Protection Convergence

The PROTECTIVE Effect of White Beech Mushroom (Hypsizygus Marmoreus) Extract against Atopic Dermatitis Skin Lesion Model using NC/Nga Mice

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Abstract

Purpose: This study was carried out to evaluate the protective effects of Hypsizygus marmoreus extract against sodium dodecyl sulfate-induced atopic dermatitis-like skin lesions in vivo.

Methods: Six-week-old NC/Nga mice were divided into a control group(Ctrl), atopic dermatitis induction group(ADIG), and H. marmoreus extract feeding group after atopic dermatitis induction(HTFG). After 3 weeks, cannabinoid receptors type 1(CB1) and type 2(CB2), and the orphan G protein-coupled receptor 55(GPR55) levels were observed to verify the modulation of the endocannabinoid system activity, whereas filaggrin and kallikrein-related peptidase(KLK) 7 expression in the stratum corneum and protease activated receptor(PAR)-2 expression in the interstitial space of epithelial tissue were observed to verify the inhibition of inflammation. Furthermore, phosphorylated extracellular signal-related kinase(p-ERK), phosphorylated mammalian target of rapamycin(p-mTOR), and E-cadherin levels were studied to verify microenvironmental regulation.

Results: Expression of CB1, CB2, and GPR55 was significantly increased in HTFG as compared to that in ADIG. The positive reaction of filaggrin in the stratum corneum was significantly higher in HTFG than in ADIG. KLK7 and PAR-2 positivity in HTFG were significantly reduced as compared to those in ADIG. The p-ERK-positive and p-mTOR-positive reactions in HTFG were significantly reduced as compared to those in ADIG.

Conclusion: H. marmoreus extract has a beneficial effect on the epithelium barrier function and can inhibit the progression of atopic dermatitis.

[Keywords] Hypsizygus Marmoreus, Atopic Dermatitis, Endocrine System, Filaggrin, Lipid Barrier

1. Introduction

Atopic dermatitis(AtD), also known as atopic eczema, is a chronic skin inflammatory disease that can occur at any age, and it affects infants as well as adults. It is known to be an intractable disease, and it is accompanied by characteristic severe itching, skin dryness, and eczema[1][2][3]. The number of AtD patients continue to increase; however, the causes and pathogenesis of AtD have not yet been clearly elucidated, and it is estimated that its onset is caused by a combination of genetics, living environment, immunity, skin barrier problems, and psychological problems[4][5]. Currently, antihistamines, topical steroids, and oral steroids are used in the treatment of AtD[6]. However, these treatments are only effective in temporarily alleviating AtD symptoms, but do not fundamentally cure AtD; therefore, there is need for better and effective treatment modalities[7][8].

β-glucan, a polysaccharide, is a physiologically active constituent present in yeast cell walls, grains, mushrooms, and others, and it can promote various physiological activities, including immunity enhancement, and skin regeneration; it exhibits antioxidant, anti-cancer, and anti-cholesterol effects, as well[9][10]. Furthermore, it is widely used as an ingredient or additive...
in cosmetics[11][12][13]. β-glucan particularly regulates immune responses by influencing immune cells, like monocytes, macrophages, dendritic cells, natural killer cells, and neutrophils, through cell receptors such as Dectin-1, Toll-like receptor(TLR)-2, and TLR-6, inducing cytokine secretion, and it also stimulates B cells to regulate immune responses[14]. Although many studies have been conducted to validate the antioxidant, anti-inflammatory, and skin regeneration effects of β-glucan, most of these studies have shown its efficacy when administered orally[15][16][17].

The endocannabinoid system(ECS) is a biological system composed of cannabinoids that regulate appetite, pain, sensation, mood, and memory[18]. It has been recently demonstrated that an increase or decrease in the ECS is associated with a variety of pathological conditions[19]. Temporarily altered ECS activation reduced symptoms of the compensatory response in the body and slowed disease progression[20]. ECS is also closely related to the regulation of cell growth, proliferation, and immune and inflammatory responses involved in the skin homeostasis[21].

The purpose of this study was to demonstrate the effects of Hypsizygus marmoreus extract in regulating epithelial inflammation caused by AtD through ECS activity. CB1, CB2, and GPR55 were observed to verify the regulation of ECS activity, and filaggrin and kallikrein-related peptidase(KLK) 7, in the stratum corneum, and protease activated receptor(PAR)-2, in the interstitial space of epithelial tissue, were observed to verify the inhibition of inflammation. Furthermore, to demonstrate the microenvironmental regulation, phosphorylated extracellular signal-related kinase(p-ERK), phosphorylated mammalian target of rapamycin(p-mTOR), and E-cadherin levels were studied. In this study, we confirm that H. marmoreus extract inhibits epithelial inflammation caused by AtD by regulating ECS activity.

2. Materials and Methods

2.1. In vivo study subjects and procedures

Six-week-old Nc/Nga male mice(Orient Bio, Republic of Korea) were acclimated for 2 weeks in a sterile breeding device, and then mice(weight 20 ± 2 g) were selected for experimental use. The mice were divided into three groups: control group(Ctrl), AtD induction group(ADIG), and H. marmoreus extract fed group after AtD induction(HTFG), with seven mice assigned to each group. For the HTFG group, 80 mg/kg of H. marmoreus extract was diluted in 0.2 mL of saline, and it was orally administered daily for 3 weeks after induction of AtD. In order to induce AtD, the skin on the back of the mouse was shaved, followed by rubbing of 1 ml sodium dodecyl sulfate(SDS, Sigma-Aldrich, USA) 20 times with a cotton swab, to disrupt the lipid barrier of the stratum corneum. The animal experiments were performed after approval from the Semyung University Animal Experimental Ethics Committee(IACUC: smece-20-11-02), and the management and use of laboratory animals were conducted in accordance with the NIH guidelines.

2.2. Immunohistochemical analysis

Immuno-histochemistry analysis was performed as described in a previous study[22][23][24]. The skin was subjected to cardiac perfusion fixation with a vascular rinse and 10% neutral buffered formalin(NBF). The dorsal skin was fixed in 10% NBF at room temperature for 24 h, and then embedded in paraffin in a conventional manner, and continuous 5-μm-thick sections were made. For immunohistochemical staining, the skin sections were first subjected to proteolysis in proteinase K(20 μg/ml) for 5 min, followed by a blocking reaction in 20% normal goat serum for 2 h. The sections were immunostained using incubation with different primary antibodies, including mouse anti-CB1(1:100), mouse anti-CB2(1:100), mouse anti-GPR55(1:100), mouse anti-E-cadherin(1:100), and mouse anti-p-mTOR(1:100) purchased from
Abcam, USA and mouse anti-filaggrin(1:200), mouse anti-KLK7(1:50), mouse anti-PAR-2(1:50), and mouse anti-p-ERK(1:50) purchased from Santa Cruz Biotec, USA, in a humidified chamber at 4 °C for 48 h. The secondary antibody, biotinylated goat anti-mouse IgG(1:100, Abcam), was linked for 12 h at room temperature, and then reacted with an avidin biotin complex kit (Vector Lab, USA) for 1 h at room temperature. Color was developed in 0.05 M Tris-HCl buffer solution (pH 7.4) containing 0.05% 3,3’-diaminobenzidine and 0.01% HCl, followed by counter staining with hematoxylin. The immunohistochemistry results were quantified as means ± standard error[25][26]. The image analysis was performed using Image Pro Plus (Media Cybernetics, USA). Skin tissues sections randomly selected from each group were observed at x 400 magnification, and then imaged with positive pixels /20,000,000 pixels. Statistical analyses were performed using the SPSS software (SPSS 25, SPSS Inc., USA), and the significance (p < 0.05) was verified using one-way ANOVA, and the Tukey HSD was performed for the post-hoc test.

3. Results and Discussion

A positive reaction for CB1 was strongly observed in the skin epithelial tissues of ADIG(53,269±2,509 /20,000,000 pixels) with a 341% increase as compared to that in Ctrl group(12,090±478/20,000,000 pixels). Furthermore, it was significantly increased in HTFG(107,874±4,037/20,000,000 pixels) by 103% as compared to that in ADIG <Figure 1>. A strong CB2 positive reaction was observed in the epithelial tissue in ADIG(34,386±1,176 /20,000,000 pixels) with an increase by 114% as compared to that in the Ctrl group(16,062±1,176/20,000,000 pixels). CB2 positivity in HTFG(130,456±4,525/20,000,000 pixels) was significantly higher(201%) that in ADIG <Figure 1>. GPR55 positive reaction was observed as strong positive in epithelial tissue of ADIG(58,337±1,992/20,000,000 pixels) with a 113% increase as compared to that in the Ctrl group(27,380±913/20,000,000 pixels), and this increase was significantly higher(68%) in tHTFG(97,866±1,610/20,000,000 pixels) than that in the ADIG <Figure 1>. Filaggrin-positive reaction was observed in the stratum corneum, with a 54% decrease in ADIG(21,327±1,906 /20,000,000 pixels) as compared to that in Ctrl(46,833±1,817/20,000,000 pixels). On the other hand, filaggrin positivity was significantly higher(287%) in HTFG(82,563±1,837/20,000,000 pixels) than that in ADIG <Figure 2>. The KLK7 positive reaction was observed as a strongly positive in the interstitial space of the epithelial tissue. It was increased in ADIG(101,351±2,697/20,000,000 pixels) by 367% as compared to that in the Ctrl(21,724±1,332/20,000,000 pixels), and it was significantly decreased by 34% as compared to ADIG in the HTFG(66,526±1,318/20,000,000 pixels) <Figure 2>. A PAR-2 positive reaction was observed as strongly positive in the interstitial space of epithelial tissue, and it was increased in ADIG(94,848±2,481/20,000,000 pixels) by 527% as compared to that in Ctrl(15,134±1,373/20,000,000 pixels). In contrast, it was significantly decreased in HTFG(40,040±1,428/20,000,000 pixels) by 58% as compared to that in ADIG <Figure 2>. Strongly positive p-ERK reaction was observed in the interstitial space of the epithelial tissue. It was increased by 11,056% in ADIG(100,652±2,486/20,000,000 pixels) as compared to that in Ctrl(8,705±322/20,000,000 pixels), and it was significantly decreased by 51% in HTFG (49,484±2,036/20,000,000 pixels) as compared to that in ADIG <Figure 3>. A strong positive p-mTOR signal was observed in the interstitial space of the epithelial tissue, with a 765% increase in ADIG(85,448±2,730/20,000,000 pixels) as compared to that in Ctrl group(9,876±295/20,000,000 pixels). Furthermore, it was significantly decreased by 66% in HTFG(29,291±1,106 /20,000,000 pixels) as compared to that in ADIG <Figure 3>. A strong E-cadherin positive reaction was observed in the intercellular space of epithelial tissue, and it was decreased in ADIG(16,763±1,264/20,000,000 pixels) by 60% as compared to that in Ctrl group(42,111±2,280/20,000,000 pixels). In contrast, it was significantly increased by 327% in HTFG(71,588±2,069/20,000,000 pixels) as compared to that in ADIG <Figure 3>.
AtD is an inflammatory skin disease characterized by proliferative eczema, erythema, and dryness, with a very high recurrence rate[27]. It is also an early hallmark of the atopic streak leading to asthma and allergic rhinitis[28]. The pathogenesis of AtD is still unknown, and it is thought to be caused by a combination of genetic, immunological, and environmental factors and skin barrier dysfunction[29]. Various keratinocyte differentiation markers, including filaggrin, involucrin, and loricrin, are low in AtD[30], and ceramide levels in the stratum corneum are also reduced[31]. The characteristic feature of AtD skin is spongiosis due to tissue remodeling and epidermal proliferation because of increased epidermal proliferation and decreased differentiation[32][33]. This structural change in the skin barrier interferes with skin homeostasis and prevents the skin from performing the normal barrier function. Therefore, it can be assumed that AtD is caused by an abnormality in the physiological balance required to maintain skin homeostasis.

ECS intervenes in the regulation of skin homeostasis by regulating cell growth, differentiation, and immune and inflammatory responses[34]. Recent studies indicated that inadequate operation of the ECS can affect skin pathologies caused by dysfunction of the skin barrier in AtD, thereby making ECS a new treatment target for a variety of skin diseases[35]. ECS comprises cannabinoid receptors(CBRs), endogenous ligands, and enzymes involving the synthesis and degradation of cannabinoids[36]. The main CBRs are CB1 and CB2, and recently, another G protein-coupled cannabinoid receptor, GPR55 was identified as a type 3 CBR[37][38]. Several studies have demonstrated the alleviation of inflammatory symptoms by CB1 agonists through the downregulation of mast cell activation[39] and the reduction of pro-inflammatory mediators derived from keratinocytes[40]. In addition, CB2 agonists inhibit skin inflammation by inhibiting the migration of inflammatory cells[41], and GPR55 found in mast cells demonstrates anti-inflammatory effects by inhibiting the release of nerve growth factors mediated by mast cells and by reducing angiogenesis[42]. In this study, CB1, CB2, and GPR55, were all significantly increased in the groups administered with the H. marmoreus extract. In ADIG, CB1 was decreased as compared to that in Ctrl, whereas it was increased in HTFG compared to that in ADIG. CB2 and GPR55 positive reactions were also decreased in ADIG compared to those in Ctrl, whereas they were increased in HTFG compared to that in ADIG. These results indicated that administration of H. marmoreus extract had an anti-inflammatory effect on the skin epithelial tissue.

In addition, a positive reaction to filaggrin was observed in this study confirming the improvement of the lipid barrier of the skin. Filaggrin is a protein that forms the granular layer of the epithelium, and it is one of the genetic markers of AtD[43]. Decrease in filaggrin due to genetic changes or acquired factors weakens the formation of cell membranes in the keratinocytes and reduces the junction between keratinocytes, thereby disrupting the skin barrier function. This facilitates the penetration of allergens from outside, causing sensitization and allergic reactions[44]. In this study, a strong filaggrin-positive reaction was observed in the stratum corneum in HTFG by 413% as compared to that in ADIG, and filaggrin levels were decreased by 72% in ADIG as compared to that in Ctrl group. This increase in the filaggrin-positive reaction indicated that the H. marmoreus extract could improve the skin barrier function by promoting keratinocyte differentiation.

The increase in pH in the stratum corneum due to various causes, increases the activity of serine proteases such as KLK7, which induces the exfoliation of keratinocytes[45]. KLK plays an important role in the skin barrier function, and its abnormal expression causes skin diseases such as AtD, and psoriasis. KLK7 is a physiological activator of caspase 14 and acts as the first step in the filaggrin degradation process[46]. It mediates the inflammatory response through PAR-2, thereby causing pruritus. PAR-2 is a G protein-coupled receptor present in mast cells, keratinocytes, and vascular endothelial cells, and it is involved in inflammatory reactions, pigment production, and skin barrier functions[47]. In this study, the KLK7 positive reaction was increased in ADIG as compared to that in Ctrl, whereas it was significantly decreased by 40% in HTFG compared to that in ADIG. The PAR-2 positive reaction also increased
in ADIG compared to Ctrl, but was significantly decreased by 63% in HTFG compared to ADIG. These results indicated that HTFG could reduce inhibition of the expression of filaggrin-like substances by regulating the process that leads to KLK7 and PAR-2 expression due to damage to the lipid barrier. Therefore, it may contribute to the recovery of the skin barrier by inhibiting the pathological process caused by damage to the lipid barrier.

According to a recent study, inflammatory cytokines, such as TNF-α, IL-1β, and IL-17A, which are tumor necrosis factors, induce mTOR activation, promote epidermal proliferation, and reduce the expression of epidermal differentiation markers[48]. In AtD skin lesions, the mTOR inhibitor, rapamycin decreases inflammatory cell infiltration and serum IgE levels, suggesting that inhibition of mTOR signaling can inhibit AtD[49]. E-cadherin expression decreased on the surface of keratinocytes of AtD lesion skin[50], followed by dissolving of the tight junctions that are crucial in maintaining the epidermis barrier function. In addition, oxidative stress directly affects epidermal keratinocytes and induces intracellular changes, leading to edema, spongiosis, and destruction of the stratum corneum. Mitogen-activated protein kinases(MAPKs), such as ERK and JNK, are essential signals for the production of cytokines, such as IL-4 and TNF-α, through the activity of nuclear factor kappa-light-chain-enhancer of activated B cells(NF-κB), which regulates the inflammatory response. IL-17, a pro-inflammatory cytokine, decreases filaggrin expression in AtD through the MAPK signaling pathway. In the present study, p-ERK-positive and p-mTOR-positive reactions were increased in ADIG as compared to those in Ctrl, whereas, they were significantly decreased in HTFG as compared to those in ADIG. E-cadherin positive reaction was decreased in ADIG compared to that in Ctrl group, and it was increased significantly in HTFG as compared to that in ADIG by 22%. Collectively, these results demonstrated that H. marmoreus extract regulated the expression of E-cadherin to maintain the epidermal structure and to inhibit phosphorylation of ERK, thereby blocking the MAPK signaling pathway.

In conclusion, H. marmoreus extract promotes the functions of the skin barrier by modulating ECS regulation, keratinocytes differentiation, and filaggrin expression in AtD, thereby restoring the skin barrier function of the stratum corneum. Therefore, on the basis of these results, β-glucan, which is abundant in grains and mushrooms, may be used in the management of AtD in the future.

**Figure 1.** Endocannabinoid system(ECS) activation effects of Hypsizygus marmoreus(HM) extract.

![Figure 1](image)

Note: HM extract promotes the ECS in HTFG. CB1, CB2, and GPR55 expression (dark brown indicated by arrows) were significantly increased in HTFG as compared to that in ADIG (p < 0.01) (bar size, 50 μm). Data of CB1, CB2, and GPR55 image analyses indicates similar results (p < 0.01). *p < 0.01, compared with the Ctrl; #p < 0.01, compared with ADIG. Ctrl, normal; ADIG, Atopic dermatitis(AD) induced with no treatment; HTFG, HM treated with AD; EP, epidermis; DE, dermis.
Figure 2. Regulation of lipid barrier and inflammation by HM extract.

Note: HM promotes lipid barrier (filaggrin) function and regulates inflammation signal proteins (KLK7 and PAR-2) in HTFG. Filaggrin expression (dark brown indicated by arrow) was significantly increased in HTFG as compared to that in ADIG (p < 0.01). KLK7 and PAR-2 expression (dark brown indicated by arrow) was significantly decreased in HTFG as compared to that in ADIG (p < 0.01). Data of Filaggrin, KLK7, and PAR-2 image analyses indicates similar results (p < 0.01). *p < 0.01, compared with the Ctrl; #p < 0.01, compared with the ADIG. Ctrl, normal; ADIG, Atopic dermatitis (AD) induced with no treatment; HTFG, HM treated with AD; EP, epidermis; DE: dermis.

Figure 3. Regulation of inflammation induced by microenvironmental factors modulated by HM extract.

Note: p-ERK and p-mTOR expression (dark brown indicated by arrow) were remarkably decreased in HTFG as compared to that in ADIG (p < 0.01). E-cadherin expression (dark brown indicated by arrow) was significantly increased in HTFG as compared to that in ADIG (p < 0.01). Data of p-ERK, p-mTOR, and E-cadherin image analyses indicates similar results (p < 0.01). *p < 0.01, compared with the Ctrl; #p < 0.01, compared with the ADIG. Ctrl, normal; ADIG, Atopic dermatitis (AD) induced with no treatment; HTFG, HM treated with AD; EP, epidermis; DE: dermis.

4. References
4.1. Journal articles


5. Appendix

5.1. Authors contribution

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