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Abdullah Alyoussef

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Attenuation of experimentally induced atopic dermatitis in mice by sulforaphane: Effect on inflammation and apoptosis

Abdullah Alyoussef

Department of Internal Medicine (Dermatology), Faculty of Medicine, University of Tabuk, Tabuk, 71471, Saudi Arabia

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Corresponding Author:

Abdullah Alyoussef, MD, DES

Associate Professor of Dermatology & Venerology (Hair AND Scalp Disorders)

Faculty of Medicine

University of Tabuk

Saudi Arabia

Phone: +966-50-4629800

aalyoussef@ut.edu.sa

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1. Abstract

Atopic dermatitis (AD) is characterized by progressive skin inflammation. In addition, sulforaphane is an isothiocyanate organosulfur compound from cruciferous vegetables. Sulforaphane was reported to ameliorate inflammatory responses. Therefore, this study was conducted to evaluate protective effects of sulforaphane in AD through affecting the balance between pro-inflammatory and anti-inflammatory cytokines and to evaluate its effect on AD-induced activation of apoptotic pathway. The method of repeated rubbing of 2,4-dinitrochlorobenzene (DNCB) on shaved dorsal skin and ears of mice was used for induction of AD. After development of AD, part of mice was injected with 1 mg/kg sulforaphane, subcutaneously three times weekly. Samples of skin was isolated for assessment of gene and protein expression of 8-hydroxy2'-deoxyguanosine, IgE, NFκB, TNF-α, IL-1β, IL-4, IL-10, Nrf2 and caspase-3. In addition, skin sections from different groups were stained with anti-caspase-3 antibodies. Mice in AD group were characterized by increased gene and protein expression of 8-hydroxy2'-deoxyguanosine, IgE, NFκB, TNF-α, IL-1β and caspase-3 associated with reduced expression of Nrf2, IL-4 and IL-10. Treatment of AD mice with sulforaphane significantly reduced the number of scratches, dermatitis score and ear thickness. In addition, sulforaphane significantly attenuated the gene and protein expressions produced by AD. Therefore, sulforaphane alleviated AD induced in mice through inhibition of oxidative stress, oxidative DNA damage, inflammation and apoptosis.

Keywords: Atopic dermatitis, caspase-3, 8-hydroxy2'-deoxyguanosine, immunoglobulin (Ig)E, interleukin (IL)-1β/4/10, nuclear factor E2-related factor 2 (Nrf2), tumor necrosis factor (TNF)- α

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2. Introduction

With increase progression in the last decades, atopic dermatitis (AD), or atopic eczema, is a widespread chronic relapsing inflammatory skin disease. AD has become a great health concern, especially in developed countries associated with impairment of the quality of life (Torres et al.

2019). Induction and progression of AD involves many genetic modifications (Alyoussef 2018). Therefore, AD is considered as a systemic disease associated with atopic and nonatopic comorbidities (Brunner et al. 2017; Ungar et al. 2017). AD might take place at any age, starting from the first two years of life (Sarkar and Kanwar 2002). Considered as the superior cause of non-fatal condition in the skin, AD is estimated to influence about 230 million worldwide according to WHO (Weidinger et al. 2018). AD covered many body organs as face, wrists, hands, ankles and feet with symptoms including edema, erythema, lichenification, excoriations, oozing and crusting (Thestrup-Pedersen 2000). It might bring out comorbidities as psychological distress, sleep loss and increased healthcare utilization or accompanied with signs and symptoms of bronchial asthma, nasal inflammation and conjunctivitis (Nedoszytko et al. 2014).

A major character of AD is skin barrier dysfunction followed by penetration of allergens into the skin (Yamazaki et al. 2017). It results from factors that spoil skin barrier function or environmental exposures as soaps, detergents and repetitive scratching (Wollenberg et al. 2018). Therapeutic objectives focused mainly of attenuating pruritus as well as controlling the disease spread. Therapeutic strategies of AD depends on application of local or systemic corticosteroids with unfavorable side effects (Boguniewicz et al. 2017). The limitation of therapeutic options of aAD leads to psychosocial comorbidities of AD including suicide, attention-deficit/hyperactivity disorder (ADHD) and schizophrenia. Patients may suffer from several problems as sleep disturbance, stigmatization from peers and difficulties in school (Pandher et al. 2021). Therefore, there is great demand to find new therapeutic agents.

Sulforaphane is 1-isothiocyanato-4-(methylsulfinyl) butane that is obtained from vegetables of the Cruciferae family such as broccoli, broccoli sprouts, cauliflower and cabbage. Sulforaphane is reported to inhibit UV-induced inflammation (Dinkova-Kostova et al. 2006; Shibata et al. 2010). Two previous studies illustrated the therapeutic effects of sulforaphane in experimentally induced AD in mice through inhibition of Nrf2/HO-1 axis (Wu W et al. 2019) or through inhibition of TARC/CCL17 and MDC/CCL22 expression (Jeong et al. 2010). In addition, sulforaphane has

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minimal side effects in patients or mice as well as it is highly soluble and bioavailable (Li Y et al. 2013). Therefore, we conducted this study to examine the therapeutic effect of sulforaphane on experimentally induced AD in mice focusing on DNA oxidative damage and the balance between pro-inflammatory and anti-inflammatory cytokines. In addition, we aimed to examine the effect of sulforaphane on AD-induced activation of the apoptotic pathway.

3. Materials and Methods

3.1.Animals

The local ethical committee approved the animal protocol. Four-week-old BALB/c mice were housed under pathogen-free conditions with normal 12-h light-dark cycle. Mice were distributed into four groups with ten mice each:

3.1.1. ***Control group.*** Dorsal hair was shaved. Exposed dorsal skin and ears were rubbed with a mixture of acetone : olive oil (3:1).

3.1.2. ***Sulforaphane treated control group.*** Mice were given 1 mg/kg sulforaphane (Sigma Aldrich Chemicals Co., St Louise, MO, USA) subcutaneously three times per week for three weeks.

3.1.3. ***DNCB-induced AD group.*** After 24 hours of shaving of dorsal hair, 100 µl of 1% 2,4-dinitrochlorobenzene (DNCB) solution in acetone : olive oil (3:1) was rubbed on back and ears for sensitization. After five days, 150 µl of 0.2% DNCB was applied on dorsal skin and ears three times a week for three weeks (Alyoussef 2015a, 2015b).

3.1.4. ***Sulforaphane treated group.*** After induction of AD, mice were subcutaneously injected with 1 mg/kg of sulforaphane three times weekly for another three weeks.

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3.2.Evaluation of symptoms of atopic dermatitis

For evaluation of atopic dermatitis, ear thickness, scratches and dermatitis score were measured. The thickness of ears was measured in a weekly basis using a micrometer. On the last two days before sacrifice, the number of scratches of each mouse were counted for 10 minutes and repeated five times. Scratches were defined as the movement using the hind paws. Finally, mice were given; scores 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for the following signs of atopic dermatitis edema, erythema/hemorrhage, scaling/dryness, and excoriation/erosion. Sum of all scores was used as dermatitis score.

3.3.Animal sacrifice and collection of samples

Animals were sacrificed by decapitation. Mice trunk blood was collected and centrifuged at 3000 rpm for five minutes. Skin samples were removed and stored at –80°C.

3.4.Immunohistochemistry

Five micrometer thickness sections were cut from the paraffin block of skin. The sections were incubated over night with anti-caspase-3 antibody (Abcam, Cambridge, MA, USA) at 4 °C. The slides were counterstained with hematoxylin. Slides were examined using Nikon Digital Camera (Japan).

3.5.ELISA determination

Commercially available ELISA kits were used for determination of skin levels of 8-hydroxy2'-deoxyguanosine, TNF-α, IL-1β, IL-4 and IL-10 (Abcam, Cambridge, MA, US) following the manufacture protocol.

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3.6.Estimation of IgE concentration

IgE was determined on Vidas instruments (bioMérieux, Inc., Durham, NC, USA) using the manufacturer protocol.

3.7.Assessment of oxidative stress

Skin levels of malondialdehyde (MDA), hydrogen peroxide and catalase were determined using commercially available kits provided by Biodiagnostic Company, Giza, Egypt.

3.8.Estimation of the activity of caspase-3

The enzyme activity of caspase-3 was determined colorimetrically using commercially available kits (Abcam, Cambridge, MA, US) following the manufacturer's procedure.

3.9.Quantitative real-time polymerase chain reaction (RT-PCR)

Gene expression of NFκB, TNF-α, IL-1β, IL-4, IL-10, Nrf2, caspase-3 and GAPDH was determined as described previously by our group (Alyoussef 2020). The specific PCR primers were summarized in Table 1.

3.10. Statistical analysis

The mean ± standard error was used. The normality of samples was checked by Kolmogorov–Smirnov test. Comparison between groups was evaluated by one way ANOVA followed by post hoc Bonferroni correction tests. SPSS version 20 was used for statistical computations. Statistical significance was predefined as $P \leq 0.05$.

4. Results

4.1.Sulforaphane attenuated AD symptoms in mice

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AD mice showed severe erythema, erosion and dryness in dorsal skins. In AD mice, we found significant increase in the number of scratches (13.89±1.17 scratches/10minutes), dermatitis score (5.1±0.42) and ear thickness (0.24±0.02 mm) compared with control mice (0.91±0.1 scratches/10 minutes, 014±0.01 and 0.036±0.005 mm, respectively). However, Sulforaphane ameliorated number of scratches (4.49±0.41 scratches/10minutes), dermatitis score (3.2±0.29) and ear thickness (0.16±0.01 mm) without affecting control group (Figure 1).

4.2.Sulforaphane reversed AD-induced increase in oxidative stress

AD showed significant elevation in the skin levels of MDA and hydrogen peroxide associated with significant reduction in the levels of catalase as well as the gene expression of Nrf2. Treatment of AD mice with sulforaphane reversed all these effects in AD mice without affecting the control mice (Figure 2).

4.3.Sulforaphane attenuated AD-induced increase in DNA oxidative damage

AD resulted in a significant increase in the skin levels of 8-hydroxy2'-deoxyguanosine as compared with the control. Treatment of AD mice with sulforaphane reduced the increase in 8-hydroxy2'-deoxyguanosine in AD mice without affecting the control mice. However, the levels of 8-hydroxy2'-deoxyguanosine in treated group still significantly higher than the control group (Figure 3).

4.4.Sulforaphane inhibited AD-induced elevation in IgE and eosinophils count

As shown in figure 4, AD significantly increased the eosinophils count and serum level of IgE as compared with the control mice. Treatment of mice with sulforaphane significantly reduced elevated eosinophils and IgE in AD group without affecting the control mice (13.98±1.14 U/ml). However, the levels of eosinophils and IgE still higher than the control group.

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4.5.Sulforaphane blocked AD-induced elevation in the inflammatory pathway

Analysis of inflammatory pathway in mice skin revealed significantly increased gene expression of NFκB, TNF-α and IL-1β and increased protein expression of TNF-α and IL-1β in skin of AD mice compared with control mice. Sulforaphane blocked all these effects (Figures 5).

In parallel, AD mice showed significant reduction in both gene and protein expression of the anti-inflammatory cytokines, IL-4 and IL-10 as compared with the control mice. Treatment of AD mice with sulforaphane significantly elevated the gene and protein expression of both IL-4 and IL-10 to be near the levels of the control group (Figure 6).

4.6.Sulforaphane inhibited AD-induced elevation in apoptotic pathway

Next, we investigated the effect of both AD and sulforaphane on skin levels of caspase-3. AD caused significant increase in the gene expression and activity of caspase-3 when compared with the control group (Figure 7). In addition, sections from skin of AD group stained with anti-caspase-3 antibodies revealed highly increased staining areas for caspase-3 (Figure 8). Treatment with sulforaphane showed significant reduction in the activity and gene expression of caspase-3 as well as the areas stained with anti-caspase-3 in skin sections stained with anti-caspase-3, but it did not affect the control group.

1. Discussion

AD is a recurrent inflammatory disease of skin that is characterized by defective skin barrier leading to increased allergen and pathogen penetration (Boguniewicz and Leung 2011). Repeated application of DNCB on mice skin leads to AD like symptoms that was confirmed by significant elevation in ear thickness, dermatitis score and number of scratches when compared with the control group. The major problem in AD is inflammatory skin disorder. In parallel, the therapeutic agents used for treatment such as steroids and antihistaminic drugs can result in severe side effects,

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limiting their clinical application. Therefore, there is a great need for scientific research to discover new therapeutic agents that could reduce the inflammatory response. In this study, we used sulforaphane, which was previously manifested to exhibit anti-inflammatory effects in many organs (Jun et al. 2020; Li et al. 2020; Ranaweera et al. 2020). However, treatment of AD mice with sulforaphane significantly attenuated DNCB-induced increase in ear thickness, dermatitis score and number of scratches. Two previous studies illustrated the ability of sulforaphane to alleviate AD experimentally induced in mice through inhibition of Nrf2/HO-1 axis (Wu W et al. 2019) or through inhibition of TARC/CCL17 and MDC/CCL22 expression (Jeong et al. 2010). However, the present study was designed to evaluate the effect of sulforaphane on oxidative stress, the balance between pro-inflammatory and anti-inflammatory cytokines and apoptotic pathway.

A major cause for cell membrane destruction is lipid peroxidation that resulted from the deteriorate effect of free radicals especially hydrogen peroxide and superoxide anion (Sivaranjani et al. 2013). Under physiological conditions, these lipid hydroperoxides are decomposed to produce highly cytotoxic aldehydes, especially MDA. However, we found significant increase in hydrogen peroxide and MDA associated with significant reduction in catalase activity in AD mice. Of note treatment with sulforaphane significantly reduced AD-induced oxidative stress that was associated with inhibition of the severity of AD symptoms. We found that sulforaphane significantly increased the gene expression of Nrf2. In line with our study, it was reported previously that the antioxidant effects of sulforaphane are attributed to its ability to activate Nrf2 protective pathway (Ren et al. 2021). The subsequent effect on DNA oxidative damage was investigated by measuring the levels of 8-hydroxy2'-deoxyguanosine in skin of mice. We found that sulforaphane significantly reduced AD-induced expression of 8-hydroxy2'-deoxyguanosine without affecting the control mice leading to DNA protection. No previous study investigated 8-hydroxy2'-deoxyguanosine in AD induced in mice.

The inflammatory effects of AD are produced by two mechanisms; the first one includes the primary defect in immunologic response with subsequent production of IgE leading to epithelial

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barrier dysfunction (Sivaranjani et al. 2013). The second mechanism involves the intrinsic defect in epithelial cells leading to barrier dysfunction (Bieber 2008). In the first mechanism which involves immunologic aspects, IgE expression produces both acute and chronic phase skin inflammations. Moreover, clinical observations suggests a relation between serum IgE and the amplitude of disease (Peroni et al. 2012). In addition, patients with AD display increased serum levels of total IgE (Ott et al. 2009). Other essential mediators of AD are TNF-α (Park et al. 2013) and IL-1β (Alshevskaya et al. 2016). Therefore, the overexpression of IgE, NFκB, TNF-α and IL-1β are hallmarks of AD (Arshad and Holgate 2001). In parallel, AD is characterized by reduction in the concentration of the anti-inflammatory cytokines such as IL-4 (Bao et al. 2021) and IL-10 (Kim et al. 2019). However, the alleviation of AD symptoms in mice with sulforaphane was associated with suppression of IgE, NFκB, TNF-α and IL-1β and increased expression of both IL-4 and IL-10. There is no previous study illustrating the ability of sulforaphane to restore the balance between anti-inflammatory and pro-inflammatory cytokines in AD, but it has been reported in other models such as sciatic endometriosis (Liu et al. 2020), LPS-activated microglia (Subedi et al. 2019), neuronal necroptosis (Qin et al. 2018) and placental cytokine production (Arita et al. 2019).

Atopic dermatitis can result in skin tissues apoptosis, which takes place via extrinsic and intrinsic pathways. In the extrinsic pathway, death occurs by ligands as pro-inflammatory cytokines, NFκB, TNF-α and IL-1β effect on cell surface via their receptors, while activation of the intrinsic pathway takes place via oxidative damage to DNA. Apoptosis was reported previously to play a major role in DNCB-induced skin damage (Alyoussef 2015a; Kim et al. 2015). Both intrinsic and extrinsic apoptotic pathways results in activation of caspase-3, which is believed to be the central executioner caspase (Caglayan Sozmen et al. 2016). We discovered elevation of caspase-3 activity and gene expression as well as increased staining of skin cells with anti-caspase-3 antibodies in AD mice. Sulforaphane significantly reduced the expression of caspase-3. There was no previous study illustrated the ability of sulforaphane to reduce caspase-3 in AD. Sulforaphane was reported previously to reduce caspase-3 activation in chromium-induced cardiotoxicity (Yang et al. 2020),

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high glucose-induced neuronal apoptosis (Tang et al. 2020) and diffuses axonal injury (Wu DM et al. 2020).

2. Conclusion

We illustrated that sulforaphane could ameliorate DNCB-induced AD in mice. Sulforaphane protection proceed via inhibition of oxidative stress, oxidative DNA damage, inflammation and apoptosis.

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Tables

Table 1: The primer sets used:

Primer	Accession number	Sequence (sense, antisense)
TNF-α	X02611	5'- TACTGAACTTCGGGGTGATTGGTCC -3' 5'- CAGCCTTGTCCCTTGAAGAGAACC -3'
IL-1β	NM_008361	5'- AGTTGACGGACCCCAAAAGAT -3' 5'- GGACAGCCCAGGTCAAAGG -3'
NFκB	NM_008689	5`-GAAATTCCTGATCCAGACAAAAAC-3` 5`-ATCACTTCAATGGCCTCTGTGTAG-3`
IL-4	NM_021283	5`-ATCATCGGCATTTTGAACGAGGTC-3` 5`-ACCTTGGAAGCCCTACAGACGA-3`
IL-10	NM_010548	5`-CGGGAAGACAATAACTGCACCC-3` 5`-CGGTTAGCAGTATGTTGTCCAGC-3`
Caspase-3	NM_009810.2	5`- TGA CTG GAA AGC CGA AAC TC -3` 5`- AGC CTC CAC CGG TAT CTT CTV -3`
GAPDH	M32599	5'- ACCACAGTCCATGCCATCAC -3' 5'- CACCACCCTGTTGCTGTAGCC -3'
Nrf2	BC026943	5`-CGAGATATACGCAGGAGAGGTAAGA-3` 5`-GCTCGACAATGTTCTCCAGCTT-3`

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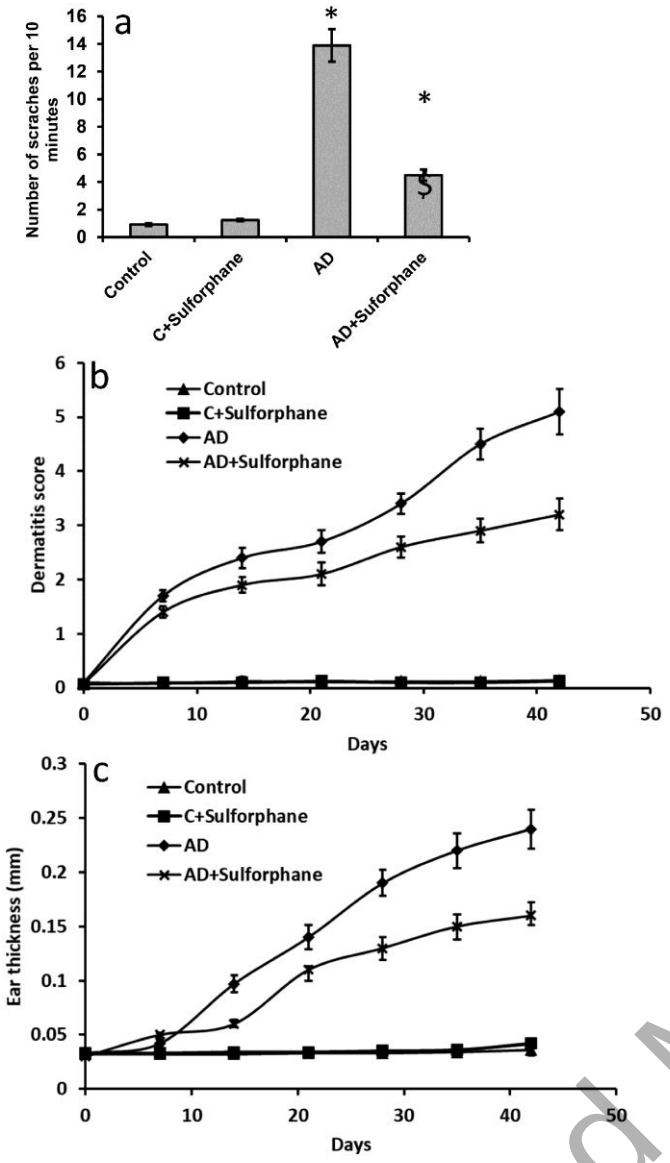

Figure 1: Effect of atopic dermatitis (AD) alone and in combination with 1 mg/kg sulforaphane on number of scratches per 10 minutes (a), dermatitis score (b) and ear thickness (c). *: significant difference compared with the control groups at $p<0.05$. $: significant difference compared with atopic dermatitis group at $p<0.05$.

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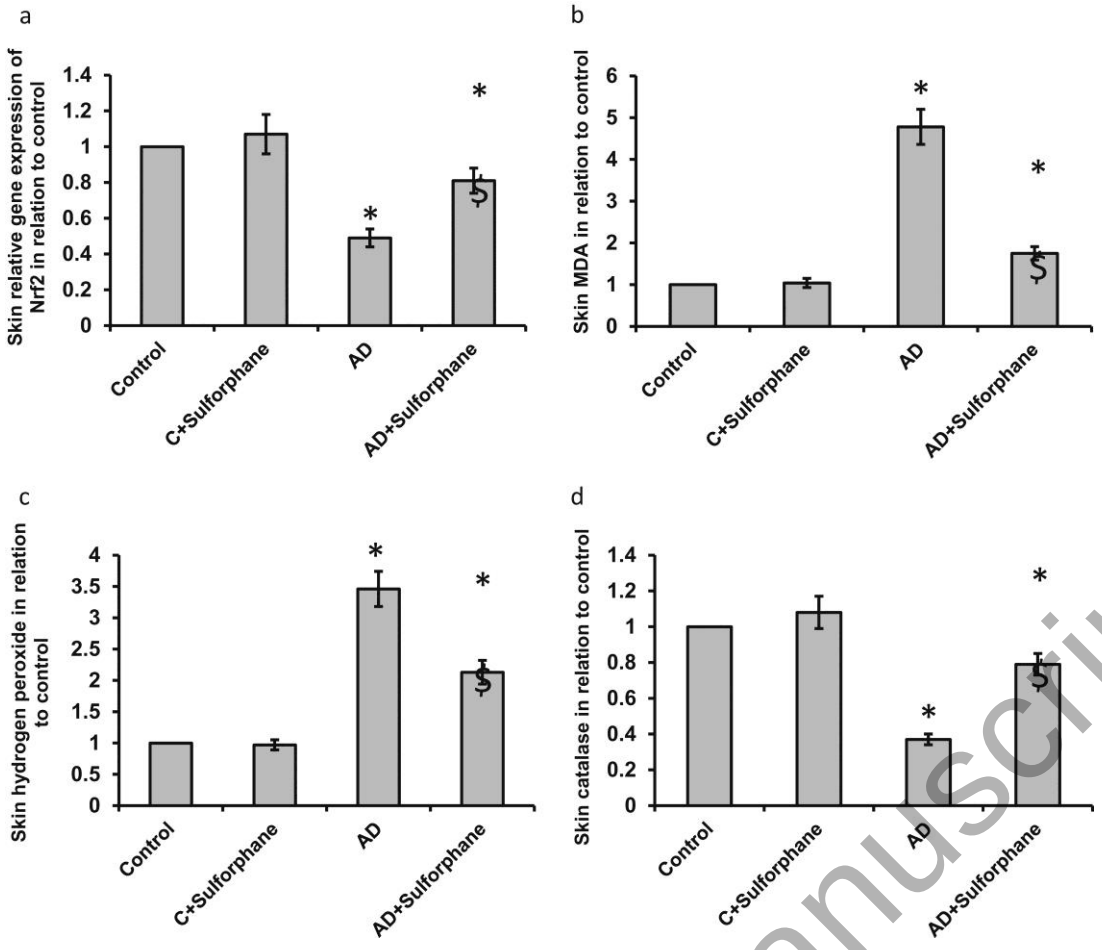

Figure 2: Effect of atopic dermatitis (AD) alone and in combination with 1 mg/kg sulforaphane on skin levels of Nrf2 (a), malondialdehyde (MDA, b), hydrogen peroxide (c) and catalase (d).. *: significant difference compared with the control groups at $p<0.05$. $: significant difference compared with atopic dermatitis group at $p<0.05$.

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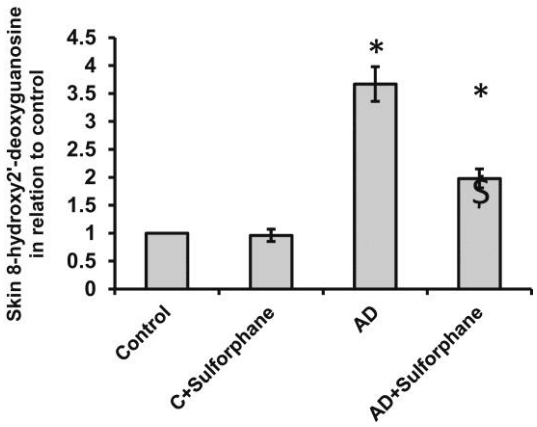

Figure 3: Effect of atopic dermatitis (AD) alone and in combination with 1 mg/kg sulforaphane on skin levels of 8-hydroxy2'-deoxyguanosine. *: significant difference compared with the control groups at $p<0.05$. $: significant difference compared with atopic dermatitis group at $p<0.05$.

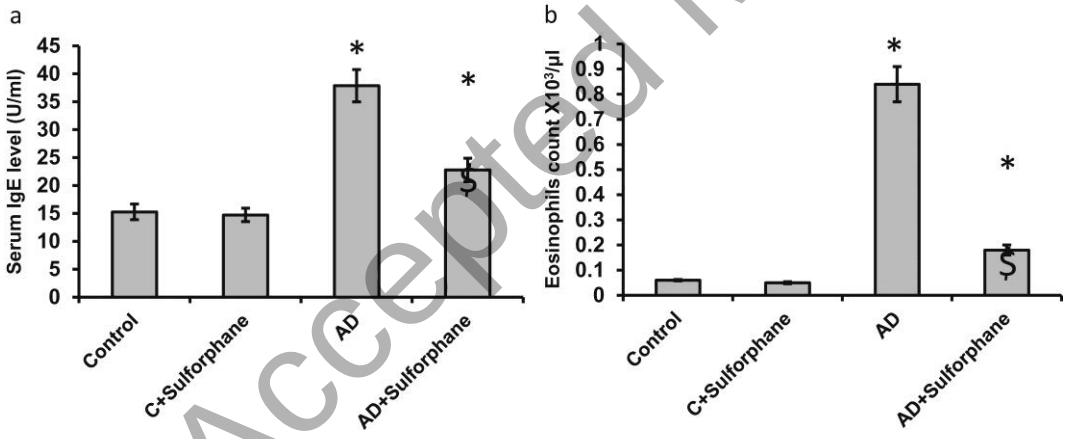

Figure 4: Effect of atopic dermatitis (AD) alone and in combination with 1 mg/kg sulforaphane on serum level of IgE (a) and blood count of eosinophils (b). *: significant difference compared with the control groups at $p<0.05$. $: significant difference compared with atopic dermatitis group at $p<0.05$.

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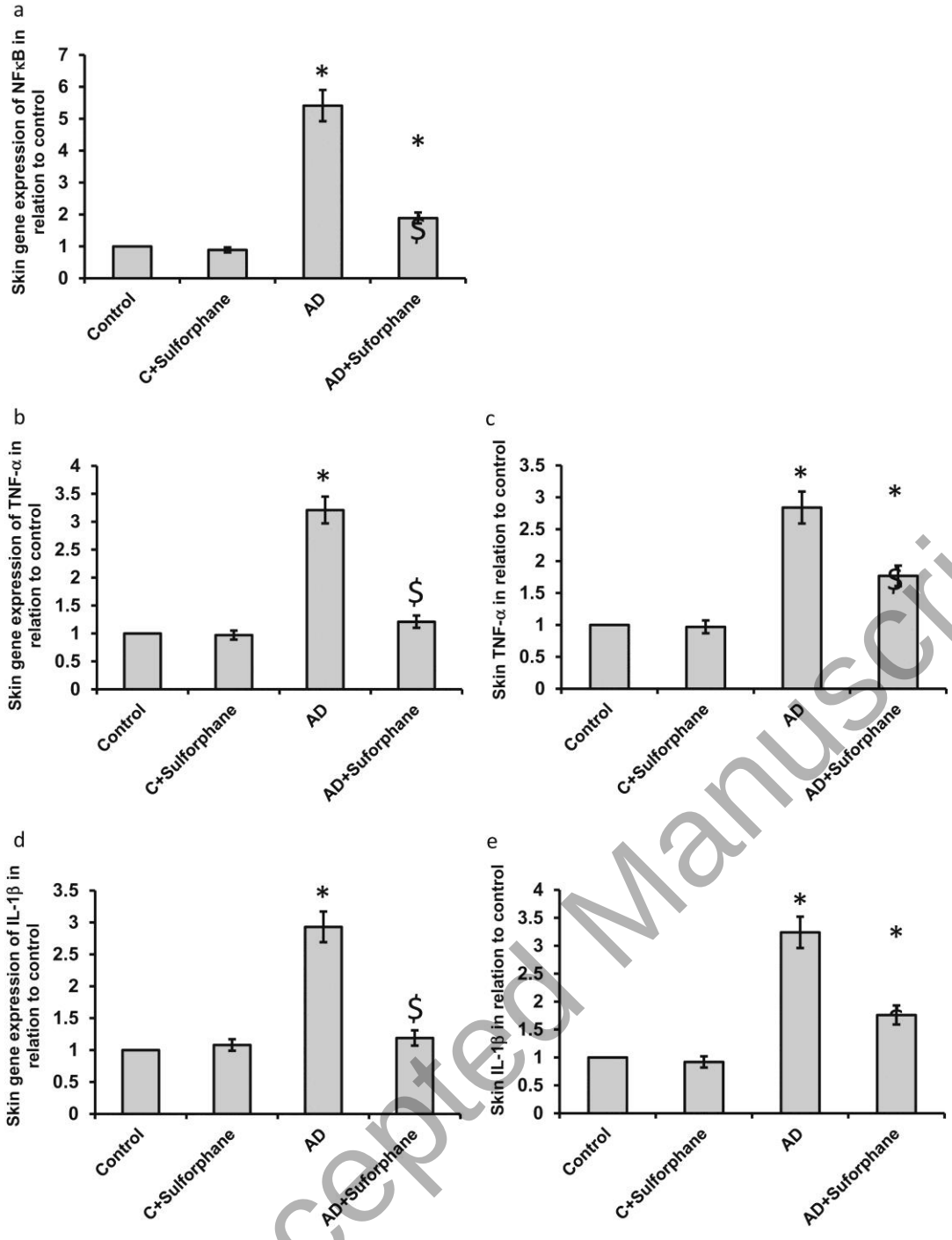

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Figure 5: Effect of atopic dermatitis (AD) alone and in combination with 1 mg/kg sulforaphane on gene expression of nuclear factor (NF)κB. a), tumor necrosis factor (TNF)-α (b) and interleukin (IL)-1β (d) as well as levels of TNF-α (c) and IL-1β (e) in skin of mice. *: significant difference compared with the control groups at $p<0.05$. $: significant difference compared with atopic dermatitis group at $p<0.05$.

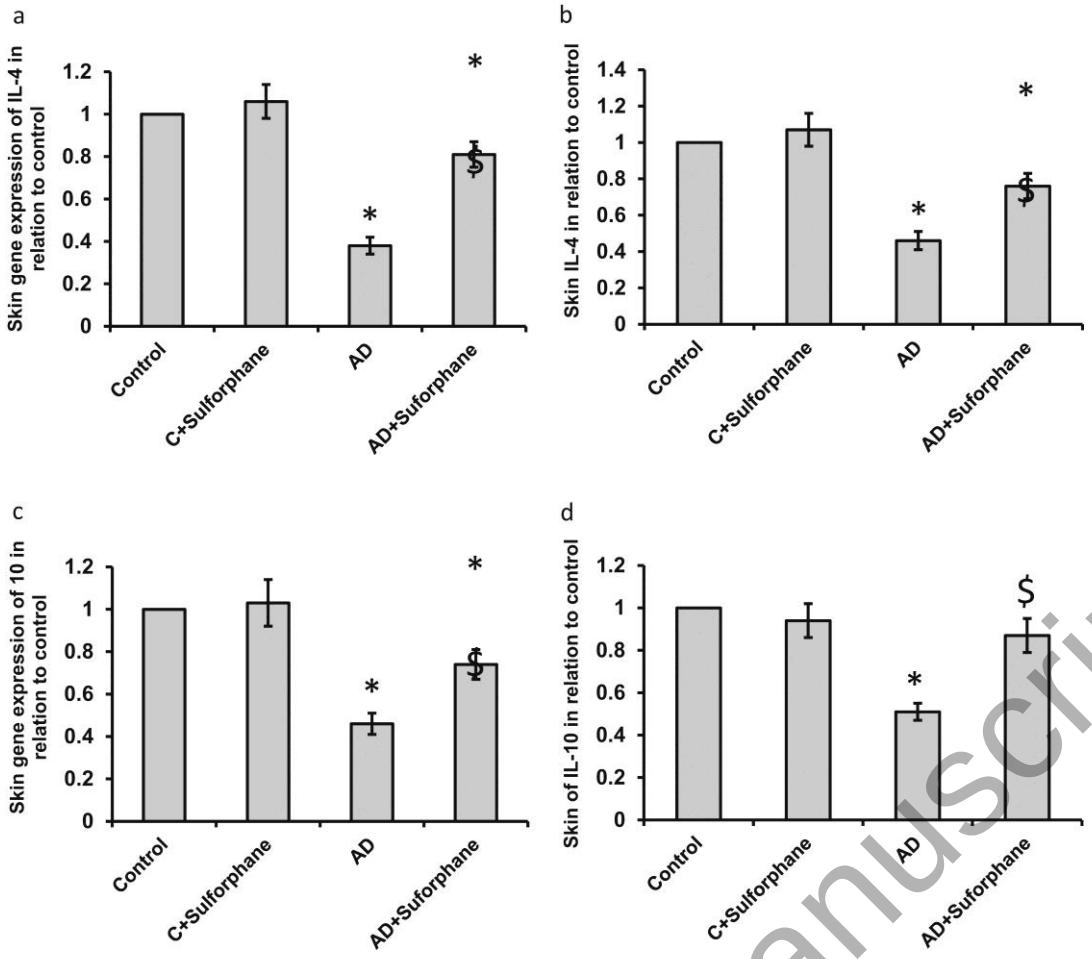

Figure 6: Effect of atopic dermatitis (AD) alone and in combination with 1 mg/kg sulforaphane on gene expression of interleukin (IL)-4 (a) and IL-10 (c) as well as levels of IL-4 (b) and IL-10 (d) in skin of mice. *: significant difference compared with the control groups at $p<0.05$. $: significant difference compared with atopic dermatitis group at $p<0.05$.

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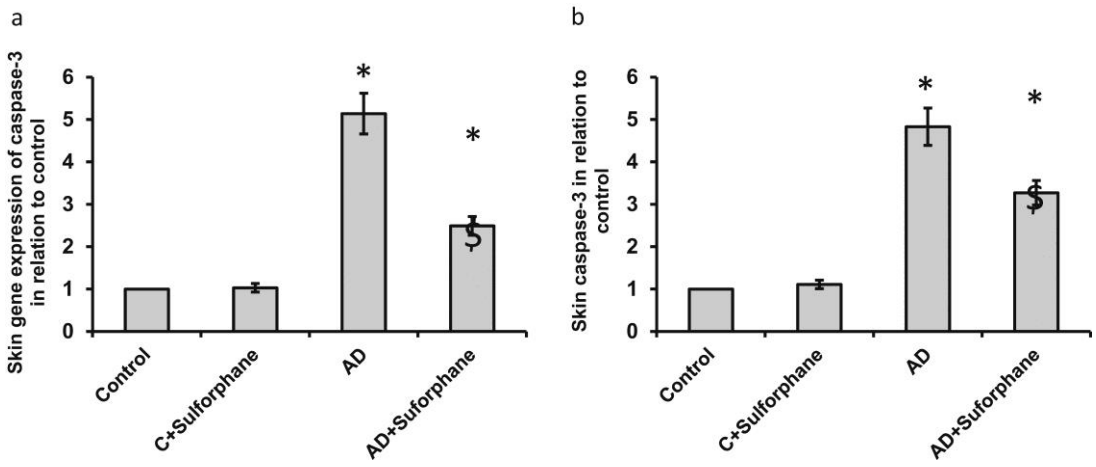

Figure 7: Effect of atopic dermatitis (AD) alone and in combination with 1 mg/kg sulforaphane on gene expression of caspase-3 (a) and activity of caspase-3 (b) in skin of mice. *: significant difference compared with the control groups at $p<0.05$. $: significant difference compared with atopic dermatitis group at $p<0.05$.

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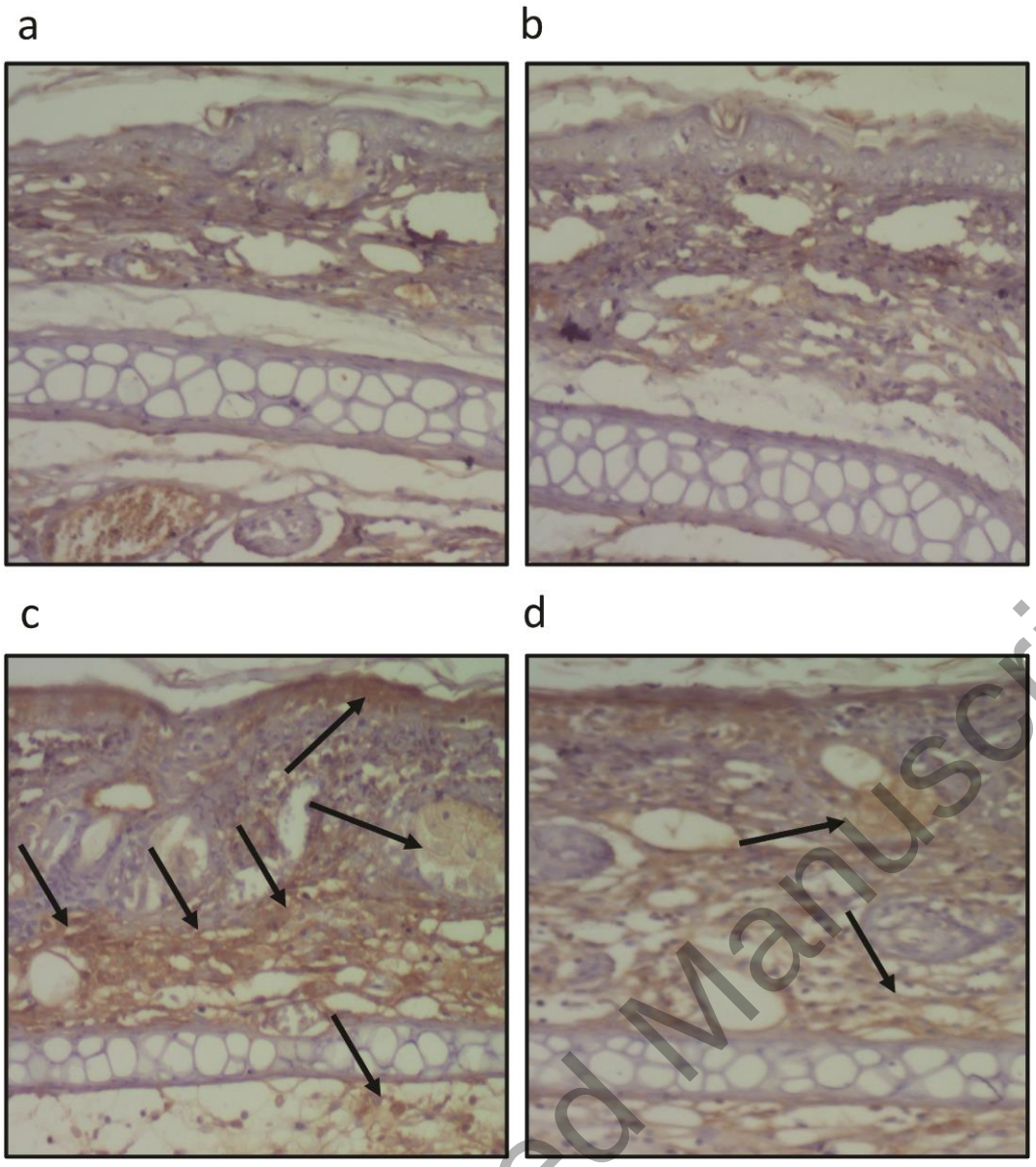

Figure 8: Effect of atopic dermatitis (AD) alone and in combination with 1 mg/kg sulforaphane on mice skin sections stained with anti-caspase-3 antibody for control (a), treated control (b), atopic dermatitis (c) and treated atopic dermatitis (d) mice.

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