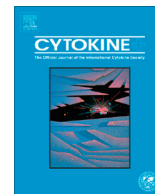




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Tannic acid modulates NFκB signaling pathway and skin inflammation in NC/Nga mice through PPARγ expression

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ABSTRACT

Polyphenolic compound tannic acid, which is mainly found in grapes and green tea, is a potent antioxidant with anticarcinogenic activities. In this present study, we hypothesized that tannic acid could inhibit nuclear factor (NF)κB signaling and inflammation in atopic dermatitis (AD) NC/Nga mice. We have analyzed the effects of tannic acid on dermatitis severity, histopathology and expression of inflammatory signaling proteins in house dust mite extract induced AD mouse skin. In addition, serum levels of T helper (Th) cytokines (interferon (IFN)γ, interleukin (IL)-4) were measured by enzyme-linked immunosorbent assay. Treatment with tannic acid ameliorated the development of AD-like clinical symptoms and effectively inhibited hyperkeratosis, parakeratosis, acanthosis, mast cells and infiltration of inflammatory cells in the AD mouse skin. Serum levels of IFNγ and IL-4 were significantly down-regulated by tannic acid. Furthermore, tannic acid treatment inhibited DfE induced tumor necrosis factor (TNF)α, high mobility group protein (HMG)B1, receptor for advanced glycation end products (RAGE), extracellular signal-regulated kinase (ERK)1/2, NFκB, cyclooxygenase (COX)2, IL-1β and increased the protein expression of peroxisome proliferator-activated receptor (PPAR)γ. Taken together, our results demonstrate that, DfE induced skin inflammation might be mediated through NFκB signaling and tannic acid may be a potential therapeutic agent for AD, which may possibly act via induction of PPARγ protein.

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1. Introduction

Atopic dermatitis (AD) is an allergic inflammatory skin disease. Although the pathogenesis of AD is complicated, infiltration of immune cells such as mast cells, neutrophils, eosinophils, and lymphocytes are elevated in disease state [1]. In addition, serum immunoglobulin E (IgE) levels are elevated in AD [2]. *Dermatophagoides farinae* (DfE) is one of the common house dust mite allergen, which is mainly associated with human AD and bronchial asthma [3]. AD patients are sensitive to DfE and upon

exposure, macrophages are known to accumulate in acutely or chronically inflamed skin [4]. The high mobility group protein (HMG)B1, a nuclear protein secreted by immune cells, such as macrophages and dendritic cells, is released into extracellular space, triggering inflammatory immune response, tumor metastasis and autoimmune disease. Extracellular HMGB1 interacts with a multi ligand-receptor for advanced glycation end products (RAGE) leading to the activation of nuclear factor (NF)κB proinflammatory signaling, thereby triggering a variety of inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)α, IL-4, IL-6 and interferon (IFN)γ [5]. Moreover, the elevated levels of cytokines can further induce NFκB activation and thereby worsening the disease condition.

The peroxisome proliferator-activated receptors (PPARs) were identified as the nuclear hormone receptor superfamily and their subtypes such as PPARα and γ are mainly involved in insulin

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sensitization in adipocytes. Moreover, PPAR γ functions as the central regulator of cellular differentiation, apoptosis, metabolic disease and lipid metabolism [6]. Recent studies reported that PPAR γ has potent anti-inflammatory activity in several animal models of both irritant and allergic skin diseases [7]. Another similar clinical study demonstrated that, oral PPAR γ agonist ameliorates skin inflammation in patients with dermatitis [8].

Immune suppressive topical or systemic steroid therapy achieves a faster improvement of AD, but their long-term use may produce a wide range of undesirable adverse effects [9]. Recently, plant polyphenols have emerged as a therapeutic alternative for the treatment of AD [10]. Tannic acid, a naturally occurring polyphenolic acid, is widely distributed in green tea, wine, grapes, and plants. It has strong antioxidant, anti-inflammatory, anticarcinogenic and antimutagenic activities [11]. In addition, the condensed tannin attenuates inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)2 expression in AD NC/Nga mouse model [12]. Another similar study reported that two condensed tannins effectively suppress Th2 cytokines in AD mice [13]. On the basis of these studies, we hypothesized that tannic acid ameliorates skin inflammation, attenuates the proinflammatory factors and inflammatory cytokines and modulate HMGB1 and NF κ B expressions in DfE induced NC/Nga mouse model of AD.

2. Materials and methods

2.1. Materials

Biostir AD, a cream containing DfE extract (DfE cream), was purchased from Biostir, Inc. (Kobe Japan). All the reagents and chemicals were of analytical grade and purchased from either Sigma or Wako, Tokyo, Japan, until mentioned otherwise. Tannic acid pure drug was purchased from Wako, Tokyo, Japan.

2.2. Experimental design

Specific pathogen-free female 6 weeks old NC/Nga mice were obtained from Charles River, Yokohama, Japan. The animals were maintained in a controlled room (temperature $23 \pm 2^\circ\text{C}$, humidity $55 \pm 15\%$, 12 h light cycle). After 1 week, the mice were randomly divided into 3 groups, untreated group-received vehicle (distilled water) (Normal, $n = 5$); DfE cream treated mice (100 mg/mouse) was divided into two groups and each received vehicle (distilled water) (AD, $n = 5$) or Tannic acid (80 mg/kg/day, per oral) (AD + Tannic acid, $n = 5$) and were allowed free access to drinking water and standard laboratory diet. The animal experiments were performed in accordance with the national guidelines and approved by the animal care committee of Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan.

2.3. Induction of AD

AD-like skin lesions were induced in NC/Nga mice using DfE cream, as we described previously [14]. Briefly, 150 μL of 4% sodium dodecyl sulfate was applied to the shaved dorsal skin and ears. After 3 h, 100 mg of DfE cream was applied topically. This procedure was carried out twice weekly for 2 weeks. Tannic acid (80 mg/kg/day, per oral) treatment [11], was started after AD induction (2 weeks from the start of the experiment) and continued for 2 weeks.

2.4. Evaluation of dermatitis severity

The dermatitis severity was measured every week, according to the eczema area and severity index scoring system: Four different

manifestations ((1), erythema/haemorrhage, (2), edema, (3), exco-riation/erosion, (4), dryness/scaling), were given individual score as per their severity (0, no; 1, mild; 2, moderate; 3, severe symptoms). The total score (minimum 0, maximum 12) for each mouse was taken as the score of the mouse. The final score was given as an average after every group scores were accumulated [14].

2.5. Histopathological studies

By the end of the study period, mice were sacrificed and their skin tissues were harvested for semiquantitative immunoblotting studies. The half of the skin was immediately frozen in liquid nitrogen for subsequent protein extraction assays. The remaining excised skin was cut into about 2 mm thick transverse slices and fixed in 10% formalin. Sections of 3–5 μm thickness were stained with haematoxylin and eosin (H&E), naphthol AS-D chloroacetate esterase and toluidine blue for detecting epidermal thickness [15], inflammatory cells such as neutrophil and mast cells, respectively. A histomorphological evaluation of all the skin sections was carried out in a blinded fashion [14].

2.6. IgE, Th2 and Th1 cytokines

By the end of the study period (day 28), blood specimens were collected from the retro-orbital sinus of every animal using a capillary tube, serum was separated and stored at -80°C until use. Serum levels of mouse IgE, IL-4 and IFN γ were measured by enzyme-linked immunosorbent assay (ELISA) kits provided by (Shibayagi, Gunma, Japan) and thermo scientific Inc. (Meridian Rd, Rockford, IL, USA) according to the manufacturer's protocol.

2.7. Protein analysis by Western blotting

Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitro-cellulose membranes [16]. Membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% tween (TBST) and incubated individually with the following antibodies: Antibodies against HMGB1, RAGE, phospho (p)-NF κ B, NF κ B, cyclooxygenase (COX)2, TNF α , TNF receptor (TNFR)1, IL-1 β , p-extracellular signal-regulated kinases (ERK)1/2, ERK1/2, and PPAR γ . All the antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) or Cell Signaling Technology, Inc. (Danvers, MA, USA) and used at a dilution of 1:1000. After washing three times with TBST, the membranes were incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at room temperature. Further, the membranes were washed three times with TBST and then developed using a chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK). The blots were scanned and the signals were quantified by densitometric analysis using Image Studio Digits ver. 4 (Superior Street, Lincoln, Nebraska, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control protein.

2.8. Immunofluorescence determination of PPAR γ

Formalin fixed, paraffin embedded skin sections were used for immunofluorescence staining. Sections underwent microwave antigen retrieval were blocked with 10% goat serum in phosphate-buffered saline (PBS), and incubated with rabbit polyclonal anti-PPAR γ antibody (Santa Cruz Biotechnology). Binding sites of the primary antibody were revealed with FITC conjugated anti-rabbit secondary antibody (Abcam Ltd, Cambridge, UK). Nuclei were counterstained with 4-6-diamidino-2-phenylindole

(DAPI) and visualized with a fluorescence microscope (AxioVs40v4.8.2, Carl Zeiss, Japan) [17].

2.9. Statistical analysis

Data are presented as a mean $\pm$ standard error of mean (SEM) and were analyzed using one way analysis of variance (ANOVA) followed by Tukey multiple comparison test or two-tailed student *t*-test when appropriate. A value of $p < 0.05$ was considered statistically significant. For statistical analysis, GraphPad Prism 5 software (San Diego, CA) was used.

3. Results

3.1. Tannic acid ameliorates clinical symptoms in DfE induced AD NC/Nga mice

As shown in Fig. 1A, application of DfE cream induced AD signs and symptoms (erythema/haemorrhage, edema, excoriation/erosion and dryness/scaling) significantly during the 2 weeks of induction period. During the course of treatment, tannic acid markedly ameliorated these phenotypes in AD skin lesions. Fig. 1B–D show the difference between individual symptoms during the course of the treatment period. There is no difference between individual symptoms and almost the similar score was attained for each symptom.

3.2. Effects of tannic acid on histopathological changes

H&E, naphthol AS-D chloroacetate esterase and toluidine blue staining of skin sections showed hyperkeratosis, parakeratosis, epidermal thickness, neutrophil accumulation and mast cells in AD skin lesions. In contrast, tannic acid treatment reduced these dermal changes compared with AD mice (Fig. 2A–C).

3.3. Effects of tannic acid on PPAR γ expression

A previous study reported that PPAR γ agonist suppressed the inflammatory cytokines in AD skin lesion [18]. Here, we have examined whether tannic acid would increase PPAR γ expression in DfE induced AD mice. As shown in Fig. 3, immunofluorescence examination showed that PPAR γ staining was decreased in DfE-induced AD mice, whereas treatment with tannic acid increased PPAR γ expression in AD skin sections. Next we confirmed PPAR γ expression in the skin tissue by Western blotting. The PPAR γ protein expression was suppressed in vehicle-treated AD mice, but when treated with tannic acid, its expression was increased dramatically (Fig. 4A). These results clearly suggest that this plant polyphenol could increase PPAR γ expression in AD skin lesion.

3.4. Tannic acid inhibits DfE induced HMGB1 and RAGE expression

Extracellular HMGB1 triggers proinflammatory cytokines in skin lesions. Thus, we examined the effect of tannic acid on

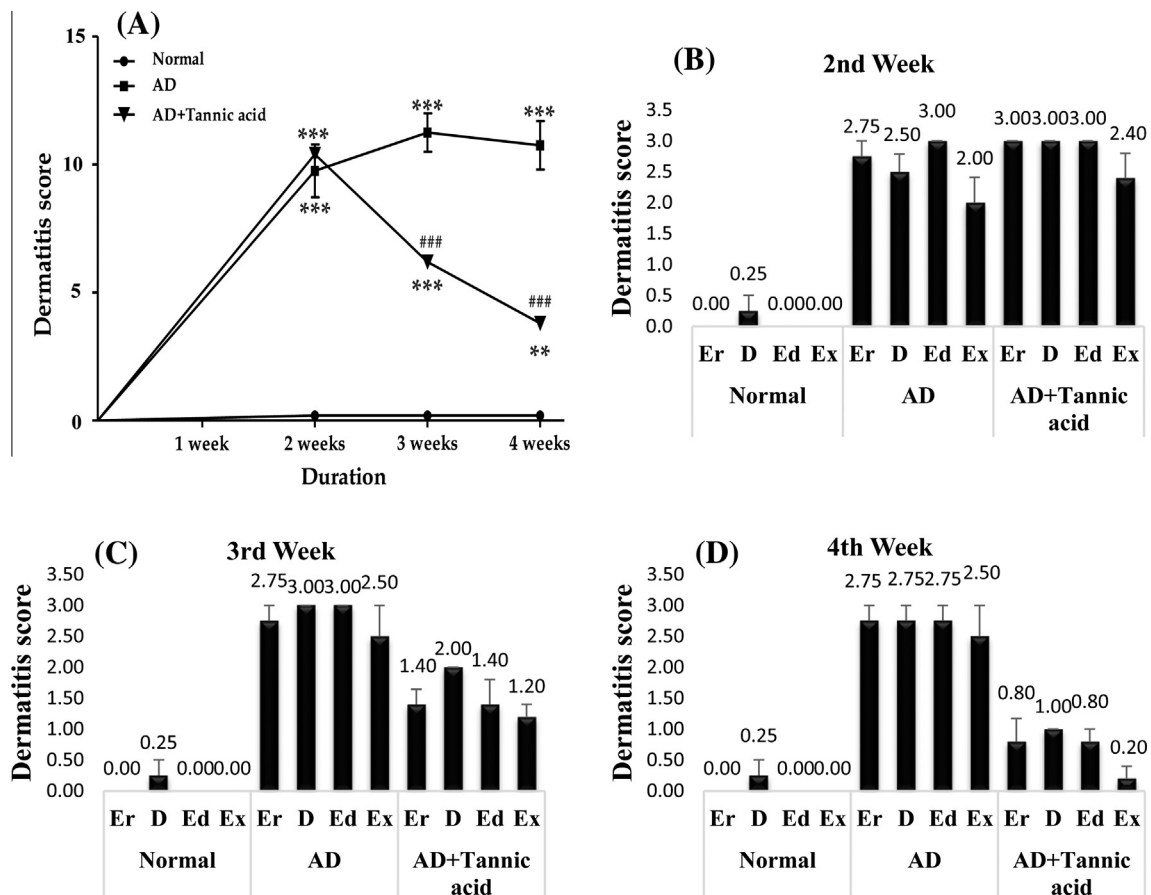


Fig. 1. (A) Effect of tannic acid on time-dependent dermatitis score of NC/Nga mice with AD-like skin lesions. (B–D) Individual score for clinical symptoms. (B) 2nd week, (C) 3rd week, (D) 4th week. Er, erythema; D, dryness; Ed, edema; Ex, excoriation. Each bar represents mean $\pm$ SEM. Normal ($n = 5$), age-matched normal NC/Nga mice; AD ($n = 5$), AD induced NC/Nga mice; AD + Tannic acid ($n = 5$), AD induced mice administered with tannic acid (80 mg/kg/day, per oral) (3rd and 4th week). *** $p < 0.001$ and ** $p < 0.01$ vs Normal; ### $p < 0.001$ vs AD (two tailed student *t*-test).

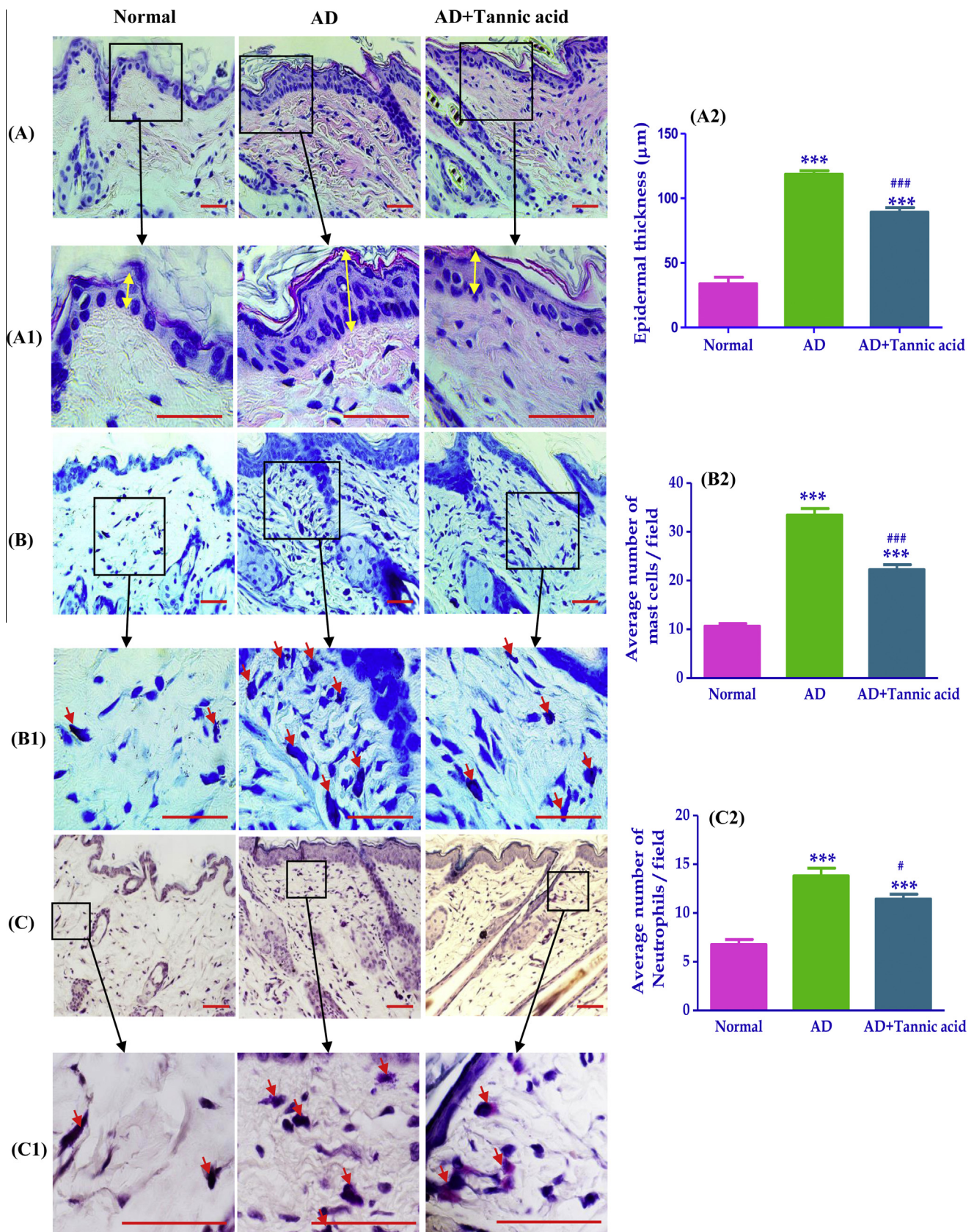


Fig. 2. (A) H&E staining of the cross-sectional tissue slices of skin showing hyperkeratosis, parakeratosis, spongiosis and epidermal thickness (40 $\times$). (A1) Individual insets of 100 $\times$ magnification, the yellow arrows indicate epidermal thickness. (A2) Quantification of epidermal thickness. (B) Skin levels of mast cells by toluidine blue staining (40 $\times$). (B1) Individual insets of 100 $\times$ magnification, the red arrows indicate mast cells. (B2) Quantification of mast cells per field. (C) Naphthol AS-D chloroacetate esterase staining of the cross-sectional tissue slices of skin showing neutrophil accumulation (40 $\times$). (C1) Individual insets of 160 $\times$ magnification, the red arrows indicate neutrophils. (C2) Quantification of neutrophils per field. Scale bar = 100 μm . Each bar represents mean $\pm$ SEM. Normal, age-matched normal NC/Nga mice; AD, AD induced NC/Nga mice; AD + Tannic acid, AD induced mice administered with tannic acid. *** p < 0.001 vs Normal; ### p < 0.001 and # p < 0.05 vs AD (Tukey multiple comparison test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

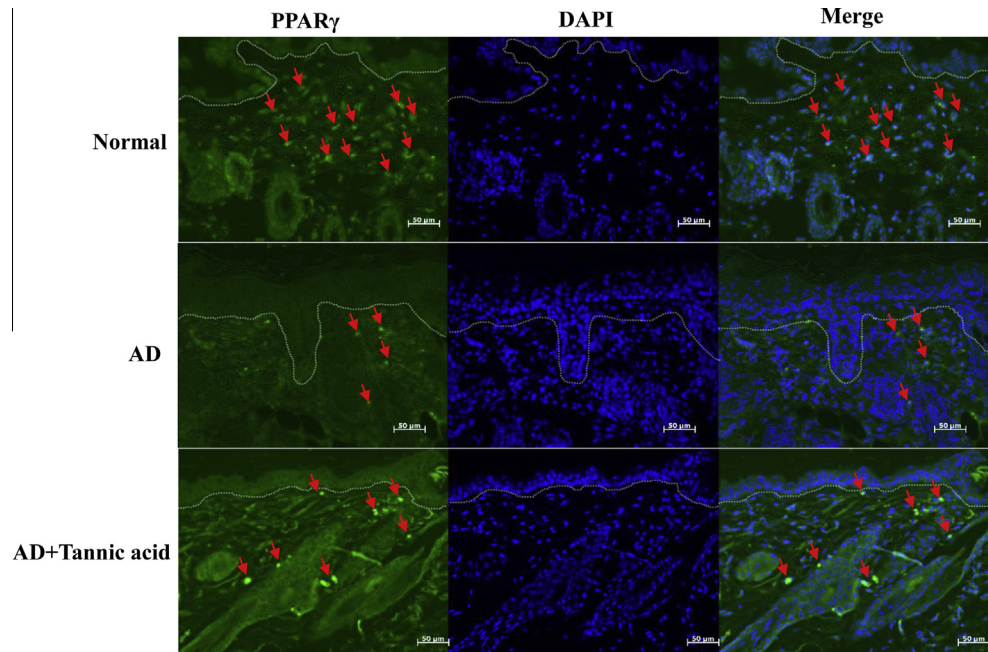


Fig. 3. Immunofluorescence determination of PPAR γ positive cells (Red arrow), Scale bars = 50 μ m; White dashed lines indicate the dermal/epidermal junction. Each bar represents mean $\pm$ SEM. Normal, age-matched normal NC/Nga mice; AD, AD induced NC/Nga mice; AD + Tannic acid, AD induced mice administered with tannic acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

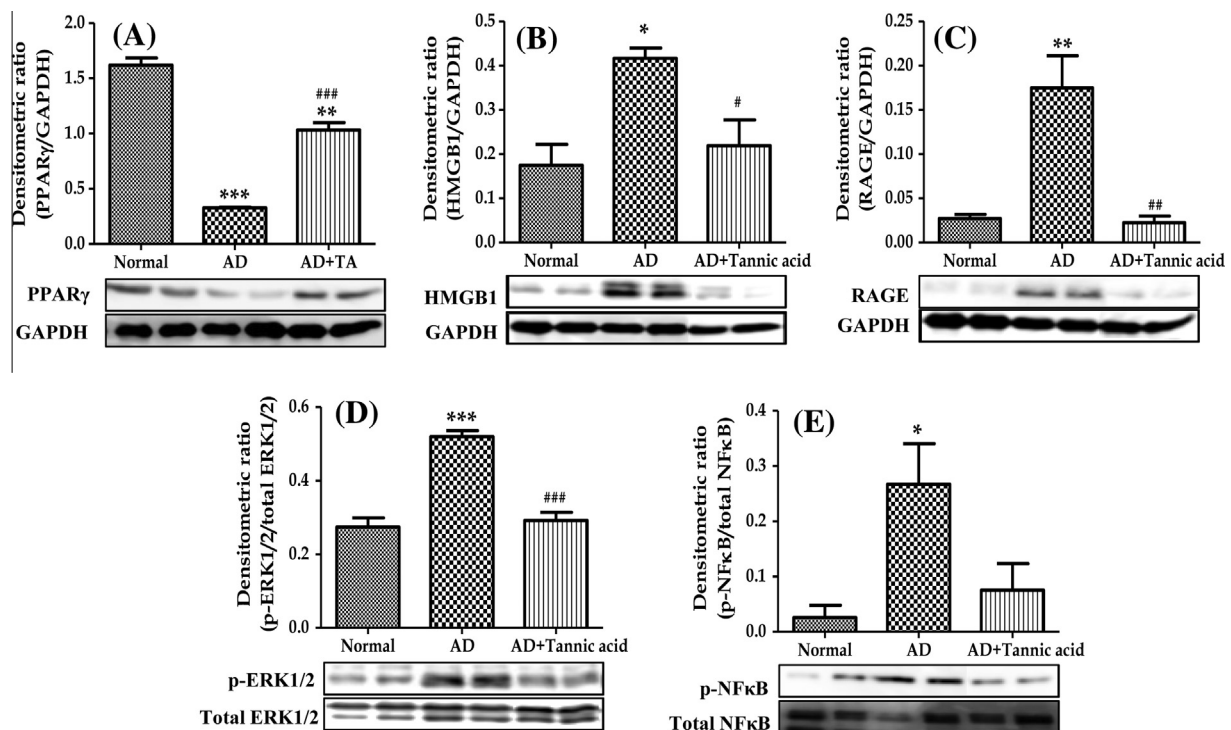


Fig. 4. Tannic acid's effects on PPAR γ , HMGB1, RAGE, p-ERK1/2, and p-NF κ B expression. Western blots show specific bands for the expression of (A) PPAR γ (ratio relative to that of GAPDH), (B) HMGB1 (ratio relative to that of GAPDH), (C) RAGE (ratio relative to that of GAPDH), (D) p-ERK1/2 (ratio relative to that of ERK1/2), (E) p-NF κ B (ratio relative to that of NF κ B). Each bar represents mean $\pm$ SEM. Normal, age-matched normal NC/Nga mice; AD, AD induced NC/Nga mice; AD + Tannic acid, AD induced mice administered with tannic acid. *** p < 0.001, ** p < 0.01 and * p < 0.05 vs Normal; ### p < 0.001, ## p < 0.01 and # p < 0.05 vs AD (Tukey multiple comparison test).

HMGB1 expression. We have found that the expression of HMGB1 and RAGE were significantly up-regulated in DfE induced AD mouse skin, whereas tannic acid treatment significantly down-regulated their expressions in AD mice (Fig. 4B and C).

3.5. Tannic acid inhibits phosphorylation of ERK1/2 and NF κ B in DfE induced AD mice

To determine whether tannic acid can modulate the activation of ERK1/2 and NF κ B in AD skin lesions, the protein levels of

p-ERK and p-NF κ B were measured. They were significantly up-regulated in vehicle-treated AD mice and these changes were significantly suppressed by tannic acid treatment (Fig. 4 D–E). These findings support that natural polyphenol tannic acid suppressed ERK1/2 and NF κ B activation in our NC/Nga mouse model of AD.

3.6. Effects of tannic acid on IL-1 β , TNF α , TNFR1, and COX2 in DfE induced AD mice

Several inflammatory cytokines are the downstream targets of NF κ B signal transduction. Our Western blot analysis indicated that the IL-1 β , TNF α , TNFR1, and COX2 protein expressions were significantly up-regulated in vehicle-treated AD mice, but significantly suppressed by tannic acid treatment (Fig. 5A–D).

3.7. Effects of tannic acid on serum IgE, IFN γ , and IL-4 in DfE induced AD mice

Next, to identify whether tannic acid could affect DfE induced serum IgE, IFN γ , and IL-4, their levels were quantified by ELISA. As shown in Fig. 6A–C, the levels of IgE, IFN γ , and IL-4 were significantly suppressed by tannic acid treatment, as compared to that of vehicle-treated AD mice.

4. Discussion

AD is a multifactorial inflammatory skin disease caused by environmental and genetic components such as infectious and non-infectious agents. At recent times, AD incidence has increased

all over the world [19], and steroids are generally used to control its symptoms, but their long term use produces undesirable adverse effects in patients with AD [20]. In these cases, the anti-inflammatory activity of natural compounds, with very low risk of adverse effects, can offer benefits in the prevention and treatment of AD lesion [21].

Several natural polyphenol compounds have been introduced in the treatment of AD [22]. Among which tannic acid, is a well-known polyphenol compound present in most of the fruits, vegetables, and plants, is reported to have potent anti-inflammatory, antioxidant, antimicrobial, antimutagenic and anticarcinogenic activities. Previous studies reported that tannic acid has promising clinical results against skin cancer [23], myocardial infarction [24], renal failure [25], hepatotoxicity/genotoxicity [26], diabetes [27] and Alzheimer disease [28]. Furthermore, tannic acid inhibited inflammatory cytokines and proinflammatory factors such as iNOS and COX2 [25]. Tannic acid and quercetin treatment suppressed Th2 related cytokines and angiogenesis in AD model; and also treatment with tannic acid alone effectively attenuated vascular endothelial growth factor expression in HaCaT cells [29]. Taken together, all of these positive results suggest that tannic acid could regulate proinflammatory factors and cytokines. Since, the effect of tannic acid on HMGB1-RAGE, NF κ B and PPAR γ expression in AD has not been studied, we have carried out the present study to show that tannic acid suppresses skin inflammation in DfE induced AD in NC/Nga mice, possibly via increased PPAR γ expression as well as suppressed NF κ B and cytokines expressions.

AD-like skin lesions were induced in NC/Nga mice by the topical application of DfE cream, which results in increased hyperkeratosis, parakeratosis, epidermal thickness, serum IgE, mast cells and

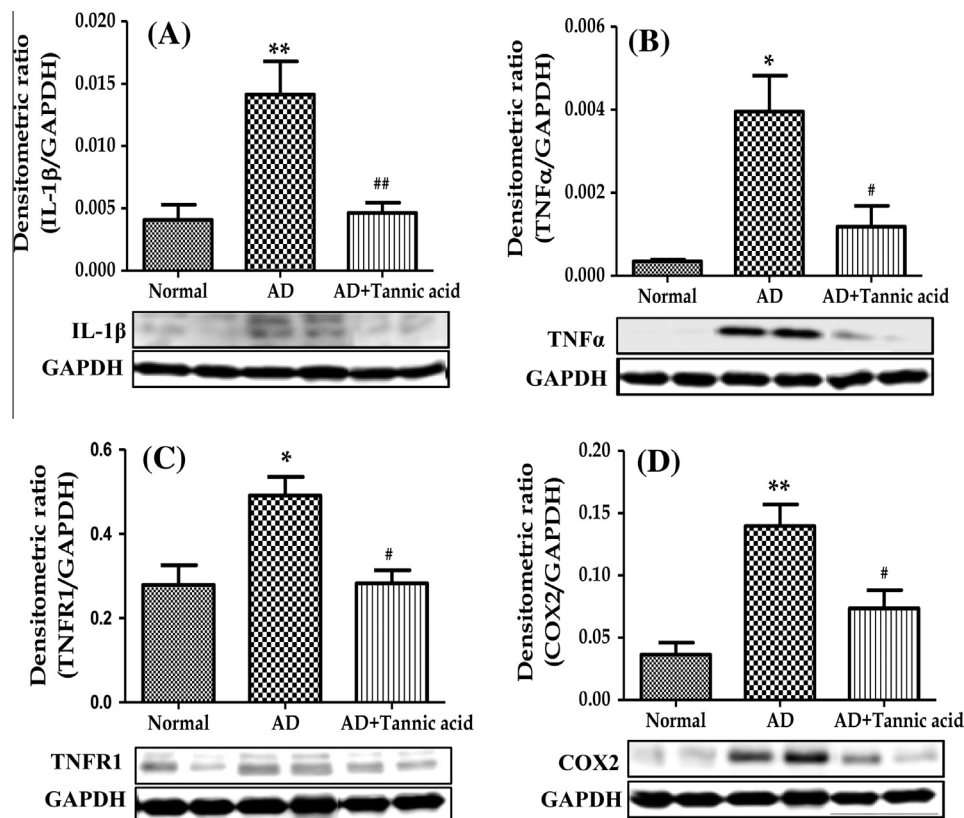


Fig. 5. Effects of tannic acid on IL-1 β , TNF α , TNFR1 and COX2 expression. Western blots show specific bands for the expression of (A) IL-1 β (ratio relative to that of GAPDH), (B) TNF α (ratio relative to that of GAPDH), (C) TNFR1 (ratio relative to that of GAPDH), and (D) COX2 (ratio relative to that of GAPDH). Each bar represents mean $\pm$ SEM. Normal, age-matched normal NC/Nga mice; AD, AD induced NC/Nga mice; AD + Tannic acid, AD induced mice administered with tannic acid. ** p < 0.01 and * p < 0.05 vs Normal; ## p < 0.01 and # p < 0.05 vs AD (Tukey multiple comparison test).

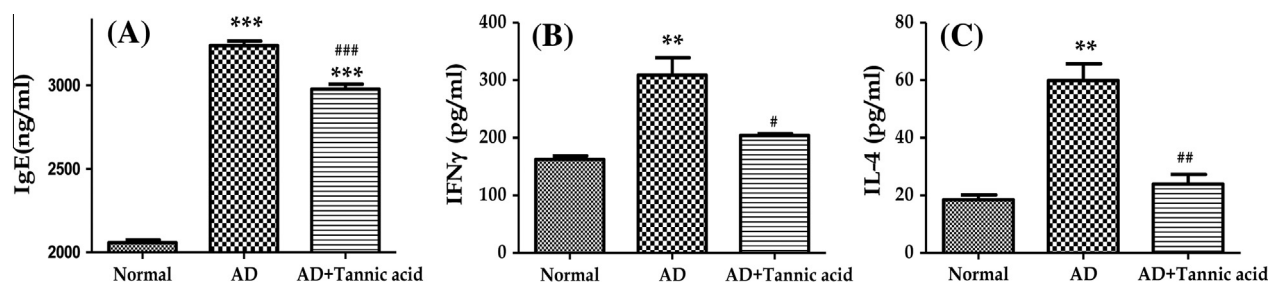


Fig. 6. Effects of tannic acid on IgE, IFN- γ , IL-4 levels. The serum concentration of (A) IgE, (B) IFN- γ , and (C) IL-4 in serum collected on day 28 was determined by ELISA. Each bar represents mean $\pm$ SEM. Normal, age-matched normal NC/Nga mice; AD, AD induced NC/Nga mice; AD + Tannic acid, AD induced mice administered with tannic acid. *** p < 0.001 and ** p < 0.01 vs Normal; ### p < 0.001, ## p < 0.01 and # p < 0.05 vs AD (Tukey multiple comparison test).

infiltrated neutrophil cells associated with inflammatory signaling, whereas oral administration of tannic acid ameliorated these changes.

PPAR γ is a member of the nuclear receptor superfamily that include receptors for thyroid, steroid and retinoid hormones. Several studies support the role of PPAR γ in immune response processes as its expression was altered by inflammatory cytokines [30] and activation of PPAR γ improves AD-like skin lesion in NC/Nga mouse model [18]. Moreover, PPAR γ activators suppressed epidermal hyperplasia in AD mice [7]. In addition, natural polyphenols such as curcumin, resveratrol and quercetin treatment activate PPAR γ , exerting anti-inflammatory effects in several animal models [6]. We have observed in the present study that, tannic acid significantly increased PPAR γ expression in AD skin lesion. We, therefore, proposed the working hypothesis of this study that tannic acid alleviates inflammation in AD model, possibly via PPAR γ expression.

Non histone nuclear protein HMGB1 is a proinflammatory cytokine associated with the development of several clinical conditions such as sepsis, lung injury, lethal systemic inflammation, arthritis, acute myocardial infarction, bronchial asthma and autoimmune diseases [31]. Very recently, we have reported that, the extracellular HMGB1 activates the inflammatory downstream danger signaling in NC/Nga mice with AD [14]. A similar scenario has been described in AD patient's serum and skin lesion where extracellular/translocated HMGB1 level was increased [14]. Moreover, the HMGB1 release was effectively inhibited by activating PPAR γ in lipopolysaccharide treated macrophage cells. Previous data suggested that PPAR γ agonist ameliorates postischemic injury and partially suppresses the inflammatory reaction via a PPAR γ dependent HMGB1 inhibiting mechanism [32]. Here, we observed that, tannic acid significantly attenuated HMGB1 expression in AD skin, which suggests the possible role of PPAR γ , behind the inhibition of HMGB1 expression in AD.

HMGB1 binding to its receptor RAGE activates multiple signaling cascades such as ERK1/2 and NF κ B, causing the activation of a variety of inflammatory cytokines and chemokines [14,33]. An interesting study reported that RAGE $^{-/-}$ mice were protected from skin tumor development [34]. It has also been demonstrated that RAGE deletion on immune cells results in significant reduction of the skin inflammatory response [35]. Proinflammatory cytokines and psoriasis were attenuated by treatment with RAGE inhibitor [36]. Moreover, PPAR γ agonist inhibits RAGE expression in endothelial cells [37]. Interestingly, in our study, tannic acid modulated the RAGE expression in the skin of AD mice. This finding confirms that HMGB1 might have interacted with RAGE to induce inflammation in AD mice.

Activated RAGE triggers ERK1/2 and downstream NF κ B signaling pathway. p65 NF κ B DNA binding is enhanced by ERK1/2 activation in endothelial dysfunction [38]. Moreover, recently, we have reported that the interaction of HMGB1-RAGE activates

ERK1/2 via a downstream NF κ B signaling pathway in DfE induced AD NC/Nga mouse model [14]. In the AD model, increased activity of the NF κ B system was reported, and its decreased activity was associated with amelioration of skin lesion and inhibition of cytokines, including leukotrienes and prostaglandins [39]. Another similar study reported that the NF κ B inhibitor treatment strongly suppressed IL-4, IL-1 β , IFN γ and TNF α in AD skin lesions [40]. Our data confirm that tannic acid dramatically attenuates ERK1/2 and NF κ B phosphorylation as well as inflammatory cytokines such as IL-1 β , IL-4, IFN γ , COX2 and TNF α in AD mice. For instance, the increased susceptibility of PPAR γ deficient mice to LPS induced endotoxemic injury and pulmonary inflammation was associated with increased activation of NF κ B and proinflammatory cytokines [41]. Another study reported that ultraviolet-B induced NF κ B activation could be attenuated by diverse PPAR γ agonists [42]. Based on these previous studies and from our findings, we can suggest that tannic acid prevents inflammatory response and this effect might be mediated by its ability to increase PPAR γ expression.

In conclusion, our results clearly suggest that DfE cream application to NC/Nga mice skin significantly down-regulates the protein expression of PPAR γ , which eventually causes AD like clinical symptoms, skin hypertrophy, keratosis, epidermal thickness, serum IgE and skin lesion via the upregulated HMGB1, RAGE, NF κ B and inflammatory cytokines. Natural polyphenol tannic acid effectively inhibits HMGB1, RAGE, NF κ B, COX2 and inflammatory cytokines such as IL-1 β , TNF α , IL-4 and IFN γ in AD mice, partly through upregulation of PPAR γ . These findings clearly suggest that tannic acid has beneficial effects against DfE induced AD in mice.

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