


ORIGINAL ARTICLE

Experimental Models of Allergic Disease

Hypochlorous acid is antipruritic and anti-inflammatory in a mouse model of atopic dermatitis

T. Fukuyama¹ | B. C. Martel¹ | K. E. Linder² | S. Ehling¹ | J. R. Ganchingco¹ |
W. Bäumer^{1,3} 

¹Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA

²Department of Population Health and Pathology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA

³Department of Veterinary Medicine, Institute of Pharmacology and Toxicology, Freie Universität Berlin, Berlin, Germany

Correspondence

Wolfgang Bäumer, Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA.
Email: Wolfgang.baeumer@fu-berlin.de

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Summary

Background: It has been reported that topical hypochlorous acid (HOCl) formulations lead to relief of itch in human patients with atopic dermatitis; however, the specific antipruritic mechanism of action remains unclear.

Objective: To confirm itch relief and reduction of lesions in a mouse model of atopic dermatitis and to elucidate possible HOCl's mode of action.

Methods: In this study, the effects of topical administration of HOCl hydrogel (0.05%) on atopic dermatitis-like lesions in NC/Nga mice model as well as in vitro effects of HOCl on dorsal root ganglia neurons and mouse bone marrow-derived dendritic cells (mBMDCs) were investigated. NC/Nga mice were sensitized with house dust mite allergen and treated topically with HOCl hydrogel both preventively and therapeutically against established lesions. Allergen challenge was continued during HOCl hydrogel application.

Results: Treatment with HOCl hydrogel prevented the development of lesions and scratching bouts during the whole observation period. When administered after full development of lesions, HOCl reduced lesions and scratching behaviour to a similar extent as a positive control 0.1% betamethasone dipropionate ointment. The reduced inflammatory response by HOCl treatment was demonstrated by reduced secretion of inflammatory cytokines in affected skin tissue from NC/Nga mice. In addition, HOCl significantly reduced IL-12 production in mBMDC. The diminished scratching behaviour was confirmed by impaired response to several pruritogens in dorsal root ganglia neurons excised from NC/Nga mice after termination of the studies. The response to the stimuli was also reduced by pre-incubation of sensory neurons from untreated BALB/c mice with 0.0001% HOCl.

Conclusions and Clinical Relevance: These data indicate a direct reduction in sensory response by HOCl, leading to significantly reduced itch and inflammation in vivo.

KEYWORDS

atopic dermatitis, dorsal root ganglia, hypochlorous acid, IgE, IL-13, IL-4, NC/Nga mice, sensory neurons

1 | INTRODUCTION

Hypochlorous acid is reported to have anti-itch and anti-inflammatory potential in human patients suffering from atopic dermatitis (AD).¹ However, the possible mechanism of action has not been well characterized. Empirically, the antiseptic potential of hypochlorous acid can be the predominant mechanism¹ as colonization with *Staphylococcus aureus* (*S. aureus*) is an important trigger factor for AD.² However, it is likely that hypochlorous acid has effects beyond antiseptic properties, particularly as a rapid onset of itch reduction is reported.¹ Sodium hypochlorite, an alkaline aqueous solution of HOCl, has been described to interact with the pro-inflammatory transcription factor NF κ B,³ leading to in vivo efficacy in a mouse model of irradiation dermatitis. Thus, it could be hypothesized for hypochlorous acid that some modulating effects beyond antiseptic properties might be responsible for the clinical efficacy seen in patients with AD.

This study was performed to determine an anti-inflammatory and anti-itch mechanism of a 0.05% hypochlorous acid hydrogel in a chronic allergen-induced mouse model, which resembles several characteristics of atopic dermatitis.^{4–6} In addition to gain information about possible immune-modulating properties and direct effect on neurons, the effect of hypochlorous acid was tested in vitro on keratinocytes, dendritic cells, and dorsal root ganglia (DRG) neurons.

2 | METHODS

2.1 | Reagents

Hypochlorous acid (HOCl) gel (0.05% AFC, pH 6 ± 0.5) was manufactured and supplied by Realm Therapeutics, Inc (Malvern, PA, USA). The HOCl is formulated into an alcohol-free/steroid-free gel containing a proprietary formulation of a gelling agent and an emollient. House dust mite allergen (*Dermatophagoides farinae*) was purchased from GREER (Lenoir, NC, USA). Allyl isothiocyanate (AITC), capsaicin, concanavalin A, compound 48/80, poly-L-lysine, laminin, lipopolysaccharide (LPS; O127:B8 and O111:B4), peptidoglycan (PGN), mineral oil, and 2-mercaptoethanol were obtained from Sigma (St. Louis, MO, USA). Collagenase, Fura-2-acetoxymethyl ester (Fura-2AM), histamine, hydrogen peroxide, phosphate-buffered saline (PBS), and SLIGRL-NH₂ were ordered from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Dispace was ordered from STEMCELL Technologies Inc. (Cambridge, MA, USA). Dulbecco's modified eagle medium with L-glutamine (DMEM), fetal bovine serum (FBS), Ca²⁺ and Mg²⁺-free Hank's balanced salt solution (HBSS), penicillin-streptomycin, and RPMI-1640 medium were obtained from Mediatech Inc. (Manassas, VA, USA). MEM eagle (EMEM) medium was obtained from Lonza Group Ltd. (Allendale, NJ, USA). Purified anti-mouse CD16/CD32 (Mouse BD FC Block™), PE-conjugated anti-mouse CD40, and BD OptEIA mouse IgE ELISA set were ordered from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). FITC-conjugated anti-mouse CD3, APC-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD11c, FITC-conjugated anti-mouse CD19,

and APC-conjugated anti-mouse IgE were ordered from Miltenyi Biotec (Auburn, CA, USA). DCs protein assay kit was obtained from Bio-Rad (Richmond, CA, USA). Recombinant mouse GM-CSF, IL-1 β , IL-2, IL-31, and β -NGF were obtained from Pepro Tech, Inc. (Rocky Hill, NJ, USA). Antibodies for Western blot were obtained from Cell Signaling, Beverly, MA, USA (MAPK and phospho-MAPK family antibody sampler kit rabbit IgG, I κ B α , and Phospho-I κ B α (Ser32) rabbit IgG and housekeeping vinculin rabbit IgG). ELISA kits for IL-1 β , IL-4, IL-6, IL-12, IL-13, KC, TARC, TSLP and TNF- α were obtained from R&D systems (Minneapolis, MN, USA). ELISA for IL-31 was obtained from eBioscience, Inc. (San Diego, CA, USA). Histamine EIA kit was obtained from Beckman Coulter, Inc. (Brea, CA, USA). β -alanine, leukotriene B4 (LTB4), serotonin, and endothelin were ordered from Tocris Bioscience (Bristol, UK).

2.2 | Mice

Female BALB/c mice were purchased from Charles River Laboratories (Raleigh, NC, USA). Female NC/Nga mice were purchased from Charles River Japan Laboratories (Tokyo, Japan). All animals were purchased at 5–6 weeks of age. Animals were kept in a controlled environment (sentinel animals, IVC cages) under the following conditions: temperature (22°C), humidity (50%), and lighting cycle (12 h/d). The mice received certified pellet diet and water ad libitum. A study protocol was approved by the North Carolina State University Animal Care and Use Committee (IACUC Protocol No. 13-111-B).

2.3 | Model of atopic dermatitis in NC/Nga mice

For sensitization, 30 μ L of house dust mite allergen (HDM) in mineral oil (10 mg/mL) was topically applied to the clipped back and 10 μ L to each pinna twice weekly. Mild tape stripping with regular office tape was performed weekly just before the first HDM sensitization until visible lesions had developed. For the preventative setting, the administration of hypochlorous acid hydrogel ($n = 10$) or hydrogel vehicle ($n = 10$) began at the start of sensitization. HOCl or vehicle was topically applied to the back and pinna twice daily. For days overlapping with sensitization, HOCl or vehicle application was carried out after the HDM application. For the therapeutic setting, application of hypochlorous acid hydrogel ($n = 8$) or hydrogel vehicle ($n = 8$) was started at day 22, when the mice showed a mean lesional score of 2.3 (see Figure 2A). Additionally in the therapeutic setting, betamethasone dipropionate (0.1% in Lipoderm, $n = 8$) was applied daily (reduced to every other day on day 26 because of significant weight loss).

Body weight, scratching behaviour, ear and back skin thickness, and clinical scores were monitored weekly. The clinical score from 0 to 4 was assigned using the following system: no symptoms, 0; mild, 1; moderate, 2; severe, 3 and extreme, 4 as described in.⁷ In this study, the mean was built from the score for skin dryness, erosions, oedema, and erythema. To further validate the scoring system, we decided for the therapeutic condition (Figure 2) to have two investigators scored the mice individually. The scores were very similar,

and the average was used for the final score. Scratching behaviour was video monitored for 60-minute period immediately after HDM sensitization every week. During the video monitoring, the mice were observed in pairs at the same cage and both mice were treated at the same dosage. A scratching bout was defined as repeated strokes with the hindlimb in the area of HDM challenge. Tissue collection from each group ($n = 5$ each) in the preventative setting was made twice, weeks 8 and 12, after initiation of the procedure. Tissue collection from each group in the therapeutic setting was carried out on day 43.

The back skin, both pinna, auricular lymph node (LN), blood, and DRG were collected from each mouse 24 hours after last HDM challenge and 1 hour after last HOCl application. Samples were processed (or stored) for histology, cytokine determination, IgE determination, FACS analysis, and functional measurement of intracellular Ca^{2+} in DRG neurons.

2.4 | Histology

Samples from pinna and back skin were fixed in 4% paraformaldehyde solution, sectioned, and stained with haematoxylin-eosin. Blinded evaluation of pinna and skin was performed with a semi-quantitative examination of cell influx and oedema (0, no influx, no oedema; 1, mild; 2, moderate; and 3, severe influx, severe oedema). Microscopic measurement of dermal and epidermal thickness was evaluated by combining 50 measurements each on 5 sections per mouse.

2.5 | Cytokine determination of skin tissues

A portion of the pinna and back skin tissue were snap-frozen in liquid nitrogen. Cytokine determination for ear tissue was performed according to Fukuyama et al (2015).⁸ Briefly, samples were homogenized under liquid nitrogen, and the homogenates were taken in 200 μL RPMI 1640 medium containing 1 mmol/L Pefabloc. The supernatants were collected, and the protein content was determined with DC protein assay kit. IL-1 β , -4, -6, -13, -31, TARC, TSLP, and TNF- α were measured by ELISA.

2.6 | Total IgE measurements in serum

Blood collected from each mouse was centrifuged at 3000 g to collect serum. The total IgE level present in serum was measured by ELISA according to the manufacturer's protocol.

2.7 | Analysis of auricular lymph node

Single-cell suspensions were prepared from the LNs removed from each mouse. Single-cell suspensions were then used to analyse cytokine production and for FACS analysis. To stimulate T-cell receptor signalling, we cultured single-cell suspensions with concanavalin A for 24 hours at 37°C in a 5% CO_2 atmosphere. The levels of cytokines (IL-6, TNF- α and TARC) in cell culture medium were measured

using an ELISA. For FACS analysis, 5×10^5 cells were incubated with 1 μg Mouse BD Fc Block for 5 minutes at 4°C, followed by incubation with the monoclonal antibodies for 30 minutes at 4°C in the dark. After washing, cells were analysed on a LSR II flow cytometer using FACSDiva software (BD Pharmingen, Becton Dickinson and Company, Franklin Lakes, NJ, USA). For each sample, 10 000 events were collected and analysed for expression of antigens.

2.8 | Isolation of DRG for cell culture

Isolation of the DRG neurons was performed according to Fukuyama et al (2017).⁹ Briefly, DRG along the whole vertebral column per mouse were excised. Isolated ganglia were enzymatically digested in dispase and collagenase. Single cells were resuspended in 50 μL DMEM medium, placed onto poly-L-lysine and laminin-coated coverslips, and further incubated in 1 mL medium at 37°C 5% CO_2 .

2.9 | Calcium imaging on DRG cell culture

All calcium imaging was performed within 24 hours in culture. Changes in intracellular Ca^{2+} in single cells were measured by digital microscopy connected to equipment for ratiometric recording of single cells as described previously.⁹ In brief, DRG cell culture was loaded with Fura-2AM and incubated for 30 minutes at 37°C 5% CO_2 . During imaging, the coverslip was continuously perfused with Locke solution at $37 \pm 2^\circ\text{C}$. Time measurement with ratiometric UV imaging at 340 and 380 nm excitation was acquired every 200 ms. DRG neurons were exposed to histamine (1 mmol/L), followed by IL-31 (100 nmol/L), β -NGF (100 nmol/L), capsaicin (1 $\mu\text{mol/L}$), and KCl (75 mmol/L), with at least 2 minutes between stimulations. To examine that HOCl can directly affect sensory neurons, the DRG of untreated age and sex-matched BALB/c mice were isolated, processed, and imaged as described above except that 5.72 $\mu\text{mol/300 } \mu\text{L}$ of hypochlorous acid (from a 0.0001% solution, pH 7.4) was applied to cell culture 1 minute prior to the addition of stimuli.

2.10 | Murine keratinocyte cell line culture

The murine keratinocyte cell line (Balb/MK2) was used in this study. Cells were cultured in EMEM medium, as previously described.¹⁰ Confluent cells were exposed to hypochlorous acid at 0.00075%, 0.0015%, or 0.003% in FBS-free medium containing Toll-like receptor ligands, LPS and PGN for 24 hours. After exposure, cytokine (CXCL1/KC and TSLP) levels in cell supernatant were determined by ELISA. Three independent experiments (with different passage and fresh test substance solutions) were performed.

2.11 | BMDC assay

Primary bone marrow-derived dendritic cell (BMDC) cultures were generated from the bone marrow of female BALB/cAnN mice cultured with GM-CSF, as previously described.¹¹ On day 8 in culture, BMDCs were exposed to 0.00075%, 0.0015%, or 0.003% HOCl in

medium containing LPS for 24 hours. It is clear that LPS is not the Toll-like agonist, which might mimic an *in vivo* situation in atopic skin, but we used LPS as it is a well-known (and characterized) stimulus for murine bone marrow-derived DCs.⁸ Cytotoxicity assay was performed to confirm the exposure of HOCl did not induce cell toxicity or death. After exposure, cytokine (IL-12 and TNF- α) levels in cell supernatant and CD11c⁺CD86⁺ surface antigen expression were determined by ELISA and FACS analysis, respectively. Three independent experiments (i.e. DCs from different animals) were performed.

2.12 | Western blot

Ten microgram/lane of total protein was loaded on Mini-Protean TGX precast gels (12%, Bio-Rad). Blotting was performed using the Trans-Blot Turbo system (7 min/25 V; Bio-Rad). The membranes were blocked with 5% milk in PBST buffer. Antibodies were used as 1:1000 dilution (except phospho-ERK1/2 as 1:2000) in 5% BSA in PBST buffer. Incubation time for primary antibodies was 2 and 1 hour for secondary antibodies (in 5% milk in PBST). Signals were developed by incubating with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA) and imaged with ChemiDoc TM (Bio-Rad).

2.13 | Statistical analysis

Data are presented as mean (\pm SD). Statistical significance of the difference was estimated at the 5% and 1% levels of probability. Student's *t* test was used to test the significance of differences between mean values of 2 groups. Comparisons for more than 3 groups were carried out using a one-way ANOVA followed by Dunnett's multiple comparison test. Comparisons of proportions were made using Fisher exact test. The data were analysed using Prism 4 (GraphPad Software, San Diego, CA, USA).

3 | RESULTS

3.1 | Hypochlorous acid prevents development of lesions and itch in NC/Nga mouse model of AD

Clinical symptoms of atopic-like dermatitis were scored according to the four major clinical symptoms of atopic dermatitis: skin dryness, excoriations, oedema, and erythema. The lesional score gradually increased in the vehicle group starting 24 days after first HDM application. **Preventative topical application of 0.05% hypochlorous acid resulted in significantly less skin lesions** compared to the vehicle group, which became statistically significant 42 days after initiation of treatment (Figure 1A). The reduction in lesions was confirmed by histology (Figure 1B-D). **Total IgE measured in serum was also significantly lower in the hypochlorous acid group** (Figure 1E). There was a steady increase in scratching bouts throughout in the vehicle group. **This increased scratching behaviour was significantly prevented by topical administration of hypochlorous acid** (Figure 1F).

There was no effect on body weight or other obvious adverse effects noticed by the topical administration of hypochlorous acid throughout the experimental period (data not shown).

After this encouraging pilot data, a new set of experiments was performed in a therapeutic setting.

3.2 | A therapeutic intervention with hypochlorous acid reduces lesions and itch

Mild tape stripping at the start of the sensitization accelerated the development of lesions compared to the sensitization in the first study. Mice were topically treated with hypochlorous acid or betamethasone dipropionate starting day 22, when all mice developed at least moderate lesions from HDM sensitization. There was a steady decrease in lesion severity in both treatment groups, and the mice were killed at day 43 for further analysis of inflammatory endpoints (Figure 2A,B). Interestingly, both treatment options led to decrease in back skin thickness as an indication of reduced lichenification (Figure 2C). The reduced back skin thickness was confirmed by histological evaluation (Figure 2D-F). Total IgE in serum and histamine concentration in skin was also significantly reduced by hypochlorous acid and betamethasone dipropionate (Figure 2G,H). Although the inflammatory response in mouse ears was only obvious after 3-4 weeks of HDM application, again, the swelling was significantly reduced in the hypochlorous acid- and betamethasone dipropionate-treated ears. This reduction in inflammatory response was confirmed by histological evaluation (reduced inflammatory cell influx and thickness of epidermis as well as dermis, Figure 3).

To further characterize the allergic inflammation in the skin, the levels of tissue cytokines in the back and ear skin were determined. Apart from pleiotropic pro-inflammatory cytokines like IL-1 β , IL-6, and TNF- α , the level of typical Th2 cytokines like IL-4, IL-13, and TARC was significantly reduced by treatment with hypochlorous acid or betamethasone dipropionate. In addition, the cytokines TSLP and IL-31, which have been described to be direct pruritogens, were also significantly reduced (Table 1, Table S1). The reduction in the inflammatory response was confirmed in the draining lymph nodes of the treated ears. There was a decreased amount of T helper cells, IgE-positive B cells, and activated dendritic cells in the draining LN of hypochlorous acid- and betamethasone dipropionate-treated mice, respectively. Re-stimulated LN cells showed a decreased secretion of IL-6, TNF- α and TARC when obtained from mice, which had been treated with hypochlorous acid and betamethasone dipropionate, respectively (Table S2). To test whether hypochlorous acid might have a direct effect on keratinocytes or dendritic cells, we pre-incubated keratinocytes and dendritic cells with different concentrations of hypochlorous acid. We detected a slight dose-dependent decrease in KC and TSLP secretion in keratinocytes cocultured with Toll-like receptor agonists (Table S3). We also observed a slight decrease in TNF- α secretion and CD11c⁺CD86⁺ expression of dendritic cells exposed to LPS. Strikingly, the IL-12 response was reduced by roughly 70% with the highest hypochlorous concentration tested (which did not affect cell viability, Table S4). To elucidate possible

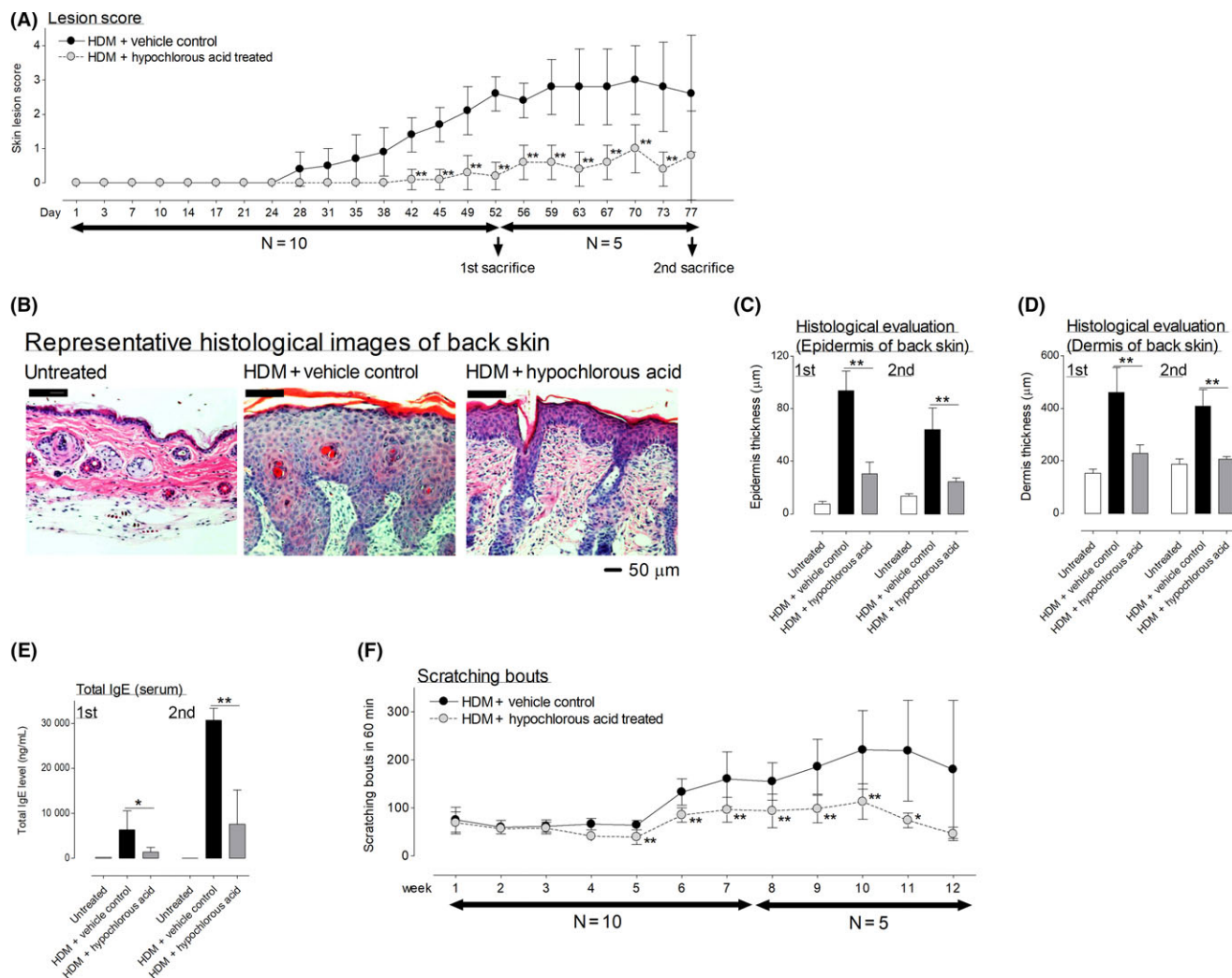


FIGURE 1 Topical administration of hypochlorous acid inhibits lesion formation and scratching behaviour in NC/Nga mice. A, Vehicle (hydrogel)-treated and house dust mite-challenged mice developed visible lesions starting at day 28, with plateau on day 52. Hypochlorous acid-treated mice developed only mild lesions throughout the challenge; B, representative histological images of back skin at 77 days after initiation of treatment; C, thickness of epidermis of treated back skin; D, thickness of dermis of treated back skin; E, total IgE levels were significantly lower in hypochlorous acid-treated mice at days 52 and 77 after initiation of treatment; F, hypochlorous acid-treated mice also show significant reduced itch behaviour, $n = 5$ -10 each group, $*P < .05$, $**P < .01$ compared to vehicle-treated mice.

pathways affected by hypochlorous acid, we measured phosphorylation of ERK, JNK, and p38 and I κ B in dendritic cells following either a 5- or 15-minute exposure to LPS. Interestingly, hypochlorous acid reduced the phosphorylation of all pathways, indicating a broad anti-inflammatory mechanism (Figure 4).

As topical hypochlorous acid hydrogel application had a significant effect on itch behaviour, we assessed whether this treatment also had an effect on the peripheral sensory neurons found in the skin. Thus, DRG, which contain the neuronal cell bodies of peripheral nerves, were obtained from mice treated with hypochlorous acid or betamethasone dipropionate to determine the *in vitro* response of those sensory neurons to histaminergic and non-histaminergic stimuli (IL-31, chloroquine, serotonin). These results were compared to DRG obtained from vehicle-only house dust mite sensitized, and age-matched untreated NC/Nga mice (Figure 5). All stimuli led to

significant less sensory nerve activation in hypochlorous acid- and betamethasone dipropionate-treated mice compared to vehicle-treated mice. Interestingly, the response to capsaicin (TRPV1 channel activator) and AITC (TRPA1 channel activator) was also reduced, which may indicate a role in reducing peripheral sensitization.

3.3 | A low concentration of hypochlorous acid reduces neuronal activation *in vitro*

Hypochlorous acid was directly applied to DRG cell culture to further elucidate whether the sensory response can be directly affected by hypochlorous acid. As previously published, hypochlorous acid can mediate the increase in intracellular calcium concentrations by inhibiting Ca²⁺-ATPase as well as activation of Ca²⁺ release channels¹² and we actually observed exposure of neurons

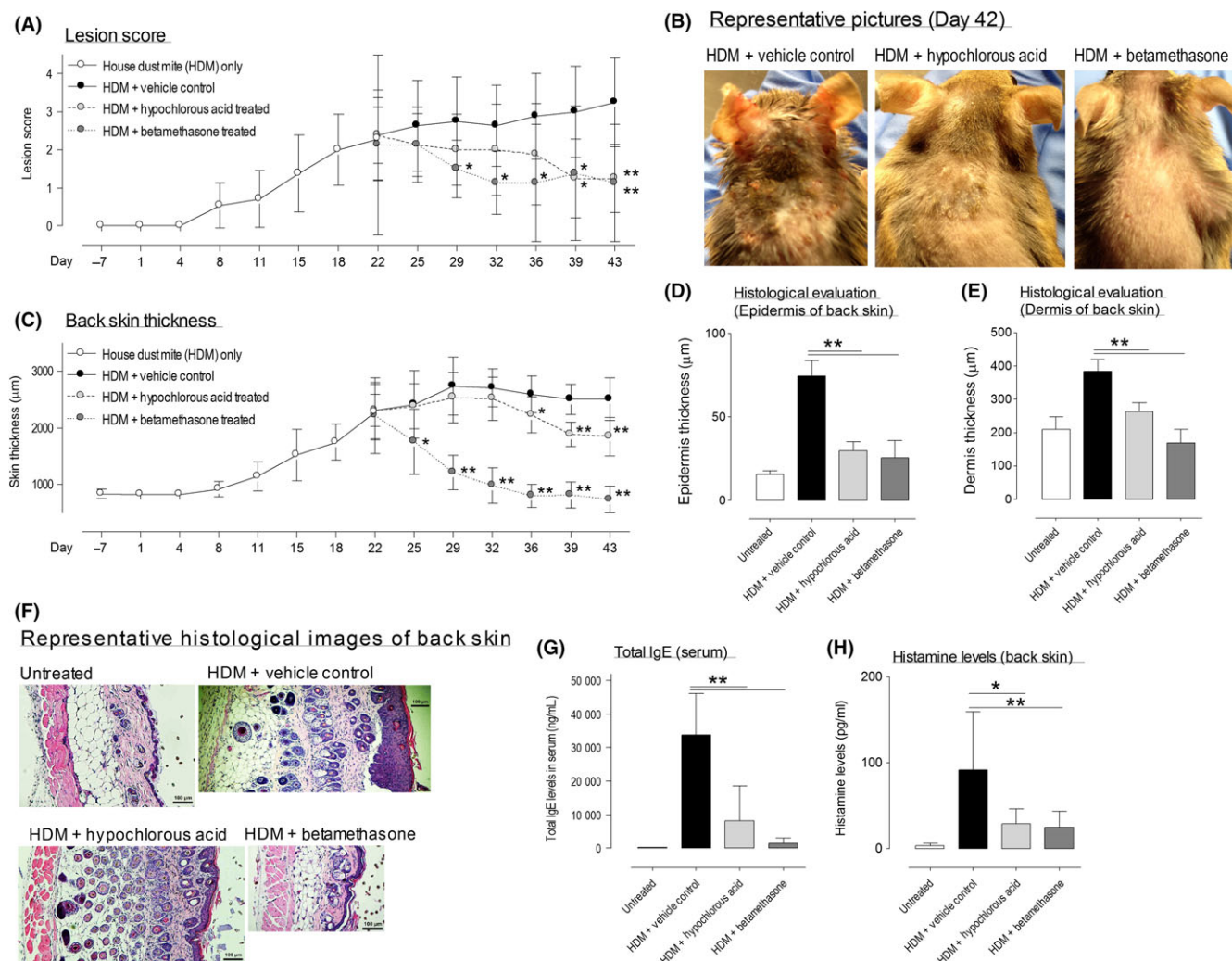


FIGURE 2 Therapeutic intervention with hypochlorous acid and betamethasone dipropionate, respectively, significantly reduced lesion formation and scratching behaviour in NC/Nga mice. A, Mice developed moderate lesions 22 days after HDM challenge. A therapeutic intervention with hypochlorous acid or betamethasone dipropionate significantly reduced the lesion score and hyperplasia at study day 43; B, representative pictures of skin lesions; C, back skin thickness was also significantly reduced by treatment with hypochlorous acid or betamethasone dipropionate. However, the effect was much more pronounced in betamethasone dipropionate-treated mice; D-F, This was confirmed by significantly reduced epidermal and dermal thickness; G, Total IgE in serum and H, histamine concentrations in skin were significantly reduced at study day 43 by hypochlorous acid or betamethasone dipropionate, $n = 8$ each group, $*P < .05$, $**P < .01$ compared to vehicle-treated mice.

to hypochlorous acid will activate neurons at a concentration range from 0.03% down to 0.00015%. However, at a concentration of 0.0001%, no significant increase in intracellular calcium was detected in DRG cell culture. This concentration was used to test several possible neuronal activators. Surprisingly, the response to nearly all stimuli (except for β -NGF and LTB_4) was significantly reduced by a 1 minute pre-incubation with hypochlorous acid. Also a significantly reduced response to AITC (but not to capsaicin) was observed (Figure 6). The stimuli used span a wide array of sensory perception pathways, like histaminergic (histamine, H4R agonist ST 1006) and non-histaminergic via cytokines (IL-2, IL-31), via Mas-related G protein-coupled receptor (MRGPR, CP 48/80), via protease-activated receptor 2 (SLIGRL-NH2), serotonergic receptors, and endothelin receptor.

4 | DISCUSSION

Atopic dermatitis (AD) is a chronic allergic skin disease characterized by inflammatory lesions and chronic itch.^{2,13} Current topical treatment options for AD include calcineurin inhibitors and glucocorticoids, but both treatment options have adverse effects.¹⁴ Hypochlorous acid and sodium hypochlorite solution ("bleach") both have been used clinically in AD patients, and although efficacy has been demonstrated for both,^{1,15} conclusive mechanisms have not been elucidated for atopic dermatitis. Sodium hypochlorite solution and the hypochlorous acid hydrogel used in this study are distinctly different. Available free chlorine can exist in the form of molecular chlorine (Cl_2), hypochlorous acid (HOCl), or a hypochlorite ion (OCl^-) depending on the pH. The available free chlorine present in bleach is mostly in the form of

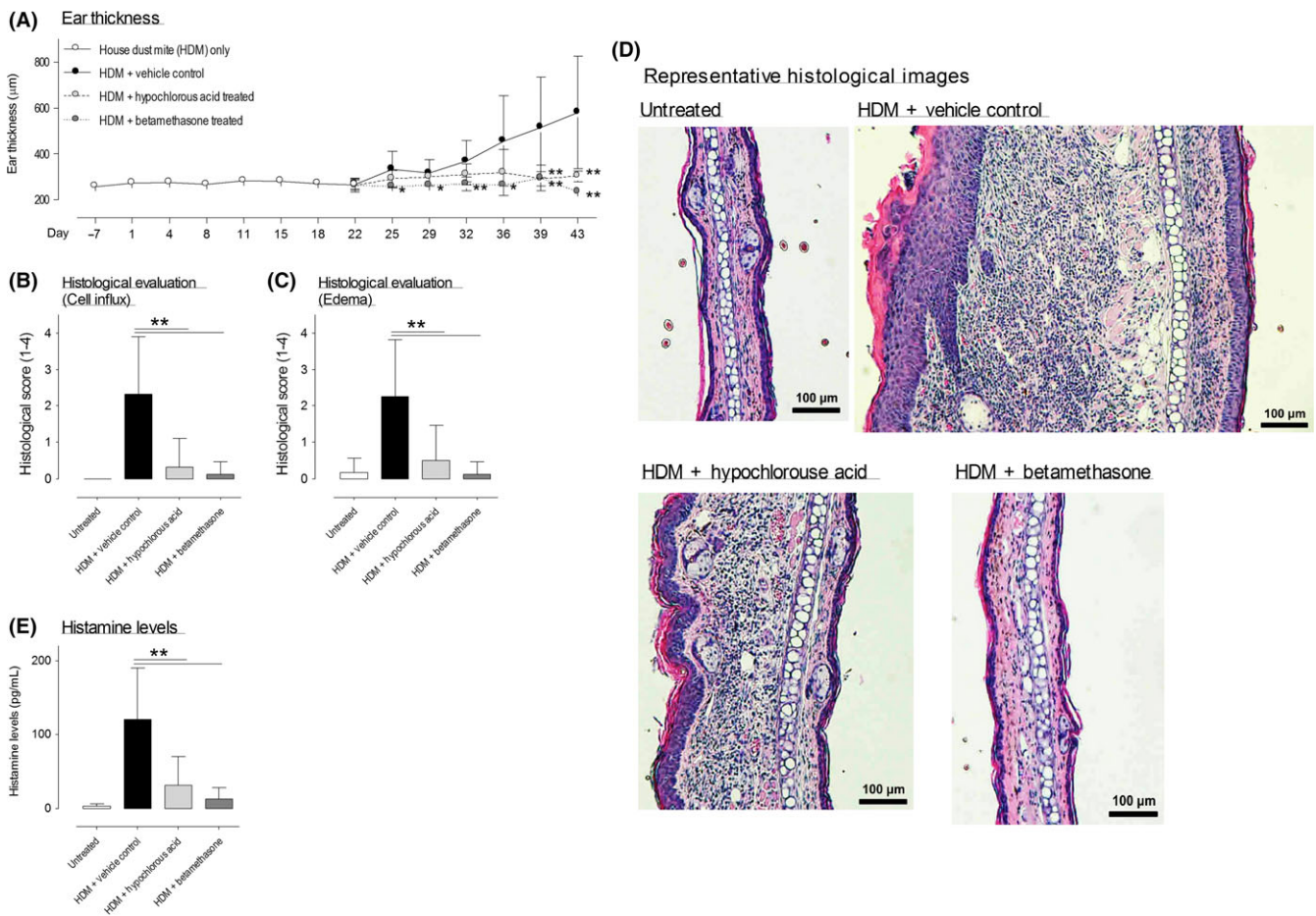


FIGURE 3 Hypochlorous acid and betamethasone dipropionate decrease HDM-induced ear inflammation in NC/Nga mice. A, HDM-induced inflammation is delayed in ear compared to back skin (Figure 2). Day 25, after initiated HDM application increased ear swelling was measured. A therapeutic intervention with hypochlorous acid and betamethasone dipropionate, respectively (started at day 22), decreased the inflammatory ear response; B-D, Histological evaluation revealed massive inflammatory cell influx and epidermal hyperproliferation. Both were significantly reduced by hypochlorous acid and prevented by betamethasone dipropionate; E, Histamine concentration in ear skin was also significantly reduced by hypochlorous acid or betamethasone dipropionate, $n = 8$ each group, $*P < .05$, $**P < .01$ compared to vehicle-treated mice.

TABLE 1 Cytokine concentration in back skin after topical application of hypochlorous acid in a therapeutic setting in NC/Nga mice. Back skin tissue was collected 24 h after last HDM challenge and 1 h after last HOCl application.

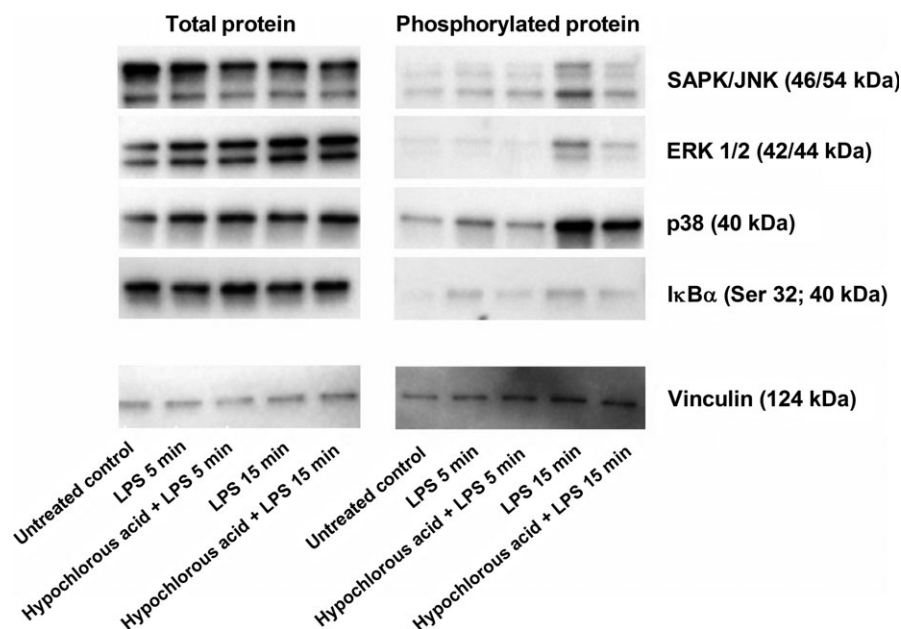
Group	Untreated	HDM + Vehicle control	HDM + Hypochlorous acid	HDM + Betamethasone
IL-1 β (pg/mg)	182 \pm 50	582 \pm 155	396 \pm 112*	279 \pm 87**
IL-4 (pg/mg)	57 \pm 27	445 \pm 154	166 \pm 62**	68 \pm 36**
IL-6 (pg/mg)	105 \pm 24	577 \pm 230	221 \pm 81**	110 \pm 41**
IL-13 (pg/mg)	46 \pm 38	187 \pm 58	101 \pm 53**	51 \pm 29**
TARC (pg/mg)	44 \pm 17	309 \pm 127	124 \pm 51**	31 \pm 12**
TNF- α (pg/mg)	204 \pm 87	970 \pm 398	612 \pm 219*	136 \pm 54**
TSLP (pg/mg)	45 \pm 15	418 \pm 183	136 \pm 55**	37 \pm 14**
IL-31 (pg/mg)	93 \pm 29	376 \pm 90	122 \pm 33**	94 \pm 39**

Results are expressed as mean \pm SD. (pg/mg protein; $n = 6-8$ per group). $*P < .05$ and $**P < .01$ (Dunnett's multiple comparison test) vs. HDM + vehicle control group.

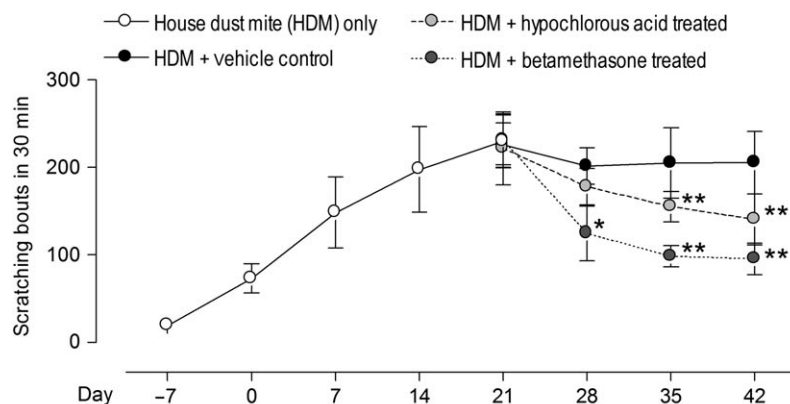
hypochlorite ion (OCl^-) in low concentrations (≤ 50 ppm, or 0.005%, based on standard dermatological recommendations) and at a pH ≥ 8.5 . The hydrogel treatment used for the present study is

formulated at a pH range 6 (± 0.5) where available free chlorine is present in the form of hypochlorous acid at a concentration of ca. 500 ppm (0.05%) with no safety concerns observed with the treated

FIGURE 4 Phosphorylation of SAPK/JNK, ERK1/2, and p38 and $\text{I}\kappa\text{B}\alpha$ in dendritic cells 5 and 15 minutes after stimulation with 1 μg LPS. Pre-incubation of dendritic cells with 0.003% hypochlorous acid for 1 hour reduced the phosphorylation of SAPK/JNK, ERK1/2, and p38 as well as $\text{I}\kappa\text{B}\alpha$ at one (5 or 15 minutes) or both time-points. Dendritic cell viability was not impaired by 0.003% hypochlorous acid incubation for up to 24 hours. Representative blot of three independent experiments.



(A) Scratching bouts



(B) Ca^{2+} influx

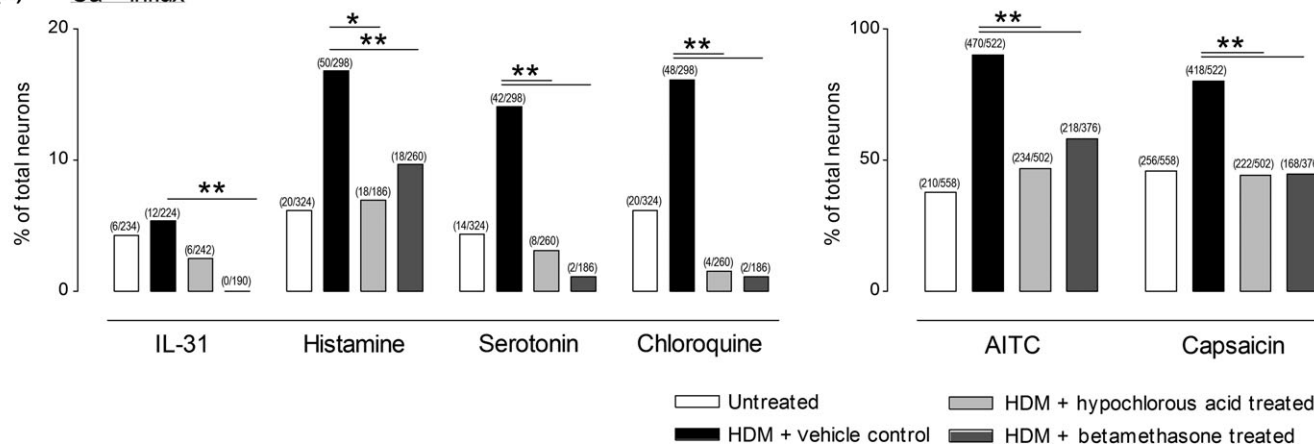


FIGURE 5 A therapeutic intervention with hypochlorous acid or betamethasone dipropionate significantly reduced scratching behaviour in NC/Nga mice and reverses peripheral sensitization. A, Scratching bouts steadily increase during repetitive challenge with house dust mite antigen within the first 21 days. Under therapeutic conditions, hypochlorous acid or betamethasone dipropionate significantly reduced scratching behaviour; B, The *in vitro* response of DRG neurons to IL-31 (1 $\mu\text{g}/\text{mL}$), histamine (1 mmol/L), chloroquine (10 $\mu\text{mol}/\text{L}$), serotonin (1 mmol/L), capsaicin (1 $\mu\text{mol}/\text{L}$), and AITC (100 $\mu\text{mol}/\text{L}$) were compared to DRG obtained from vehicle-treated, HDM-challenged mice and age-matched untreated NC/Nga mice. All stimuli led to significant less sensory nerve activation in dorsal root ganglia isolated from hypochlorous acid- or betamethasone dipropionate-treated mice compared to vehicle treated, $n = 8$ each group, $*P < .05$, $**P < .01$ compared to vehicle-treated mice.

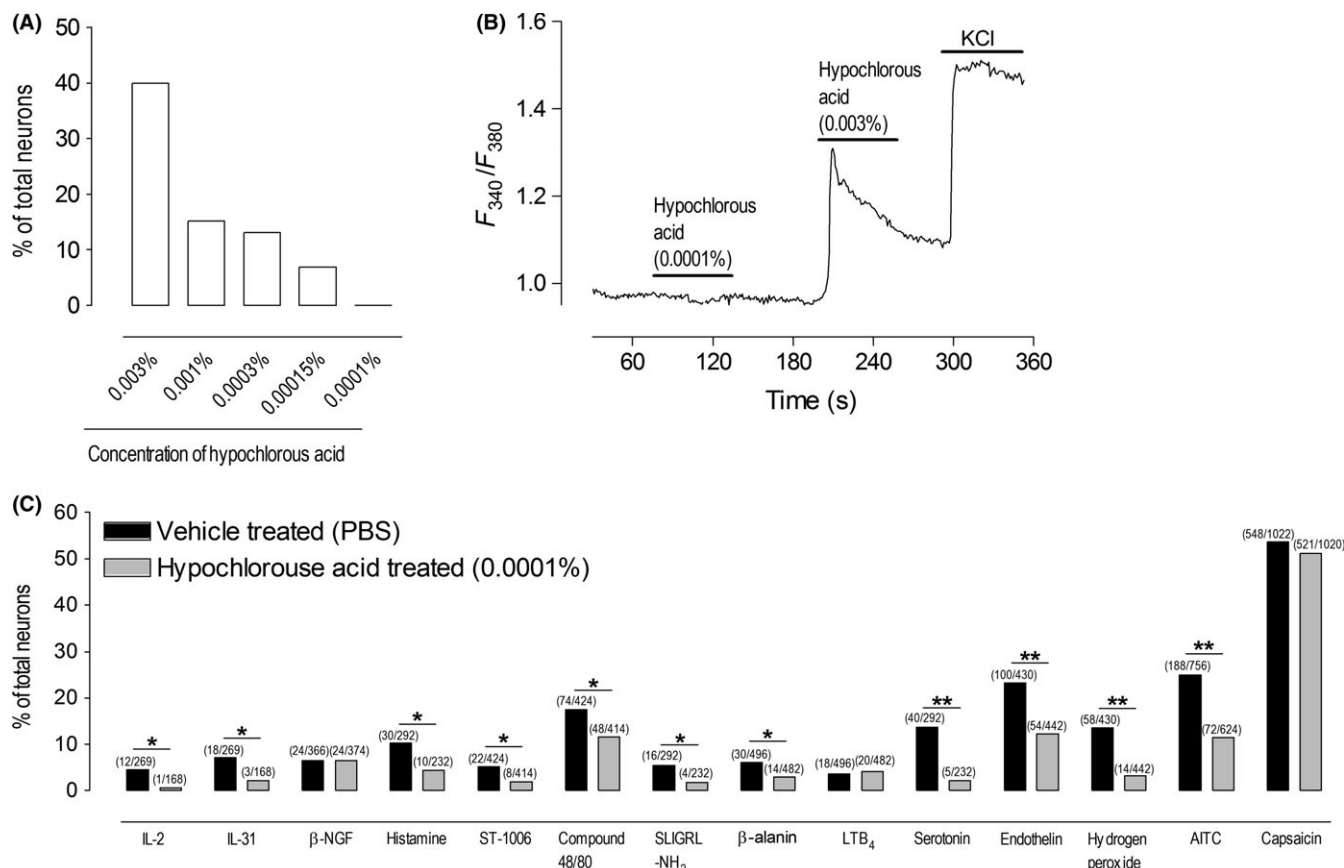


FIGURE 6 Hypochlorous acid directly interferes with neuronal activation in vitro. A and B, Concentrations of 0.00015% to 0.003% lead directly to sensory activation as determined by calcium influx. However, 0.0001% incubation did not induce a calcium signal; C, Neurons, pre-incubated with 0.0001% hypochlorous acid, respond significantly less to stimuli like IL-2 (1 μ g/mL), IL-31 (1 μ g/mL), histamine (1 mmol/L), ST 1006 (1 mmol/L), compound 48/80 (100 μ mol/L), SLIGRL-NH₂ (1 mmol/L), β -alanine (1 mmol/L), serotonin (1 mmol/L), endothelin (10 μ mol/L), hydrogen peroxide (0.03%), and AITC (10 μ mol/L). However, no change was seen in response to β -NGF (1 μ g/mL), LTB₄ (10 μ mol/L), and capsaicin (1 μ mol/L).

mice in this study. Thus, the reported effects of this study are specific for hypochlorous acid, not for hypochlorite.¹⁶ This high concentration of hypochlorous acid cannot be reached using bleach. Thus, the presented study reveals several new findings, but specifically the possible benefit of topical hypochlorous acid treatment in atopic dermatitis patients. We demonstrated that topical application of hypochlorous acid prevents the itch and inflammatory response in a translational mouse model of atopic dermatitis, it reduces the lesions comparable to a potent (WHO class 2) topical corticosteroid and significantly reduced the induced lichenification and itch in the therapeutic setting. In addition, we observed a reduced ex vivo response to various stimuli in dorsal root ganglia cells of mice treated with hypochlorous acid.

In vehicle-treated mice, we observed a constant increase in scratching behaviour within the first 3 weeks of sensitization with house dust mite antigen (Figure 2). It has been described that a steady increase in scratching behaviour is partly mediated by higher expression of receptors and higher responsiveness to pruritic stimuli in chronic dermatitis models.^{17,18} In the current study, we confirmed this in our chronic AD NC/Nga mouse model. Compared to age-matched non-sensitized NC/Nga mice, the DRG of house dust mite

antigen-challenged NC/Nga mice responded at a higher magnitude to IL-31 (IL-31 receptor A, oncostatin M receptor¹⁹), histamine (histamine H1 and H4 receptor²⁰), chloroquine (MRGPR A3/X1²¹), serotonin (5HT7²²), capsaicin (TRPV1 channel), and AITC (TRPA1 channel).²³ To our knowledge, this is the first report of enhanced excitability of DRG in this widely used mouse model of AD. Interestingly, this enhanced responsiveness to the various stimuli was blunted by the therapeutic administration of hypochlorous acid and betamethasone dipropionate which reflected the significantly reduced scratching behaviour observed in vivo (Figure 2). These results indicate a prevention or reduction in sensory neurotransmission. However, it is not clear whether this is a direct effect on neurons or secondary to the anti-inflammatory potential of the drugs. Furthermore, we determined direct effects on immune cells (dendritic cells) and keratinocytes; both play a pivotal role in the initiation and maintenance of atopic dermatitis.^{24,25}

For both cell types, a partly reduced response to innate stimuli was observed. Interestingly, the strongest impact of hypochlorous acid was seen in IL-12 production in dendritic cells and for this cell type, we could demonstrate a broad reduction in phosphorylated

MAPK and NF κ B activation. These results have implications in the chronic manifestation of AD, as IL-12 is a trigger for a Th1 shift which is observed in the chronic phase of AD.²⁶

However, the overall reduction of inflammatory response in keratinocytes and DCs was moderate; thus in the next step, we studied possible direct effects of hypochlorous acid on sensory nerve cell response to a broad array of stimuli determined by calcium response in these peripheral neurons. We stimulated directly the DRG as this is frequently used as a measure for peripheral sensory perception and its modulation.^{17,19-22} Unexpectedly, the response to nearly all stimuli was reduced by a low-concentration (0.0001%) hypochlorous acid solution. This concentration did not activate the neurons directly as determined in pilot experiments and we can exclude cytotoxic effects on neurons, as the response to LTB₄, β -NGF, and KCl was not altered by pre-incubation with 0.0001% hypochlorous acid solution. (Figure 6).

Although the exact mechanism of action is still not entirely clear, we can conclude that the broad reduction in MAP kinases as well as NF κ B pathway may lead to reduced secretion in cytokines relevant for the maintenance of atopic dermatitis (IL-4, IL-13, TARC²), and cytokines described to play a major role in the mediation of itch (IL-31, TSLP^{19,27}).

NC/Nga mice have been widely used for elucidating mechanisms and new therapeutic options of AD^{28,29} as lesions can be induced with a relevant allergen (house dust mite antigen) and the phenotype resembles several similarities with the human counterpart (lichenification, Th2 cytokines, enhanced IgE levels, constant itch). These data give some new mechanistic insights into the anti-itch properties of hypochlorous acid. However, the precise cellular target in neurons, keratinocytes, and dendritic cells remains to be discovered.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

ORCID

W. Bäumer  <http://orcid.org/0000-0002-4346-5911>

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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