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Abstract

Atopic dermatitis (AD) is an inflammatory skin disease characterized as a Th2-predominant immune response. Patients are susceptible to infection. Ozone is used for wound treatment due to its anti-infection features. However, it is unclear whether ozone can recovery AD lesions. In this study, mice were repeatedly challenged with the triplex allergens of staphylococcal enterotoxin B, ovalbumin and calcipotriol ointment on the back to develop AD lesions and were treated with ozonated oil. We found that ozonated oil significantly inhibited inflammation and healed the lesions in 7 days. Ozonated oil inhibited NGF expression as compared to the groups treated with vehicle or PBS (P<0.01).The serum proteins and lesional transcripts of Th2 cytokines including IL-4 and IL-31 were lower in the ozonated oil treated group than the groups treated with vehicle or PBS (P<0.05). The IL-10 level was increased with treatment of ozonated oil (P<0.01). On the other hand, the expressions of Th1 cytokines including IL-2, TNF-α, and IFN-γ in the serum were not regulated by ozonated oil. Our results showed that ozonated oil could suppress inflammation in an AD murine *via* decreasing Th2-dominant cytokines response and increasing IL-10 expression. These suggest that ozonated oil may be a potential remedy for AD.

KEYWORDS

Atopic dermatitis; cytokines; immune regulation; ozone

Main text

Introduction

Atopic dermatitis (AD) is a skin disease characterized as common chronic and recurrent inflammation. It shows eczematous skin lesions and intensive pruritus and affects 10-30% of children and 2-10% of adults depending on the population(1). Recent data have shown that its prevalence is increasing, especially in low-income countries(2). Genetic, immunological and environmental factors are the main contributors that drive cutaneous inflammations in AD(3). Patients with AD have an increased susceptibility to infection or colonization with bacteria, fungi, and viruses, most notably Staphylococcus aureus, resulting in development and exacerbation of eczematous symptoms(3,4). Traditionally, sustained exposure of pathogens tailors immune responses and drives the development of specialized T helper (Th)2-bias cytokine environment(5). Topical corticosteroids (TCS) and calcineurin inhibitor are the first-line therapy for AD. However, they can be associated with significant adverse effects for chronic applications(6,7). Systemic immuno-modulatory therapies for AD are being developed(8).

Ozone, e.g. O3, has been widely recognized as one of the common bactericidal, antiviral and antifungal agents. It has been empirically used for treatment of chronic wounds, such as trophic ulcers, ischemic ulcers and diabetic wounds (9, 10). The possible mechanisms underlie the decrease in bacterial infection, amelioration of impaired dermal wound healing or increase in oxygen tension in the wound area(10).We previously tested ozonated water treatment for two cases of skin

methicillin resistant S. aureus (MRSA) infection. We have found that ozonated water can effectively kill MRSA and both MRSA cases have been completely healed with ozone therapy (11). In this study, we attempted to investigate whether ozone administration can be a potential therapy for AD through its immune regulation and anti-infection effect.

Materials and methods

Animals and materials

The research was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of the Third Xiangya Hospital, Central South University. Ozonated oil with a peroxide value of 2000 to 2200 mmol-equiv/Kg and its vehicle, camellia oil, were provided by Hunan Health Care Technology Co. LTD(Hunan, China). Ovalbumin (OVA) and staphylococcal enterotoxin B(SEB) were from Sigma (St Louis, MO, U.S.A) and dissolved in sterilized phosphate-buffered saline (PBS). Calcipotriol ointment(CO) and depilatory cream were purchased from Leo Pharmaceutical Co.

Sensitization and challenge procedures

Eighteen female 6-8-weeks BALB/c mice were used in this study. The animals were anesthetized by intraperitoneal injection of chloral hydrate. The hair on the back of mice was removed using depilatory cream. A small patch slot, containing 100μl of PBS, or mixture of 1.25μg of SEB, 50μg of OVA and 10mg of CO, was secured to the shaved skin area with a transparent bio-occlusive dressing, respectively. The drugs were applied repeatedly at every other day for 15 days (Figure 1).

Administration of ozonated oil

As shown in Fig 1, on the day of sixteenth, the challenged mice were treated by ozonated oil daily for one week. Ozonated oil was 0.1ml/cm^2. In the treatment control groups, the mice were treated by PBS or vehicle oil without ozonation.

Histological analysis

On the day of 23rd, the skin specimens were scanned by reflectance confocal microscopy (RCM) for the measurement of thickness of epidermis under anesthesia. The skin tissues were removed, fixed in 10% buffered neutral formaldehyde and embedded in paraffin. The sections were cut for 4 μm thickness followed by hematoxylin and eosin (H&E) staining.

Detection of mRNA levels of cytokines in skin

Real-time PCR was used to determine the mRNA levels of cytokines, including IL-4, IL-31, IL-2, TNF-α, IFN-γ, IL-10 and NGF, in the skin. Total RNAs of each lesion

were extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Complementary DNA was then synthesized from one microgram total RNA using random hexamer and MMLV reverse transcriptase (Thermo Scientific). The cDNA amplification was carried out using SYBR Green Master Mix kit according to the manufacturer's protocol. Target genes were amplified by using specific oligonucleotide primer and β-actin gene was used as an endogenous control (Table 1). Data were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$). Three separate experiments were performed for each lesion.

Measurement of serum cytokines

Serum cytokines levels were analyzed using the commercial ELISA kit from Bio-Plex Pro™ (Bio-Rad, U.S.A.)

Statistical analysis

Values are expressed as mean±SEM. Statistical analyses were performed using ONE-WAY ANOVA and LSD test. A 95% confidence limit was taken as significance ($P<0.05$).

Results

Ozonated oil suppressed allergic skin inflammation

The BALB/c mouse model of AD were established according to the schematic (Fig 1) and the references(12-14). The skin appeared as erythema, subcutaneous nodules, infiltrated erythema, and ulceration at sites of epicutaneous sensitization with the triplex allergens of OVA, SEB and CO. Swelling and erythema receded in one week in the ozonated oil site as compare to the sites treated by PBS or vehicle (Fig 2a). The dermal layer contained decreased numbers of nucleated cells in the skin sites treated by ozonated oil as compared to the sites treated with PBS or vehicle (Fig 2b). The thickness of epidermal layer was (20±0.89)μm in the ozonated oil treated sites, which was significantly lower than vehicle treated [(36.75±1.58)μm] or PBS-treated [(39.3±1.37)μm] sites(Fig 2c, p<005).Since NGF (nerve growth factor) is a marker for AD severity (15-17), we analyzed NGF expression profile. Ozonated oil treatment significantly decreased the expression levels of NGF in the PBS or vehicle treated sites (Fig 2d, p<0.01), A similar pattern of NGF protein levels in serum from all three groups was identified by ELISA(Fig 2e). These findings showed that ozonated oil inhibited allergic skin inflammation and improved the healing of lesions in 7 days.

Ozonated oil inhibited expressions of Th2 cytokines in the allergic skin and serum

The mRNA levels of Th2 cytokines, IL-4 and IL-31, were highly expressed in PBS or vehicle treated sensitized skins (Fig 3a, 3b). The mRNAs of IL-4 and IL-31were significantly decreased after ozonated oil treatment as compared to PBS and vehicle

groups (Fig 3a, 3b) (P<0. 05). Similarly, ozonated oil intervention resulted in a significant decrease in serum Th2 cytokines(Fig 3d, 3e)(P<0.05). There was no significant difference between the PBS and vehicle treated groups.

Ozonated oil inhibited Th1 cytokines transcripts in the lesional sites but not protein levels in the serum

Similar protein levels of serum Th1-type cytokines including TNF-α (Fig 3f) and IFN-γ (Fig 3j) were shown among all three groups. However, there were significantly lower mRNA levels of TNF-α, IFN-γ and IL-2in ozonated oil treated lesional atopic skin as compared with PBS or vehicle treated atopic skin (Fig 3c, 3g, 3h, p<0.05). There was no significant difference in mRNA levels between the control groups. There was no significant difference of serum IL-2 levels between the ozonated and vehicle treated groups, though both were significantly higher than that in the PBS treatment group (Fig 3k, p<0.01), suggesting vehicle but not ozonated oil accounted for the decrease in serum IL-2. Therefore, Th1-type cytokines TNF-α, IFN-γ and IL-2 were not regulated by ozonated oil.

Ozonated oil increased expression of immunosuppressive cytokine IL-10

We next investigate whether ozonated oil treatment was associated with expression of the immunosuppressive cytokine IL-10. Level of IL-10 mRNA was significantly higher in ozonated oil treated group compared with PBS and vehicle treated atopic skins (Fig 3i, p<0.01). Serum IL-10 level was significantly increased in the ozonated oil treated

group as compared to the both control groups (Fig 3l, $p<0.01$). There was no significant difference of IL-10 levels between the two control groups (Fig 3i, 3l).

Discussion

The prominent features of AD are eczematous skin lesions and intensive pruritus. Recent studies have explored the role of the nervous system in the pathogenesis of AD. The course of AD is associated with an altered pattern of cutaneous in nervation and abnormal expressions of neuropeptides including NGF, substance P (SP) and vasoactive intestinal peptide (VIP) in the lesional skin. NGF shows an increase in both serum and lesions of AD patients, which has been proposed to serve as a biomarker of disease severity (15-17).Our results showed that NGF was highly expressed in AD model, which was similar in AD patients. Furthermore, ozonated oil can decrease NGF expression in serum and atopic skin, suggesting its function of ameliorating disease severity.

Ozone has been used topically for the treatment of war wounds, anaerobic infections, herpetic infections, trophic ulcers and burns, cellulitis, abscessed, anal fissures, and gingivitis(18,19). Hee et al. reported that application of ozonated oil can accelerate acute cutaneous wound repair in the guinea pig model by promoting collagen synthesis and fibroblast proliferation at the injury site and by increasing the expression of growth factors such as PDGF, TGF-β, and VEGF(20). In our observation, ozone water can improve MRSA infection skin(11). Furthermore, ozone can affect the expression of

pro-inflammatory cytokines, such as IL-1 and TNF-α, and the adaptive inflammatory responses including COX-2 gene activation in keratinocyte via activation of NFκB (21). These findings suggest that ozone can induce the keratinocyte proliferation and differentiation resulting in affecting skin biology(9). However, the ozonated oil does not penetrate through the mucous membranes and act on any specific receptors. Its action is indirect. O3 reacts rapidly with antioxidants and polyunsaturated fatty acids (PUFA), resulting in formation of intracellular second messages, such as hydrogen peroxide (H_2O_2) and 4-hydroxynonenal. These second messengers finally activate nuclear transcriptional factors, such as NFκB, nuclear factor of activated T-cells (NFAT), activated protein-1 (AP-1), as well as further modulation of interferons and cytokines(19, 21). The present study shows that ozonated oil recovers the skin lesion quickly within one week, possibly through the second message H_2O_2. However the detailed mechanism is unknown and will be our future direction.

In AD, even non-lesional skin shows impaired barrier function. Increased water loss causes the skin to dry and induces inflammation by initiating a cytokine cascade. Increased entry of allergens and irritants also causes atopic skin to be more susceptible to irritant contact dermatitis. Moisturizers can improve impaired barrier function associated with AD via multiple mechanisms. For example, they reduce the risks of cracking by increasing skin hydration, relieve pruititus, and exerting antimicrobial and anti-inflammatory effects(22, 23). Therefore, even the vehicle, camellia oil, used in the present study can also contribute to the improvement in AD.

The classical Th1/Th2 paradigm explains the immune responses in AD. The Th2 responses lead to an increased production of cytokines, primarily IL-4 and IL-31, which then promotes AD pathology (10, 21). In the present study, we have shown that ozonated oil potentially inhibits Th2 response in the AD model. On the other hand, serum Th1-dominant cytokines levels are not regulated by ozonated oil, but their mRNA levels are inhibited in the ozonated oil treated atopic skin. These results allow us to postulate that lesional skin exhibited a mixed inflammatory cytokine patterns that may be a result of self-trauma and secondary infection. The reason for inconsistency between the Th1 cytokines mRNA levels in lesional skin and protein levels in serum is unknown and subject to further investigations.

Tregs preferentially express the critical immunosuppressive cytokines TGF-β1, IL-10，and IL-35(24). The reduced recruitment of Tregs to the skin is associated with severe inflammation and keratinocyte proliferation (25). Recently, it has been reported that Treg cells are deficient in AD lesions (26). Meanwhile, IL-10-producing regulatory B cells (B10 cells), which have been shown to suppress excessive inflammation in various inflammatory and autoimmune diseases, are also found to be decreased in patients with severe AD. Depletion of B10 cells in humans results in an improvement of AD (27). In the present data, IL-10 expression is increased in ozonated oil treated group, suggesting that regulatory cells are potentially activated or their numbers are modulated by ozone. In addition, Bocci has reported that ozone may induce IL-10 and TGF-β release(28). Therefore, our finding suggests that ozone can potentially contribute to regulating immune responses in AD model via inducing IL-10 expression.

In conclusion, the present results have shown that ozonated oil can effectively improve the atopic skin via restoration of Th1/Th2 balance and increasing expression of immune suppressive cytokine 1L-10. Our results suggest ozonated oil can be potentially a new therapeutic strategy for treatment of AD.

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Disclosure statement

The authors have no conflicts of interest to disclose.

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Tables

Table 1 Primers for real-time PCR

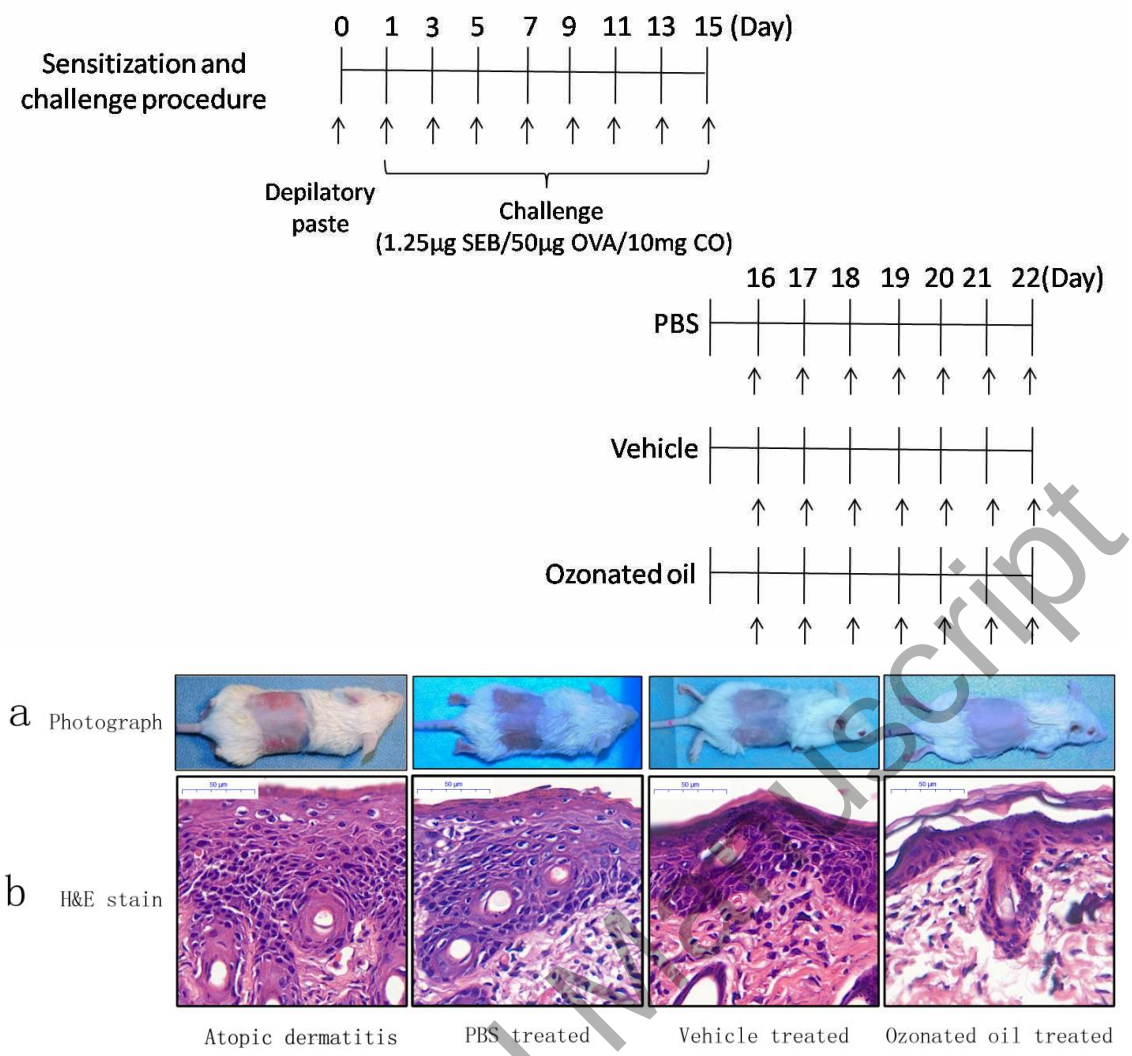
Gene	Primers	Sequence (5'-3')
IL-4	UP	GCCATATCCACGGATGCGACAA
	DN	GGTGTTCTTCGTTGCTGTGAGG
IL-31	UP	CATCTCGGTCATCATAGCACATC
	DN	TTCATCATATTTCCAGGCACAG
IL-2	UP	AGATGAACTTGGACCTCTGCG
	DN	ACTCATCATCGAATTGGCACTC
TNF-α	UP	GGAACTGGCAGAAGAGGCACTC
	DN	GTAGACAGAAGAGCGTGGTGGC
IFN-ɣ	UP	CTCAAGTGGCATAGATGTGGAAG
	DN	TGCTGATGGCCTGATTGTCT
IL-10	UP	TGGACAACATACTGCTAACCGAC
	DN	AGTGGGCTTCAGGGACAGAG
NGF	UP	ATAAAGGTTTTGCCAAGGACG
	DN	AGTGGGCTTCAGGGACAGAG
β-actin	UP	CCTGGGGCATCACTTCTACC
	DN	GACCCATTCCCACCATCACA

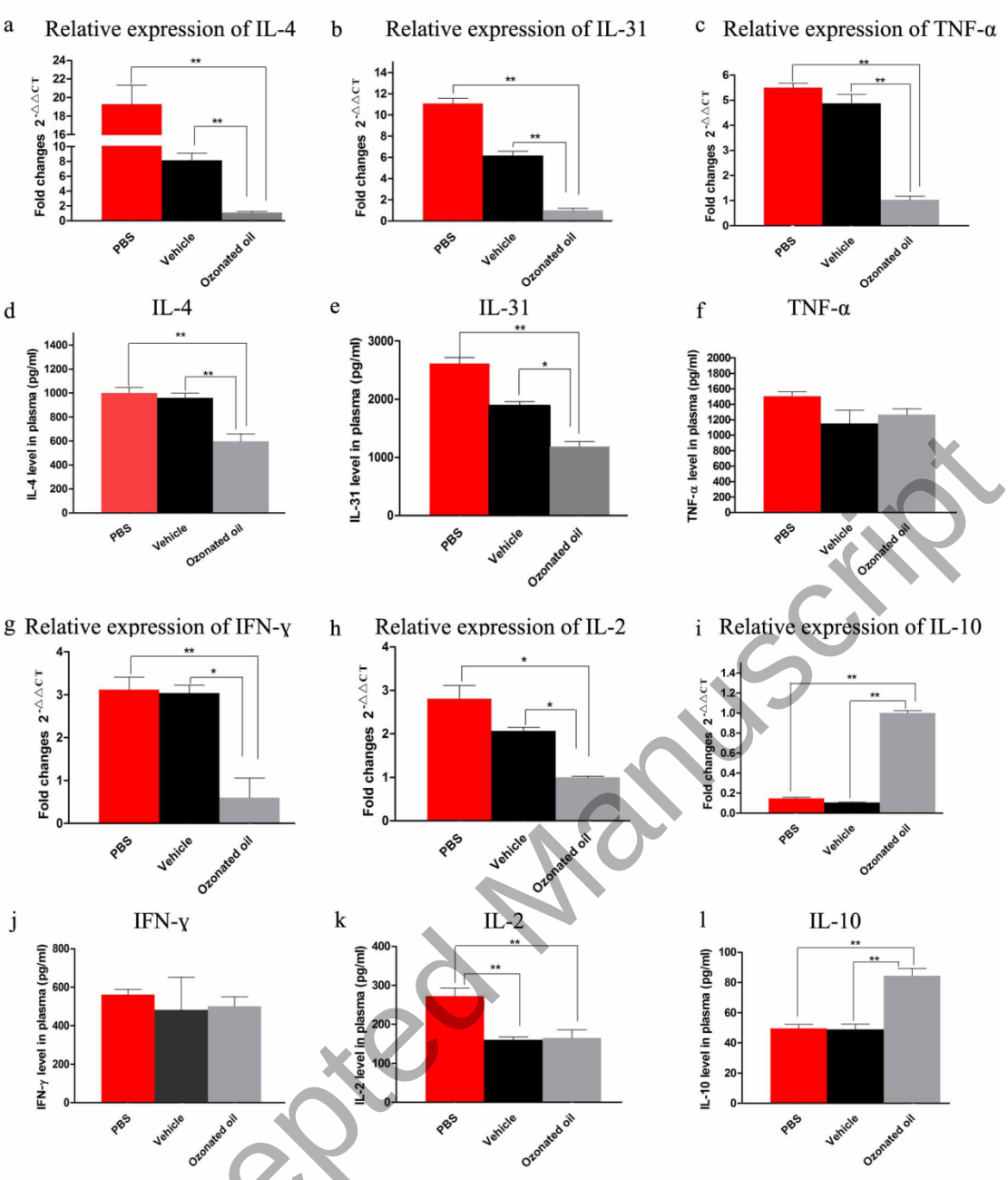
Figure captions

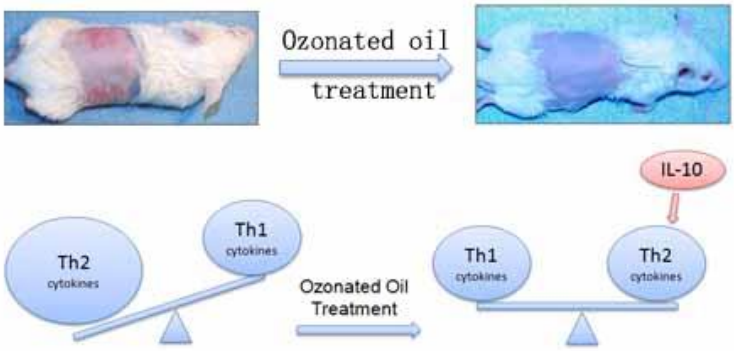
Fig 1.Outlinefor induction of atopic dermatitis model and the treatments. Mice were challenged on the shaved skin with a triplex allergen, SEB/OVA/CO, on every other day for 15 days to induce an AD skin lesion. Mice were then received ozonated oil, PBS or drug vehicle on the shaved skin once a day for one week, respectively. SEB, staphylococcal enterotoxin B; OVA, ovalbumin; CO, calcipotriol ointment.

Fig 2. Representative photographs and histological features of treated skin lesions. (a) Photos of treated mice. (b) Histological features of treated skin sites in BALB/c mice. Skin sections were stained with H&E and examined at 200× magnification. Scale bar, 50μm. (c) Epidermal thickness detected by reflectance confocal microscopy in treated skin sites. Expression of NGF mRNA (d) and protein (e) in ozonated oil, PBS, or vehicle treated sensitized skin sites and serum. The columns and error bars represented the mean ±SEM (n=6 animals per group). *P<0.05; **P<0.01.

Fig 3.The mRNA (a, b, c, g, h, i) and protein(d, e, f, j, k, l) levels of cytokines in ozonated oil, PBS, or vehicle treated sensitized skin sites and serum, respectively. The columns and error bars represent the mean ±SEM (n=6 animals per group).* P<0.05. * * P<0.01.







Ozonated oil correct Th1/Th2 imbalance via increasing suppressor cytokine IL-10 in atopic dermatitis(AD)-like murine model. Ozonated oil is supposed to improve the atopic skin via anti-infection, relieving itching, promoting wound healing and immune regulation. Ozonated oil is a potent new tool for the topical treatment of AD.