be an effective, viable, safe, long-term and convenient solution to inhibit the development of SD.

**Experimental procedures**

**Study design**

Throughout the study, the subjects were asked to use, every other day, for 4 weeks, the certain shampoos under investigation, and to avoid use of any other hair products that might interfere with the results, as follows: group A and B, the placebo shampoo; group C, placebo shampoo + 2% ketoconazole lotion; group D, placebo shampoo + 0.5 mg ml⁻¹ rhTβ4. Each volunteer underwent dermatological examination, physiological parameters assessment, and microbiome sampling before (T0, day 0), during (T1, day 7; T2, day 14; T3, day 28), and after (T4, day 42; T5, day 168) the intervention. For 14 days prior to the initiation of the study, as well as 140 days after the 4-week intervention period, subjects were instructed to wash their hair every other day with the placebo shampoo only. Our study and all experiments were in line with the basic principles of ethics of the International Declaration of Helsinki and were reviewed by the Ethics Review Committee (GDIRB [2018]5-5).
Clinical evaluation and physiological measurement

Qualified subjects were classified as healthy and scalp SD groups according to severity of three cardinal symptoms of SD, namely, scaling, erythema, and active folliculitis. The symptoms of scaling were scored based on a board-certified dermatologist's evaluation using the Adherent Scalp Flaking Score (ASFS): 0 = no scaling, 2 = slight scaling, 4 = some scaling, 6 = moderate scaling, 8 = heavy scaling, and 10 = very heavy scaling. The symptoms of erythema were given a score of 0 or 1: 0 = absence of erythema, 1 = presence of erythema. The entire scalp was divided into eight sections and the clinical severity score of SD was determined by summing up the scores of each section. The groups were divided according to the final ASFS and erythema score [healthy group: ASFS < 10, erythema score = 0, with no active folliculitis (n = 21); scalp SD group: ASFS ≥ 10, erythema score ≥ 1, or with active folliculitis (n = 71)]. In the intervention trial, the healthy control group was assigned as group A, and the scalp SD group was then randomly divided into group B (n = 25), group C (n = 21), and group D (n = 25).

The lesion site with the highest ASFS from the SD group subjects and one random site from the healthy group subjects were sampled and analyzed. The physiological measurements were performed under a controlled environment with relative humidity between 40% and 60% and temperature at 25°C. All subjects stayed at this environment for 30 min before the measurements. The ASFS and the Maximum Erythema Area (MEA) were assessed at T0–T5. Additionally, scalp physiological parameters were measured as follows: transepidermal water loss (TEWL) was examined by Vapometer (Delphin, Kuopio, Finland); hydration was examined by Corneometer CM 825 (Courage + Khazaka, Koln, Germany); sebum secretion was examined with a Sebumeter SM815 (Courage + Khazaka).

Bioinformatics processing

Sampling, DNA extraction, sequencing, and species identification were performed as previously described (Lin et al., 2021). Briefly, the microbial samples of healthy and SD scalps were collected by swabbing for 30 s with Catch-all Sample Collections Swabs (QEC091H, Epicentre, Madison, WI, USA). DNA was then extracted using the Oral Swab DNA Rapid Extraction Kit (Centrifugal column type) (Beijing BioTeche, Beijing, China) strictly following the manufacturer's specifications. Next, PCR amplification was performed and the PCR amplicons were sequenced using Illumina HiSeq (PE 250). Specifically, the 514F (GTGCCAGCMGCC GCGGTAA) upstream primers and 805R (GGACTACHVGGGTWTCTAAT) downstream primers were used to amplify the 16S rRNA gene V4 region from bacteria. Correspondingly, the ITS1-F (CTTGGTACATTAGAGGAAGTAA) upstream primers and ITS1-R (GCTGCGTTCTTCATCGATGC) downstream primers were used to amplify the ITS1 region from fungi. The sequencing data from the high-throughput Illumina MiSeq platform was processed using a standard QIIME (Quantitative Insights Into Microbial Ecology) analysis, and all of the sequences were clustered into Operational Taxonomic Units (OTUs) based on their taxonomic relatedness. The α diversity within community was evaluated using the PD_whole_tree index and Shannon index, while the β-diversity between communities was analyzed using the weighted and unweighted UniFrac represented in a PCoA plot. Furthermore, the LEfSe [Linear discriminant analysis (LDA) effect size] analysis was used to compare the microbial abundance that were differentially altered between the healthy controls and SD patients.

Statistical analyses

Statistical analyses in this study were performed using Python (V3.7) and R (V3.6.3). Epidemiological data, subjective scalp scores, and physiological scalp parameters were analyzed by one-way analysis of variance (ANOVA) and Analysis of Covariance (ANCOVA). For microbial analysis, the Wilcoxon test was used to compare the relative abundance of predominant microorganisms between different groups. Data are shown as mean±SEM. n.s., not significant, P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. R: a language and environment for statistical computing.

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Conflict of interest

The authors declare no competing interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Bacterial diversity comparison between the normal and SD scalps. A. PD_whole_tree and Shannon index of bacteria between the healthy controls and scalp SD patients. B. Principal coordinate analysis (PCoA) of the bacterial community structures based on weighted UniFrac distance matrix. Each point on the PCoA plot represents a scalp microbiome sample (red = SD, and green = healthy).

C. LEfSe results showing significantly different bacterial taxa between the healthy control and scalp SD patients.

Fig. S2. PCoA based on weighted Unifrac distance of bacteria among the different groups from baseline (T0) to Day 168 (T5). Each p-value was calculated by one-way ANOVA comparing to A_T0. n.s., not significant, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Table S1. Demographic data of participants in the study.

Table S2. Clinical severity score and skin functional parameters at different follow-up points in four groups. Measurements are presented for the entire sample as means (standard deviations).