

Beneficial Effects of Liposome Containing Arthrocen (ROCEN) on Atopic Dermatitis in Mice

Ramin Goudarzi

Division of Research and Development, Pharmin USA, LLC, San Jose, California, United States of America

Maryam Eskandarynasab

Tehran University of Medical Sciences

Ahad Muhammadnejad

Tehran University of Medical Sciences

Ahmad Reza Dehpour

Tehran University of Medical Sciences

Alireza Partoazar (✉ partoazar@yahoo.com)

Tehran University of Medical Sciences

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Title:

Beneficial effects of liposome containing arthrocen (ROCEN) on atopic dermatitis in mice

Author names and affiliations:

Ramin Goudarzi¹ (Pharm D), Maryam Eskandarynasab² (MS), Ahad Muhammadnejad³ (PhD), Ahmad Reza Dehpour^{2,4} (Pharm D, PhD), Alireza Partoazar^{2,4} (PhD)

1. Division of Research and Development, Pharmin USA, LLC, San Jose, California, United States of America
2. Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
3. Cancer Biology Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran.
4. Experimental Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran

Corresponding author:

Alireza Partoazar, Ph.D., Assistant professor of Medical Nanotechnology.
Experimental Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran.
P.O. Box: 13145-784, Tehran, Iran.
Tel/Fax: +98-21-66402569;
E-mails: partoazar@tums.ac.ir, partoazar@yahoo.com

Abstract

Objective: Atopic dermatitis (AD) is a chronic inflammatory skin disease caused mainly by the immune stimulus. The current study aimed to investigate the effects of liposome containing arthrocen (ROCEN) and its comparison with betamethasone (Beta) on mice subjected to AD. **Methods:** First of all, the risk assessment of ROCEN sensitization was done, then mice were subjected to oxazolone (Oxa) for chronic AD induction and treatment. Scratching and wiping behaviors related to dermatitis were evaluated in animals treated topically with ROCEN. The histological and immunohistochemistry analysis of interleukin-8 (IL-8) and tumor necrosis factor- α (TNF- α) were conducted to the dorsal skin of treated rats. **Results:** The results showed that cutaneous administration of ROCEN on sensitized mice for 5 weeks, alleviated significantly scratching and wiping symptoms, erythema, scaling, and edema in animals' skin. Moreover, histological indices showed that ROCEN reduced effectively leucocyte infiltration and improved skin healing parameters in AD mice. Immunohistochemically markers of IL-8 and TNF- α were hindered significantly by ROCEN in dermal tissues of mice. **Conclusion:** ROCEN potentiated alleviation of the AD symptoms rather than betamethasone drug in an experimental model.

Keywords: Atopic dermatitis, avocado and soybean, Oxazolone, Eczema, IL-8, TNF- α .

Introduction

AD is an increasingly prevalent chronic inflammatory skin disease worldwide [1]. The most common symptoms of AD-like hand eczema are intense itching, a typical distribution of mild to severe rash, edema, hemorrhage, and erosion [2]. AD is a multifactorial disease associated with a variety of immunological mechanisms that environmental and even neuropsychological factors can play role in the development of this disease [3-5].

One of the characteristic features of AD is the involvement of various inflammatory cells including lymphocytes, eosinophil, neutrophils, with a predominance Th₂ cell response [6, 7] An allergen exposure can stimulate keratinocytes and release cytokines like TNF- α , resulting in the excitation of proinflammatory cytokines production including IL-1 β , IL-6, and IL-8 from other keratinocytes and dermal cells [7]. It can be directed to the allergic phase via recruitment and activation of leukocytes and dendritic cells and presenting the allergen to specific T-cells. It is also indicated that IL-8 cytokine production by Th₂ cells activated by allergic contact response can contribute to activation and regulation of various immune cells in inflamed tissues [7, 8].

Chronic AD, eczema, is mostly treated with corticosteroids like Beta [9] while their long-term usage can bring serious side effects including hyperglycemia and dermal and epidermal injuries [10]. Therefore, new formulation and drugs are always the interest of modern medicine to improve the life quality of AD patients.

Arthrocen (ASU), an oil extract of avocado and soybean, has shown that it downregulates inflammatory mediators such as TNF α , IL8, and PE₂ while increases the expression of transforming growth factor-beta (TGF- β), production of collagen, and aggrecan in connective tissue [11, 12]. Therefore, evidence reports suggest that topical ASU may regulate inflammatory cells and cytokines associated with AD symptoms and improve tissue impairment due to eczema [13].

However, because of the very low water solubility of Arthrocen, the use of pharmaceutical carriers such as liposomes is recommended for effective skin delivery of lipophilic molecules of ASU [14-16]. Liposome carriers not only are versatile tools in drug delivery but also have anti-oxidative, anti-inflammatory, and wound repair properties [17-19]. Recently, we have used the topical formulation of

liposomal ASU to enhance the drug efficacy in wound healing, inflammation reduction, and pain alleviation in animal models [13, 16]. In the study by Goudarzi et al., ROCEN formulation has caused potentially TGF- β 1 production in burnt tissues of rats resulting in wound healing acceleration and thermal pain alleviation with a long-lasting effect [13]. Hence, we also aimed to investigate the effects of the topical application of ROCEN and its comparison with Beta drug on AD mice model.

Materials and methods

Drug preparation

Liposomal formulation of ASU was prepared according to our recent study.[13] Briefly, lecithin (10% w/v), PG (15% v/v), cholesterol (1% w/v), and ASU (2% w/v) were dissolved in ethanol. Then phosphate buffer saline was added to the mixture at 40°C and under stirring condition (700 rpm). Eventually, the suspension was homogenized with mild power for 5 min to obtain ROCEN formulation. Oxazolone (Oxa) was obtained from Sigma-Aldrich, (USA) Arthrocen manufactured by Pharmin USA, LLC (USA) and Betamethasone %0.1 was prepared from Sobhan Daru Pharmaceutical Company (Iran).

Animal

The 6-8 weeks female BALB/c mice (mean weight 30 \pm 2 g) were procured from Tehran University of Medical Sciences (Tehran, Iran). The animals were placed under controlled environmental conditions at 23 \pm 1 °C with 50% humidity and 12 h dark/light cycles. The animals were subjected to acclimatization for at least 1 week before the experiment.

Safety evaluation of ROCEN

In this study, a primary delayed-type contact hypersensitivity test, MEST, was considered for evaluation of possible allergic contact dermatitis due to ROCEN application. Briefly, mice were divided into 3 groups consisted of 10 rats including control, ROCEN, formaldehyde (positive control). The test substance was judged to be a nonirritant agent before MEST and then the test was carried out according to a standard protocol that it has been depicted briefly in Figure 1. A positive sensitization response is brought up to have happened if the one or more ear test of mice was at least 20 % thicker than the control ear [20].

Induction of dermatitis and treatment

AD was induced in mice according to the Goindi et al. method [21]. Firstly, female BALB/c mice were anesthetized with ketamine (50 mg/kg) and xylazine (10

mg/kg) and the hair on the upper back of each animal was shaved one day before the experiments. AD was induced by topical application of 10 μ L Oxa (5% w/v in ethanol) on the shaved dorsal skin daily for one week. Then, the same experiments were followed daily by gently rubbing of 60 μ L Oxa (0.5% w/v in ethanol) with a frequency of 30 \times per min on the dorsal skin at the four weeks. 90 min after Oxa application, drug was administered topically by rubbing with a frequency of 50 \times per min on the animals' back of treated groups. In the control group, only 60 μ L ethanol (without Oxa) was applied to the dorsum of mice.

Experimental design

The mice were randomly divided into the four groups with 6 animals in each experiment and defined as follows: group: vehicle, Oxazolone (Oxa) group: Oxa 60 μ g/ day + Water 100 μ g/ day, ROCEN group: Oxa 60 μ g/ day + ROCEN 100 μ g/ day, Beta group: Oxa 60 μ g/ day + Beta 1%, 100 μ g/ day. In all experimental days (0-35), evaluation of behavior-related skin dermatitis was done according to the below protocols. One day after the last dose of the drug (36th day), animals were euthanized with CO₂ and then their dorsal skins separated for histological analysis. A brief description of the experimental is individual in Figure 2.

Evaluation of skin severity

The severity of AD on the dorsal skin was evaluated once a week according to four symptoms of erythema, scarring/dryness, edema, and excoriation/erosion. The symptom of erythema was scored as 0 (none), 1 (very slight (light pink)), 2 (well-defined (dark pink)), 3 (Moderate to severe (light red)), 4 (Severe (dark red)). The other relative symptoms were scored by numbers of 0 (none), 1 (mild), 2 (moderate), 3 (severe). An experimental score was defined as skin severity score achieved by the sum of the relative data of dermatitis symptoms for each experiment [22].

Measurement of scratching/wiping behavior

Mice were placed individually in the acrylic cages with the four cells in dimensions of 15cm \times 10cm \times 10cm. The small openings were created in each cell to allow air circulation. The camera recorder was embedded in front of the cages to record animals' behavior. The adaptation of mice to the experimental conditions was done by handling, restraining, and placing them in containers several times before the experiments began at each experimental day. On the days of experiment (7-14-28-35), 90 min after drug application, the animals' behavior was recorded for 20 min while non-experimenters present in the observation room. Scratching, grooming,

and licking behaviors were assessed according to the frequency of each behavior during 20 min experiment which counted from video records [23].

Histopathology and immunohistochemistry

Tissue samples were taken from dermatitis areas of mice dorsum one day after the treatment. Skin samples were fixed in 10% (v/v) formalin buffer with PBS and then dehydrated and embedded in paraffin. The section of samples was prepared with 5µm thickness and stained with hematoxylin and eosin for histopathology analyses. An expert pathologist assessed as blindness the sections for epithelial thickness, collagen deposition, angiogenesis, leukocyte infiltration, mononuclear cells (MCs), lymphocytes (Lymphs)/Plasma cells (PCs) using a light photomicroscope. The tissue slides were also subjected to immunohistochemical assay of TNF- α (orb39299, biorbyt, Cambridge (United Kingdom)), IL-8 (orb39299, biorbyt, Cambridge (United Kingdom)) using commercially available antibodies according to the manufacturers' instructions, and being analyzed by pathologist.[24]

Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) to compare the significant differences among each group. $P < 0.05$ was considered as statistically significant.

Results

Safety of ROCEN

MEST was carried out to further assess the sensitization potential in ROCEN treatment and the results were shown in Figure 3ABC. In the positive control group, topical application of formaldehyde caused a significant increase in ear thickness and redness region of mice ($p < 0.0001$). It was indicated that ROCEN did not cause any sensitivities in the ear thickness and redness region of mice.

Skin severity reduction by ROCEN

After the repeated application of Oxa, skin dryness occurred first, followed by mild erythema and edema. Finally, the skin became thick and severe erythema, edema, and scarring were observed, and these symptoms could not be detected in the control group (Figure 4A). As shown in Figure 4B, the dermatitis scores of the Oxa group increased rapidly and became significant every week after the first elicitation compared to the control group ($p < 0.0001$). However, in the ROCEN Group, skin lesions were minimally observed compared to Oxa and beta groups (p

<0.05). The application of Beta as a standard drug caused significant improvement of dermatitis only on days 28 ($p < 0.05$) and 35 ($p < 0.01$) (Figure 4B).

Itching control by ROCEN

The scratching number was calculated at days of 7, 14, 28, and 35 for 20 min. Oxa group was showed a significant increase of scratching, grooming, and licking events 30 min after the Oxa application compared to the c group at all days ($p < 0.05$) (Table 1). The scratching and the wiping scores in the ROCEN treatment were significantly lower ($p < 0.05$) than the Oxa group at all-time intervals. The remains of the analyzes are shown in detail in Table 1. These results suggest ROCEN improve the effects of eczema even better than Beta group (Table 1).

Histological and immunohistochemical analysis

Histological changes in mice dorsal skins were compared between Oxa and treated groups on the 36th day of the experiment. lesional skin in Oxa group showed a significant ($p < 0.05$) epithelial thickness, collagen deposition, angiogenesis, and leukocyte infiltration (Table 2 & Figure 5A-I). The histological parameters were significantly ($p < 0.05$) improved by ROCEN via the increased epithelial thickness reduced collagen deposition, and decreased angiogenesis in comparison with the untreated Oxa group. Furthermore, the infiltration of inflammatory cells such as MCs, lymphocytes, eosinophil, and neutrophil was significantly ($p < 0.05$) reduced by ROCEN (Figure 5C) and the ROCEN group works better than the beta group. The number of basophils did not show any significant differences among the groups (Figure 5A – II). Immunohistochemical staining revealed significantly ($p < 0.001$) overproduction of IL-8 and TNF- α in the sensitized dermis after topical application of Oxa compared to the control as depicted in Figure 6A-C. Topical ROCEN application decreased significantly ($p < 0.05$) IL-8 and TNF- α staining compared with Oxa.

Discussion

AD or eczema as a common chronic inflammatory skin disorder is associated with chronic itch, pruritus, inflammation, and cell proliferation [1]. Treatment of patients' AD with systemic oral corticosteroids or even topical corticosteroids like Beta may be potentially at risk of side effects of the drug [10, 25]. In this study, we used topical Beta as a mainstay drug in the treatment of atopic eczema for comparison with ROCEN as a new anti-AD formulation on a murine model.

Topical application of ROCEN hydrogel moisturize the skin and also to be its safe components without irritability, can be reasonable for its long term application in allergic dermatitis. ROCEN has been prepared from natural oil [11] and lecithin in the liposomal structure that is well known as drug supplementation without any adverse effect in pharmacy [13]. Treatment of AD with ROCEN not only has had anti-inflammatory and analgesic effects in experimental reports but also due to its permeability property may play role in barrier homeostasis of the skin [13, 16].

In the current study, treatment with topical ROCEN was associated with the improvement of all important dermatitis symptoms and hindering the itching and wiping behaviors in mice subjected to eczema. Itching is the major symptom of inflammatory AD that impairs the patients' life quality [26, 27]. Itching stimulates scratch reflex and increases the desire to scratch, resulting in exacerbation of the skin lesions, secondary infections, and sleep disturbances in the patient. Therefore attenuation of itching is believed to be one of the fundamental approaches in controlling skin lesion progression in an eczematous state [28].

Histopathology of eczematous lesions is characterized by epidermal hyperplasia and dermal inflammatory cell infiltration with increased numbers of monocyte, macrophages, mast cell, basophil, eosinophil [29, 30], and neutrophil in an early inflammatory reaction [31].

Angiogenesis occurs physiologically in the wound healing and during inflammatory diseases [32] while mast cells in AD lesions may stimulate angiogenesis via the release of proangiogenic factors [33]. Besides, fibrocystic accumulation in the skin fibrogenesis can lead to the production of cytokines that induce collagen deposition in the lesion site [34-36]. It has been indicated that suppress of fibrogenesis causes reduction of proangiogenic mediators resulting in decreasing angiogenesis and activation of T cells [37].

In this study, ROCEN daily application modulated significantly angiogenesis and collagen deposition and reduced leucocyte infiltration such as eosinophil rather than Beta cream in skin tissue of AD mice.

In our study, TNF- α [38] and IL-8 [1, 39] as proinflammatory cytokines involved in allergic contact dermatitis were evaluated histochemically after 36 days of topical treatment with ROCEN. It has been proposed that IL-8 is a non-specific mediator with mitogenic activity in keratinocytes that induced inflammation or immunogenic after stimulation with contact sensitizers and irritants [39]. Moreover,

the upregulation of TNF- α along with other inflammatory cytokines contributes to the changes in epidermal abnormality and skin barrier dysfunctionality. This results in thickened skin and scale formation [1, 40]. Our immunohistochemically data indicated that both TNF- α and IL-8 have downregulated by ROCEN in lesion tissues related to AD. Therefore, according to the results, it can be suggested that improvement of tissue indices and reduction of inflammation in dermatitis by ROCEN may be associated with the down-regulation of IL-8 and TNF-a cytokine factors.

Conclusion

This study supported evidence of the therapeutic benefits of topical ROCEN even better than Beta in control of AD. Current results suggest that ROCEN can reduce tissue damage, dermatitis symptoms like pruritus, and downregulation of inflammatory cytokines associated with a contact stimulus. Topical ROCEN may be applicable in the clinic instead of current AD therapeutics to minimize side effects.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Tehran University of Medical Sciences in agreement with the standards for the use of laboratory animals (No. 1398.226). No data concerning clinical studies or involving human subjects in this study.

Consent for publication

All authors agree to publish the work in this journal.

Availability of data and materials

All data are available in this paper.

Declaration of interest: No conflict of interest exists concerning the submitted manuscript. It is confirmed that all methods have been performed in accordance with the relevant guidelines and regulations.

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Authors' contributions

1. Ramin Goudarzi: Conceptualization, Writing, and Reviewing.
2. Maryam Eskandarynasab: Animal study, methodology.
3. Ahad Muhammadnejad: Histopathology evaluation, Data curation.
4. Ahmad Reza Dehpour: Data validation, Pharmacologist.
5. Alireza Partoazar: Supervision, Drug preparation, Resources.

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Table 1. Scratching/wiping frequency data in treated AD mice. Data are defined as the mean±SEM; n = 6 for all groups. a,b,c,d: Oxa compared with control group. A,B,C,D: treated groups compared with Oxa group. 1,2,3,4: ROCEN compared with Beta group. Aa¹; p<0.05, Bb²; p<0.01, Cc³; p<0.001, Dd⁴; p<0.0001.

Behavior type	Date	Oxa	Sham	Rocen + Oxa	Beta + Oxa
No. of scratching (per 20 min)	7 th day	42.8 ±6.8 ^C	9.6±3.8	14±4.3 ^b	18±0.3 ^a
	14 th day	18.2±4.1 ^B	9±4.5	10.5±3.5 ^a	13±3.2
	28 th day	108±16.1 ^D	7.5±1	29.6±6.1 ^{d*}	54.25±18.5 ^b
	35 th day	113.6 ±14 ^D	10± 3	32.6±10.3 ^{d1}	50.7±15.3 ^c
No. of grooming (per 20 min)	7 th day	29.8±8.8 ^A	9.6±3.8	8.2±3.9 ^{b1}	16.3±2.1 ^a
	14 th day	21.2±1.9 ^A	14±1.8	18.4±2.7	18±1.4
	28 th day	67.4 ±6.5 ^D	6±7	10.5 ±1.9 ^{d1}	30.5 ±2.7 ^d
	35 th day	78 ±12.5 ^D	12.5± 2.5	53.6 ±10.3 ^a	39.7 ±8.2 ^b
Time of licking(s) (per 20 min)	7 th day	341.8 ±7 ^D	74±12.1	115±41.3 ^{d2}	229.4±71.3
	14 th day	174±29.5	107±48.6	129.6±25.1	214.3±131
	28 th day	294.6 ±39.2 ^D	34.3 ± 4.5	40.3 ±1.9 ^{d3}	234.2 ±19.5 ^a
	35 th day	340 ±40.4 ^D	12.5 ± 2.5	58.1 ±22.3 ^{e2}	229.5 ±8.5

Table 2: The dorsal skin histological changes in mice. a,b,c,d: Oxa compared with control group. A,B,C,D: treated groups compared with Oxa group. 1,2,3,4: ROCEN compared with Beta group. Aa1; p<0.05, Bb2; p<0.01, Cc3; p<0.001, Dd4; p<0.0001.

Experiment	Epithelial thickness	Collagen deposition	Angiogenesis	Leucocyte infiltration
	(μm)	%	No./ mm ²	No./mm ²
Oxa	67.74 ± 7.68 ^C	52.49 ± 4.4 ^B	4.8 ± 0.54 ^A	1250 ± 85.63 ^C
Sham	159.5 ± 33.84	37.01 ± 3.98	3.63 ± 0.66	913.33 ± 76.88
ROCEN+ Oxa	139.8 ± 12.42 ^b	34.11 ± 3.74 ^b	3.8 ± 0.23 ^a	806 ± 51.92 ^{d2}
Beta + Oxa	107.77 ± 9.77	43.9± 4.14	6.05± 1.1	1045 ± 51.85 ^a

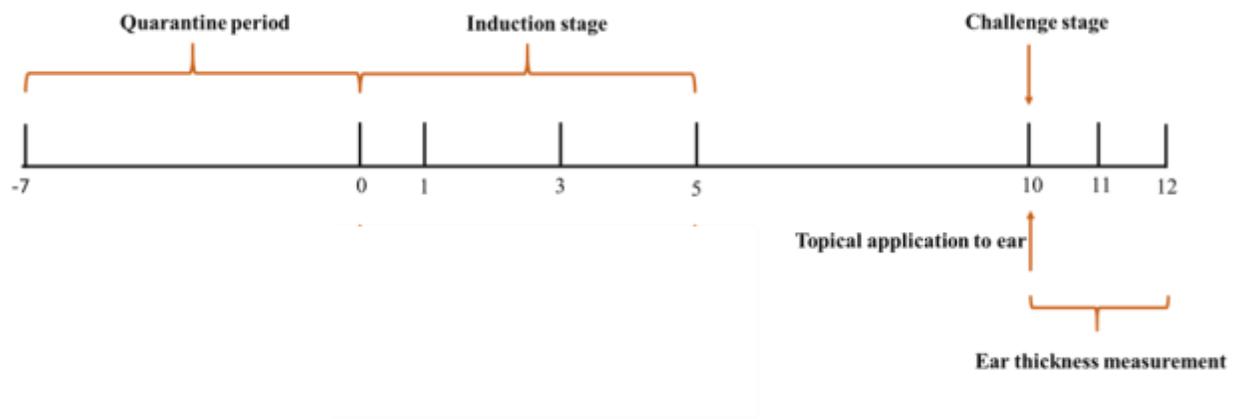


Figure 1: The line chart of the MEST protocol for allergenicity assessment.

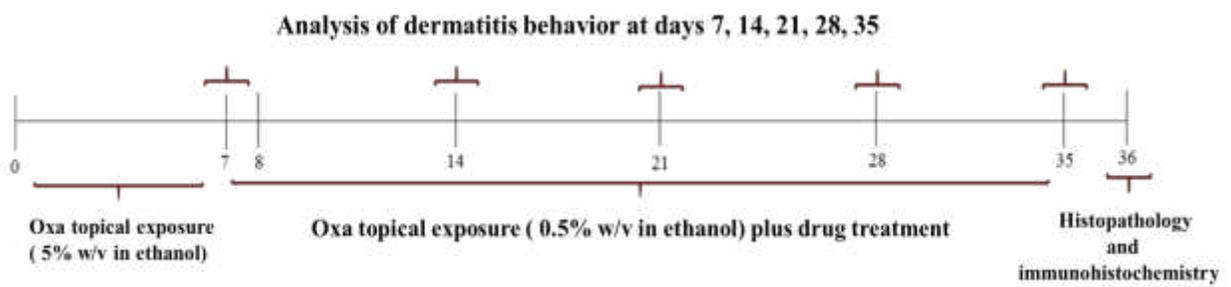


Figure 2: Summary of experimental dermatitis protocol.

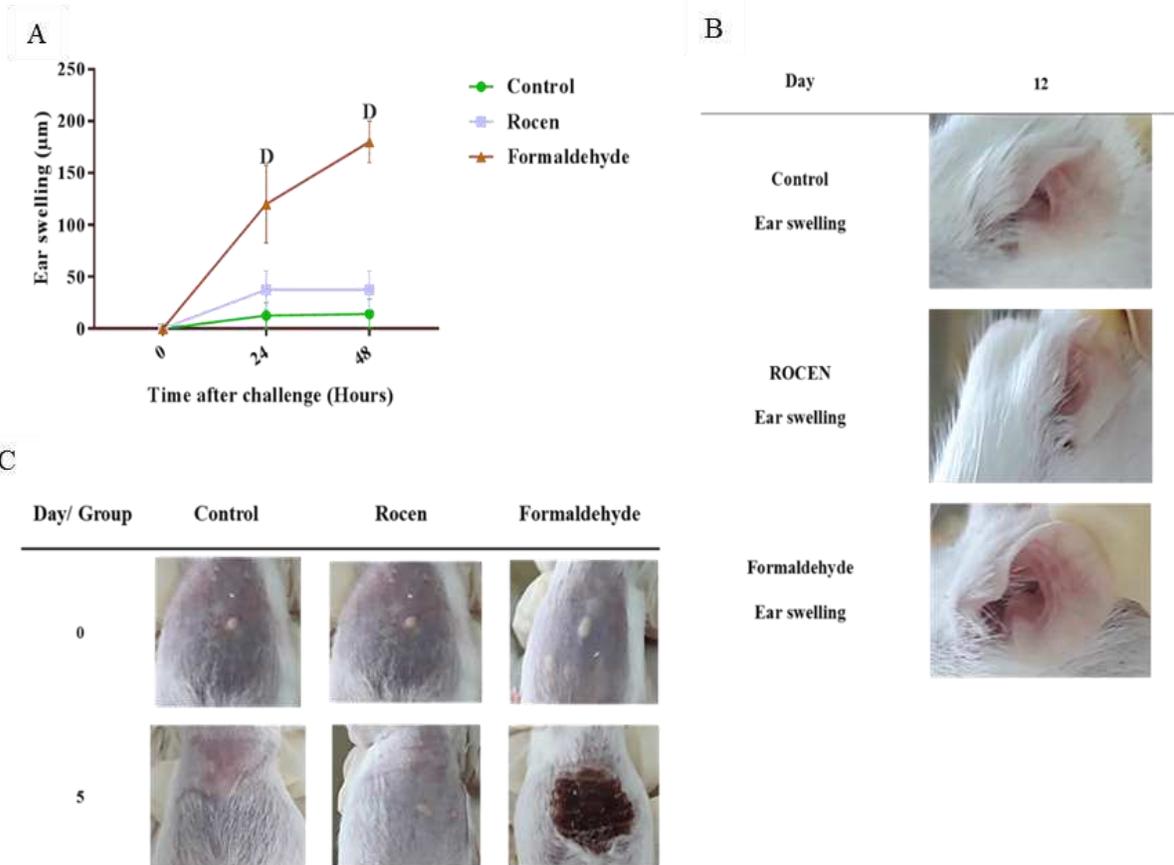


Figure 3: The demonstration of ROCEN sensitization evaluation using MEST. A. Swelling measurement of the left ear of the mice did not show any irritation via ROCEN application. n =10 for all groups. D when treated groups compared with control group, (D; P-value < 0.0001). B. The representative mice ear shows a considerable ear redness in formaldehyde group (sensitized mice). C. The mice were shaved ventral abdomen on day 0 for induction of sensitization by applying two intradermal injections (40 µl) of adjuvant and 100 µl of water (control), ROCEN, formaldehyde (as dermatitis lesion) were applied topically by rubbing gently.

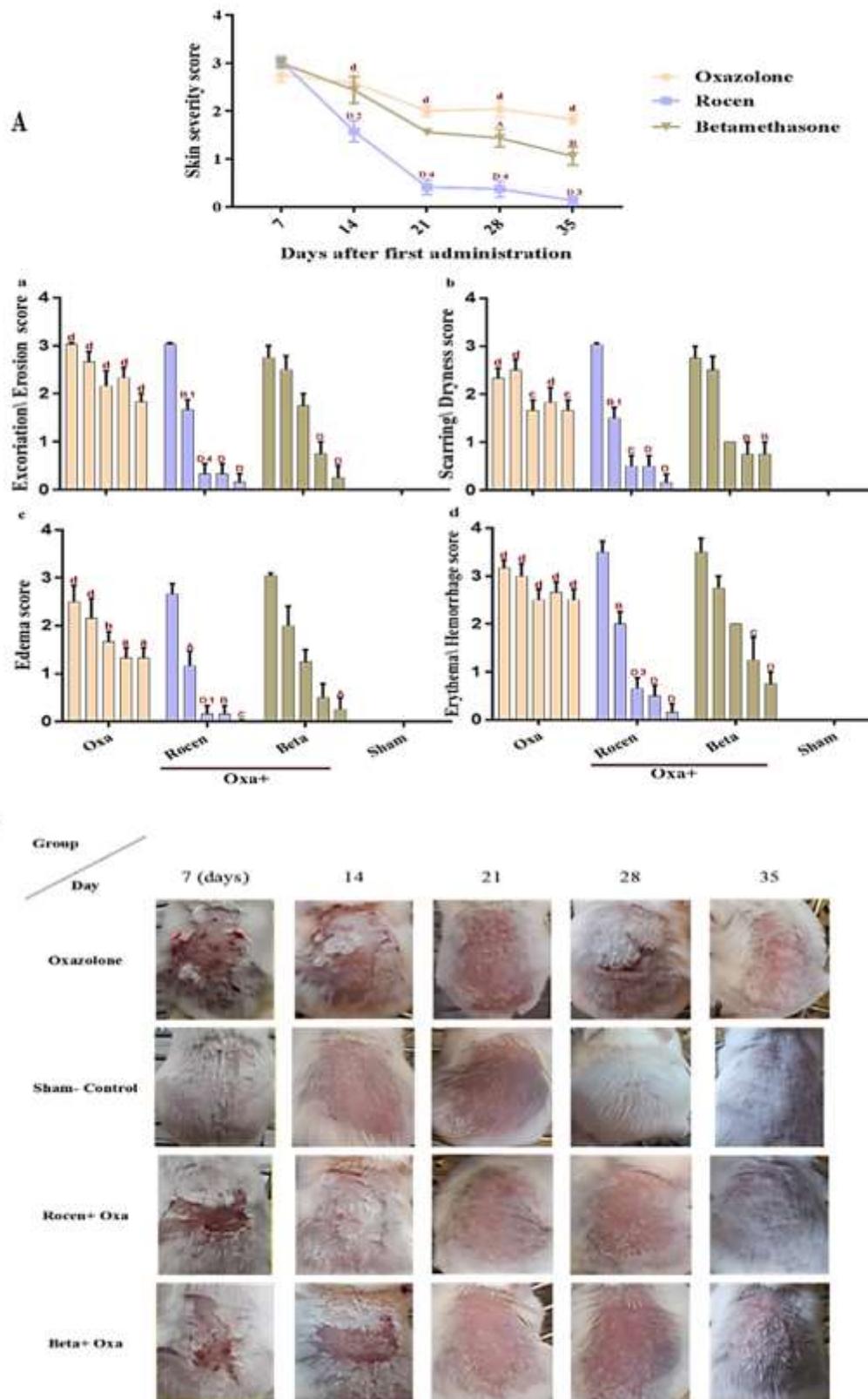


Figure 4: Indicative skin severity data of AD-like skin lesions treated by ROCEN in mice. A) skin severity scores indicate a significant dermatitis healing by topical ROCEN rather than Beta at relative time points. The severity score was achieved by the sum of the relative dermatitis data (a, b, c, and d scores) at each experiment and data represented by mean \pm SEM. B) Macroscopic features indicate that Oxa administration causes severe AD lesions at different time intervals while ROCEN and Beta treatments recover the lesion progress in mice. a, b, c, d: when Oxa group is compared with control group. A, B, C, D: treated groups are compared with Oxa group. 1, 2, 3, 4: ROCEN group is compared with Beta group. Aa¹; p<0.05, Bb²; p<0.01, Cc³; p<0.001, Dd⁴; p<0.0001.

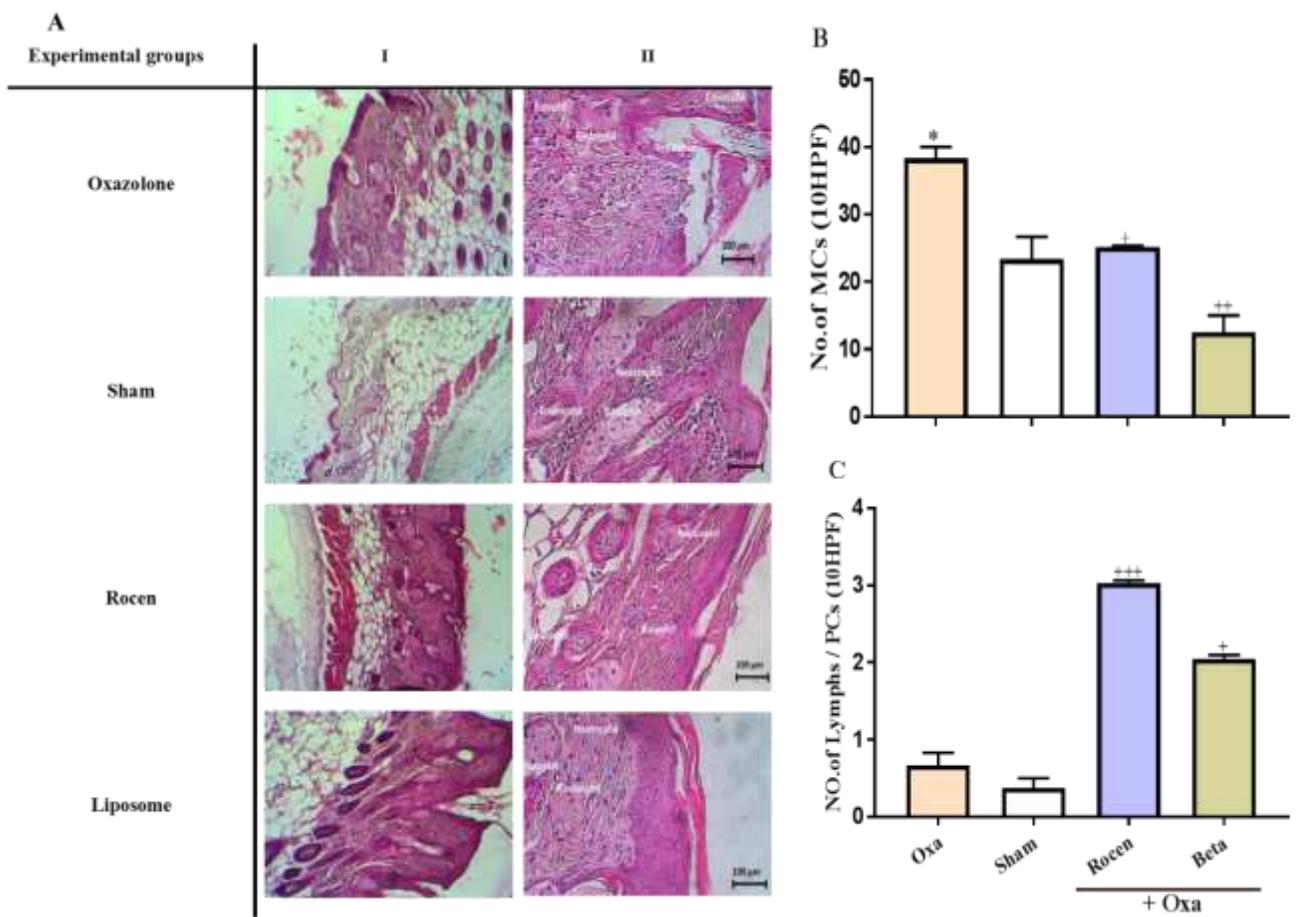


Figure 5: (A) I. Histopathological pictures of Oxa-induced chronic dermatitis and relative treated groups. II. The neutrophil, basophils, and eosinophil's cells were showed with a light microscope. B. The number of MCs and (C) Lymphs/ plasma cells were defined as the mean \pm SEM; n = 6 for all groups. * When Oxa compared with sham group, and + treated group compared to Oxa group. * &, +; p < 0.05, ***, &, +++; p < 0.001.

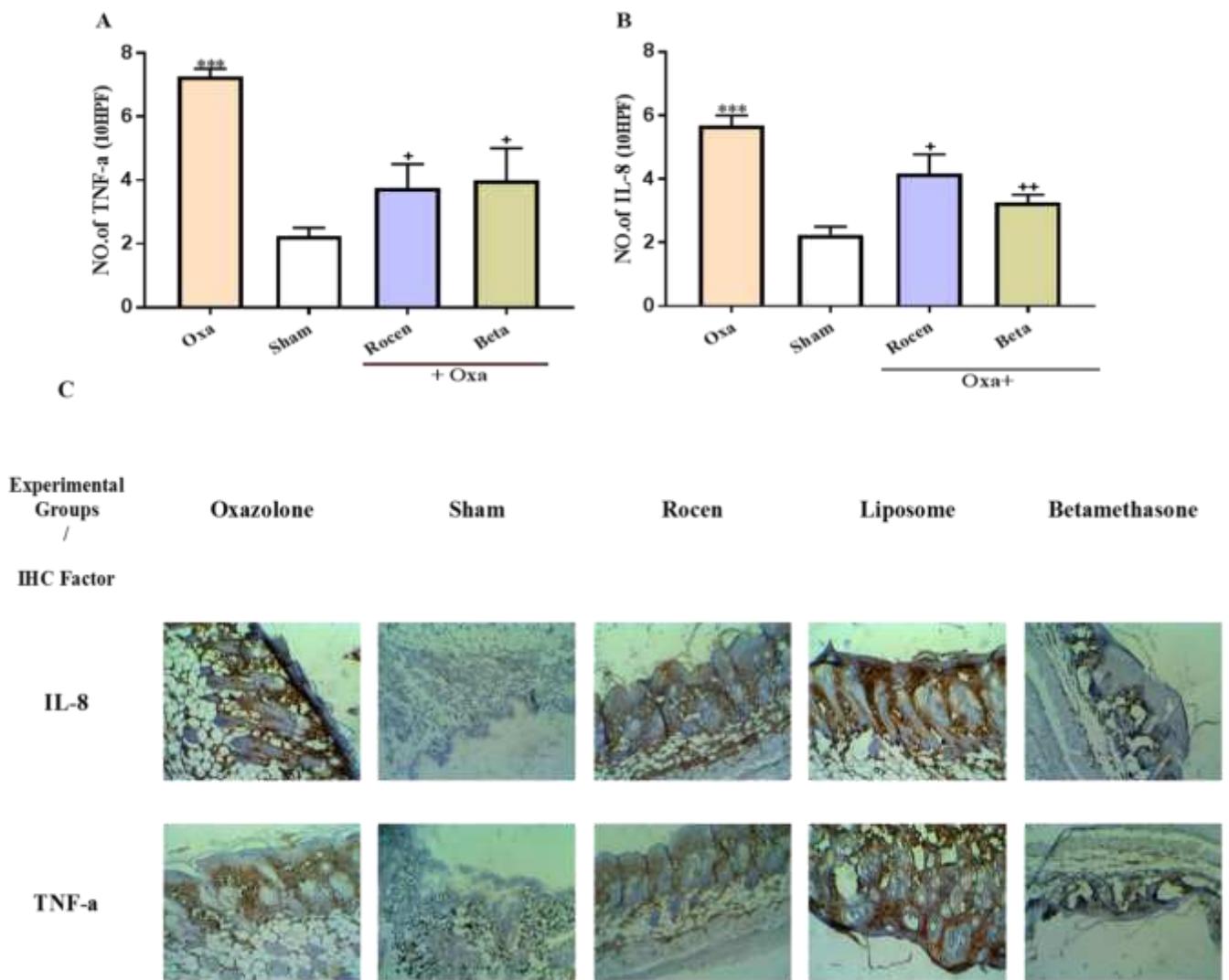


Figure 6: Data immunohistochemistry of cytokines related to topical treatment of AD. TNF- α (A) and IL-8 (B) analyzed by immunohistochemistry were defined as mean \pm SEM with n=6. C) Photomicrograph of IHC section of the dorsal skin showing IL-8 and TNF- α . * Oxa compared with control group, + treated group compared with Oxa group (one-way ANOVA). * & +; $p < 0.05$, ** & ++; $p < 0.01$.

Figures

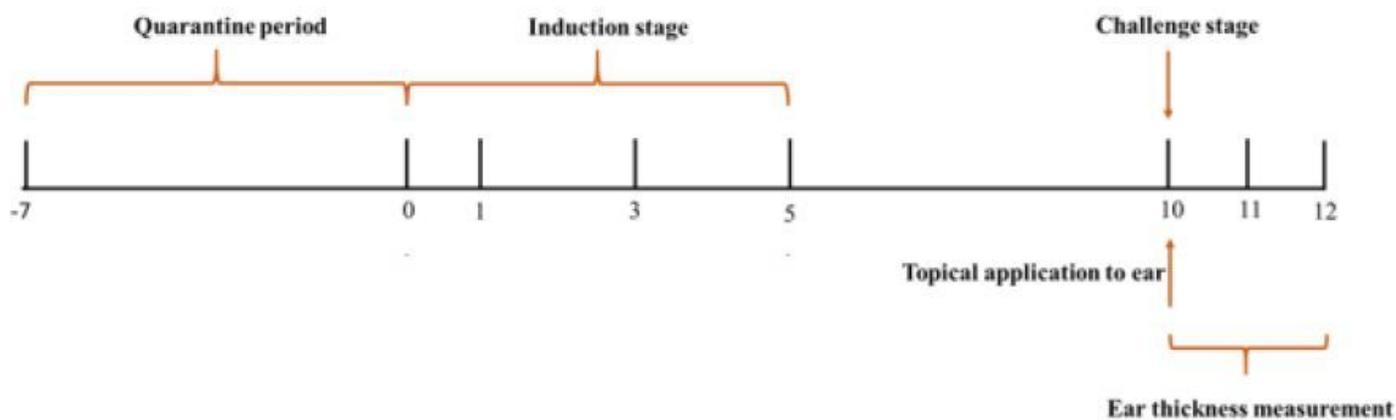


Figure 1

The line chart of the MEST protocol for allergenicity assessment.

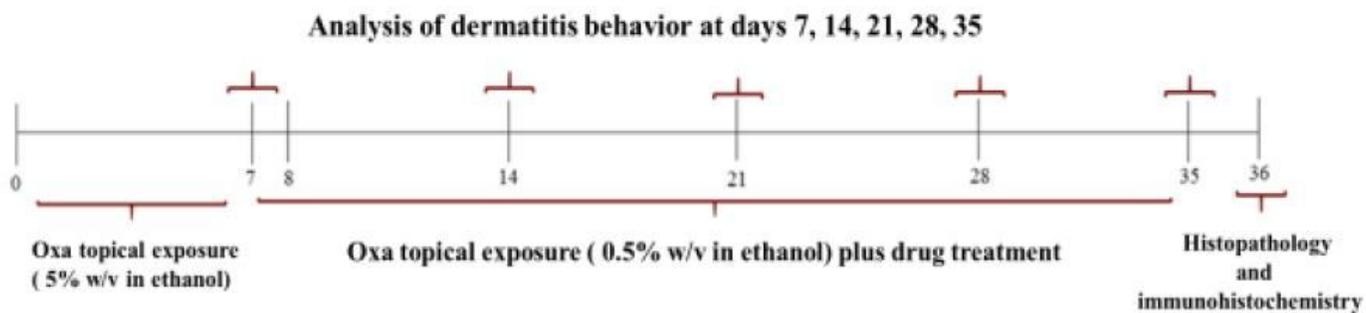


Figure 2

Summary of experimental dermatitis protocol.

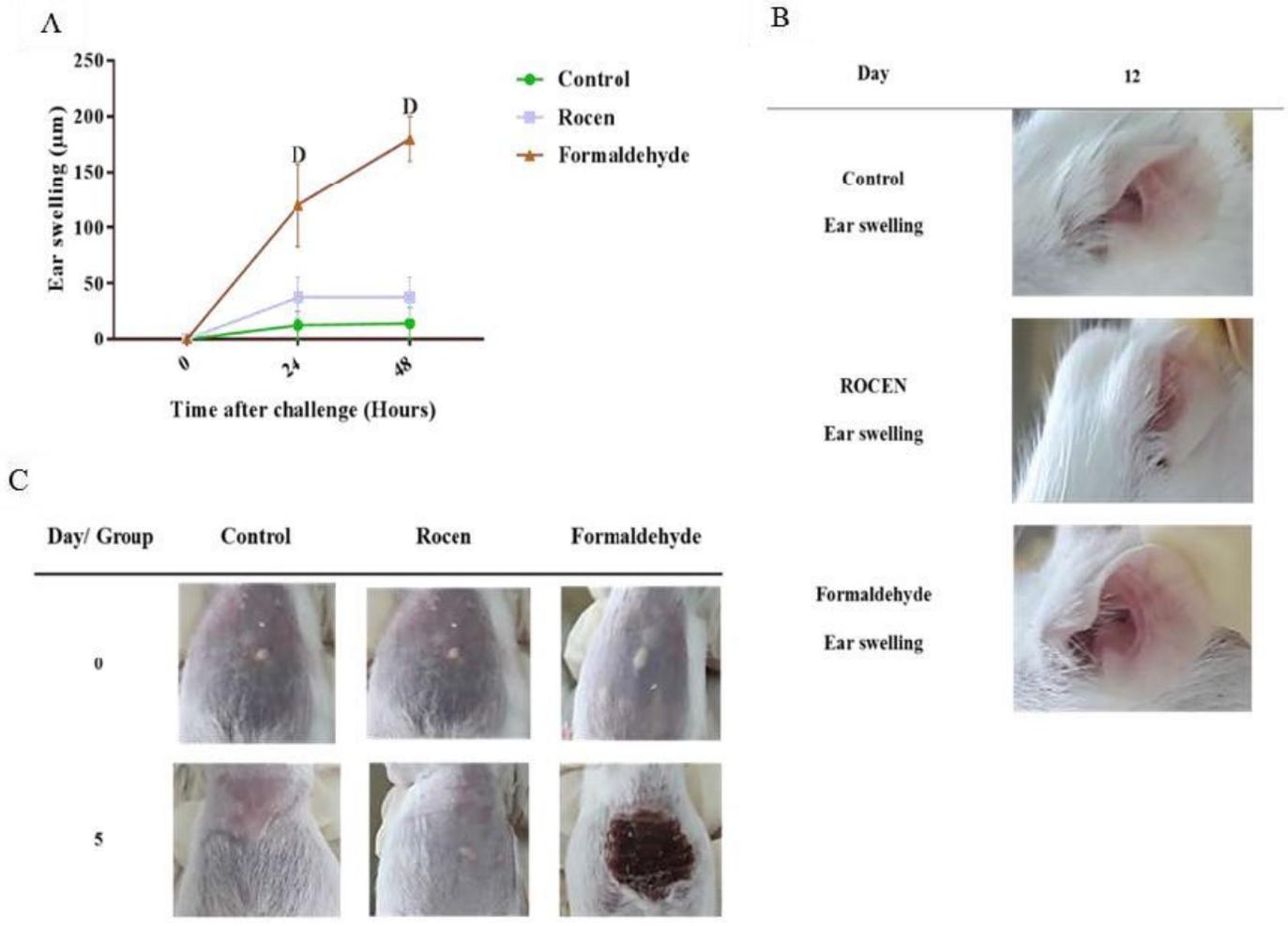


Figure 3

The demonstration of ROCEN sensitization evaluation using MEST. A. Swelling measurement of the left ear of the mice did not show any irritation via ROCEN application. n =10 for all groups. D when treated groups compared with control group, (D; P-value \leq 0.0001). B. The representative mice ear shows a considerable ear redness in formaldehyde group (sensitized mice). C. The mice were shaved ventral abdomen on day 0 for induction of sensitization by applying two intradermal injections (40 μ l) of adjuvant and 100 μ l of water (control), ROCEN, formaldehyde (as dermatitis lesion) were applied topically by rubbing gently.

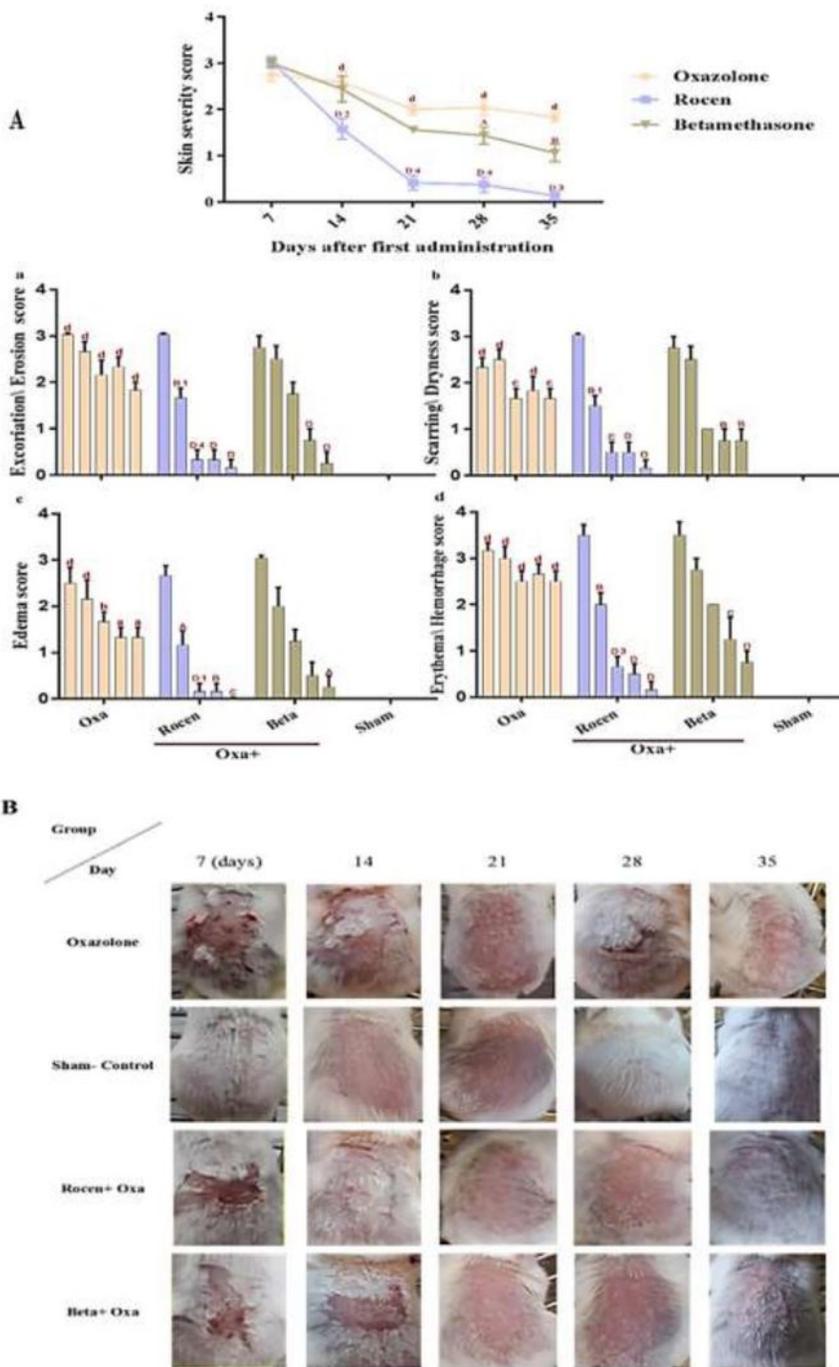


Figure 4

Indicative skin severity data of AD-like skin lesions treated by ROCEN in mice. A) skin severity scores indicate a significant dermatitis healing by topical ROCEN rather than Beta at relative time points. The severity score was achieved by the sum of the relative dermatitis data (a, b, c, and d scores) at each experiment and data represented by mean±SEM. B) Macroscopic features indicate that Oxa administration causes severe AD lesions at different time intervals while ROCEN and Beta treatments

recover the lesion progress in mice. a, b, c, d: when Oxa group is compared with control group. A, B, C, D: treated groups are compared with Oxa group. 1, 2, 3, 4: ROCEN group is compared with Beta group. Aa1; p < 0.05, Bb2; p < 0.01, Cc3; p < 0.001, Dd4; p < 0.0001.

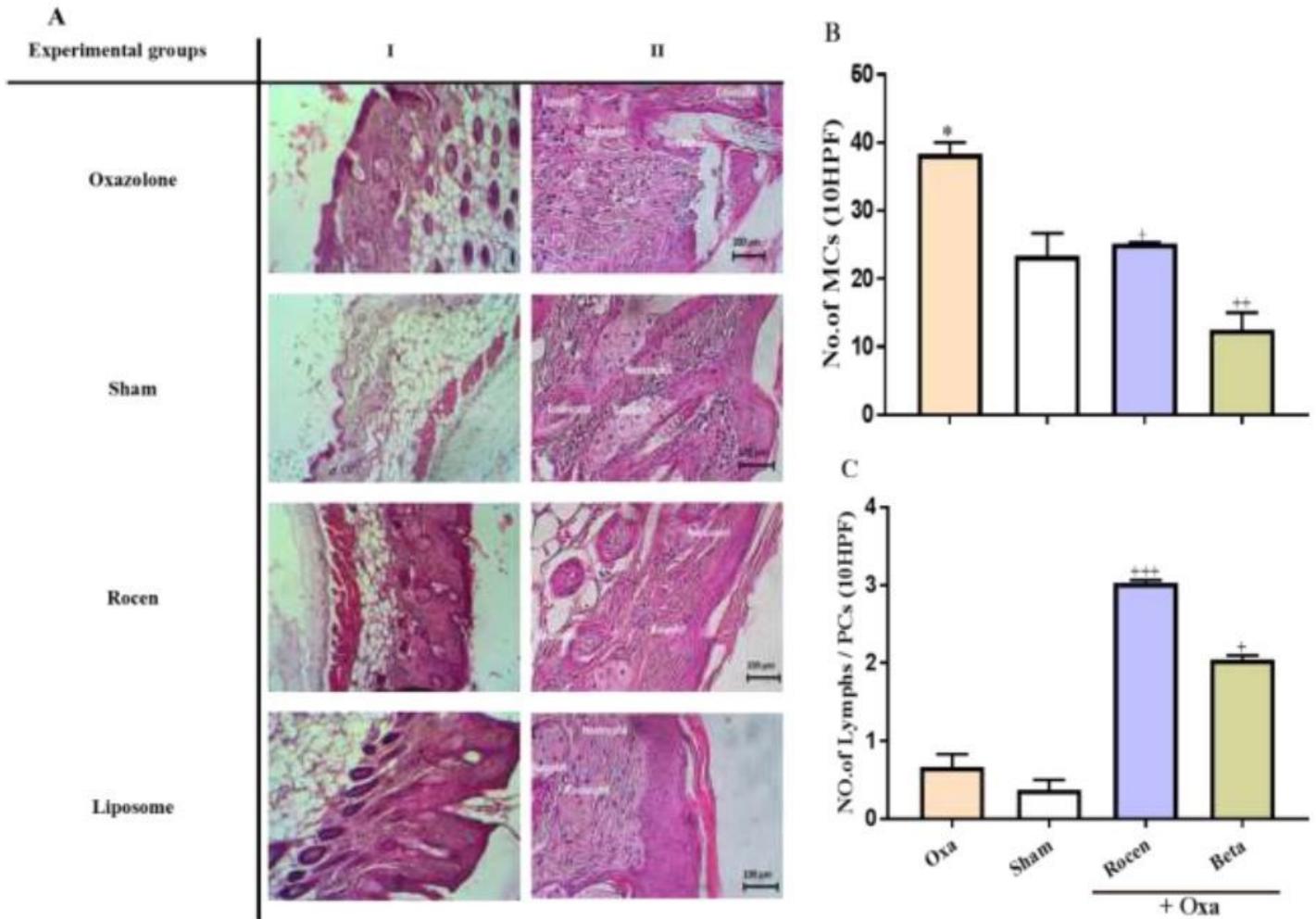


Figure 5

(A) I. Histopathological pictures of Oxa-induced chronic dermatitis and relative treated groups. II. The neutrophil, basophils, and eosinophil's cells were showed with a light microscope. B. The number of MCs and (C) Lymphs/ plasma cells were defined as the mean \pm SEM; n = 6 for all groups. * When Oxa compared with sham group, and + treated group compared to Oxa group. * & +; p < 0.05, ***, & +++; p < 0.001.

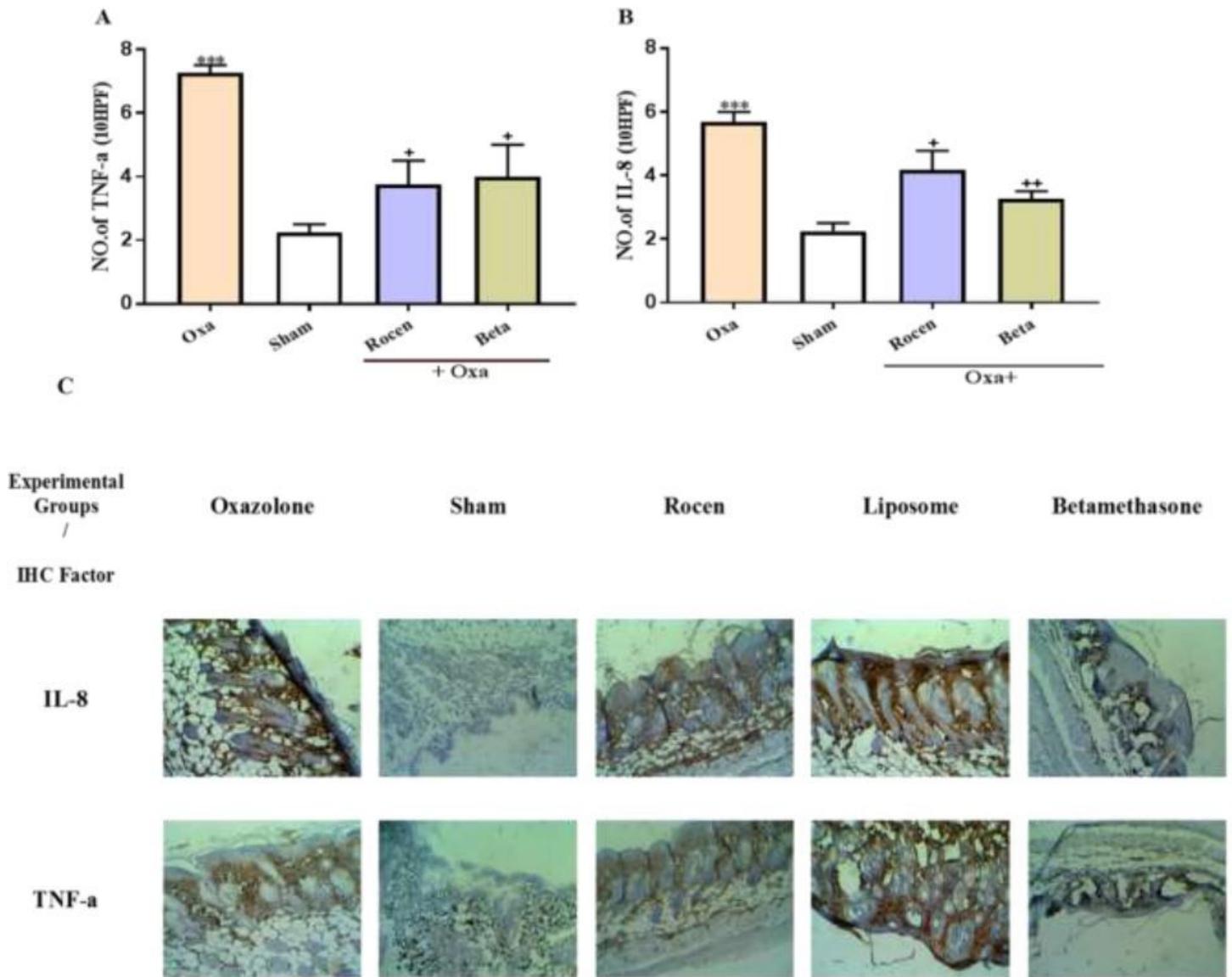


Figure 6

Data immunohistochemistry of cytokines related to topical treatment of AD. TNF-α (A) and IL-8 (B) analyzed by immunohistochemistry were defined as mean±SEM with n=6. C) Photomicrograph of IHC section of the dorsal skin showing IL-8 and TNF-α. * Oxa compared with control group, + treated group compared with Oxa group (one-way ANOVA). * & +; $p \leq 0.05$, ** & ++; $p \leq 0.01$.