

Topical nanocrystalline silver cream suppresses inflammatory cytokines and induces apoptosis of inflammatory cells in a murine model of allergic contact dermatitis

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Summary

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KCB and PJS are full time employees of NUCRYST Pharmaceuticals Inc.

Background Nanocrystalline silver has both antimicrobial and anti-inflammatory properties. However, the exact mechanisms underlying these activities are not known.

Objectives The objectives of this study were to assess the anti-inflammatory effects of nanocrystalline silver using a murine model of allergic contact dermatitis, compare the effects with those of tacrolimus and a high potency steroid, and to relate the effects to modulation of pro-inflammatory cytokines and apoptosis of inflammatory cells.

Methods Dermatitis was induced on the ears of BALB/c mice using dinitrofluorobenzene. Topical treatment, including vehicles, 1% nanocrystalline silver cream, tacrolimus ointment and a high potency steroid, was applied once a day for 4 days. Ear swelling was measured and the erythema was evaluated daily. After 4 days of treatment the mice were killed and samples from the ears were collected for histological and immunohistochemical examination, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) staining and extraction of total RNA for reverse transcriptase polymerase chain reaction (RT-PCR).

Results Significant reductions of ear swelling, erythema and histopathological inflammation in mice ears were observed after 4 days of treatment with 1% nanocrystalline silver cream, tacrolimus ointment or a high potency steroid with no significant difference among them. Both RT-PCR and immunohistochemical staining of sections from ear biopsies demonstrated that nanocrystalline silver, tacrolimus and steroid significantly suppressed the expression of tumour necrosis factor (TNF)- α and interleukin (IL)-12. TUNEL staining demonstrated a significant increase in the numbers of apoptotic cells in material from the group treated with nanocrystalline silver when compared with that from groups treated with vehicle, tacrolimus or steroid.

Conclusions This study demonstrates that nanocrystalline silver inhibits allergic contact dermatitis in mice, similar to steroid and tacrolimus. Nanocrystalline silver suppresses the expression of TNF- α and IL-12 and induces apoptosis of inflammatory cells; mechanisms by which nanocrystalline silver may exert its anti-inflammatory effects.

Allergic contact dermatitis (ACD) is regarded as a prototype of T-cell mediated delayed-type hypersensitivity reactions.¹ During the sensitization phase, epicutaneously applied hapten is taken up and processed by epidermal Langerhans cells, and then presented to naïve T cells in the draining lymph nodes.

The T cells then convert to hapten-specific T cells. In the elicitation phase, re-application of the same hapten results in the recruitment of hapten-specific T cells, which react to the hapten presented by Langerhans cells, release cytokines and chemokines and attract other inflammatory cells which play major

roles in the pathogenic process of ACD. Numerous studies have revealed that multiple cell types, chemical mediators, cytokines and chemokines are involved in the regulation of this process in ACD.^{2,3} Among these, the pro-inflammatory cytokines TNF- α and IL-12 have been reported to play major roles in the initiation and progression of these processes.^{2,4-6}

Animal models of ACD are reliable and reproducible models for clinical ACD. In addition, T cells are thought to play a major role in the pathogenesis of other inflammatory skin diseases including atopic dermatitis and psoriasis.

Although several therapeutic agents, including topical steroids and immunomodulators, are available to treat inflammatory skin diseases, they often lack durable efficacy and are associated with adverse effects.⁷⁻¹¹ Therefore, a safe and better treatment for inflammatory skin diseases is needed.

Nanocrystalline silver has been demonstrated to have exceptional antimicrobial properties.¹² ActicoatTM dressings coated with nanocrystalline silver are successfully used in the treatment of burns and for wound healing.¹³⁻¹⁶ Our recent studies demonstrated that topical nanocrystalline silver in an emollient cream exhibited anti-inflammatory activity in a guinea pig model of ACD and the anti-inflammatory activity of topical nanocrystalline silver cream was as effective as that of topical steroids and immunomodulatory agents.¹⁷ However, the exact mechanisms underlying this activity are not known. The objective of this study was to assess the anti-inflammatory effects of nanocrystalline silver using a murine model of ACD, compare the effects to those of tacrolimus and a high potency steroid, and to study the role of nanocrystalline silver in the induction of apoptosis of inflammatory cells, and modulation of pro-inflammatory cytokine expression.

Materials and methods

Animals

Eight- to ten-week-old female BALB/c mice, weighing 16–18 g, were purchased from Charles River Laboratories (St. Constant, Canada). The animals were maintained under conventional conditions and supplied with rodent chow and water *ad libitum*. The animal study protocol was approved by our Institutional Animal Care and Use Committee.

Induction of allergic contact dermatitis

ACD was induced by the method described previously¹⁸ with minor modifications. Briefly, the back skin of each mouse was painted with 100 μ L of 0.5% of dinitrofluorobenzene (DNFB) (Sigma Chemical Co, St. Louis, MO, U.S.A.) in 4 : 1 acetone : olive oil. Five days later, the mice were challenged on both sides of the ears with 20 μ L of 0.2% of DNFB solution. The animals were rechallenged after 2 days to yield extensive disease. One day after the rechallenge the animals began to be treated with the test compounds. The extent of ear swelling and erythema were used as a measure of ACD. Erythema was scored on a scale of 0–4 (where 0 was absent

and 4 was severe pinkish red colour of the ear). Ear thickness was measured before challenge and then pre- and post-treatment with a digital micrometer.

Treatments

Groups of 15–30 animals were treated with a topical application of approximately 100 mg of the following test compounds, once a day for 4 days: high potency steroid (halobetasol propionate cream, 0.05%, Ultravate[®], Bristol-Myers Squibb Company, Princeton, NJ, U.S.A.), tacrolimus ointment (Protopic[®] ointment, 0.1%, Fujisawa Health Care Inc., Deerfield, IL, U.S.A.), and 1% nanocrystalline silver (NPI 32101) cream. Nanocrystalline silver was produced by physical vapour deposition using a process of magnetron sputtering and consisted of 96.1% silver with crystallite size <50 nm. The 1% nanocrystalline silver was incorporated into an emollient cream. The emollient cream vehicle and/or ointment vehicle for tacrolimus (kindly supplied by Fujisawa Health Care Inc.) were used as placebo. Results using cream and ointment vehicles were statistically identical. Therefore, for clarity, only the results for placebo cream are shown in most cases.

Histopathology and immunohistochemistry

Formalin fixed, paraffin-embedded sections of the mouse ears were used. Five-micrometre sections were stained with haematoxylin and eosin using standard methods and examined by a board certified veterinary pathologist unaware of the treatment of the samples being evaluated. Histological changes in each of three layers, epidermis, dermis and subcutis were graded on a scale of 0–4 as described previously.¹⁷

For immunohistochemistry, a Universal streptavidin/biotin detection system (Thermo Shandon, Pittsburgh, PA, U.S.A.) was used. After dewaxing, and hydration, sections were treated with protein blocking agent to reduce non-specific binding of antibodies. The tissues were then incubated with rabbit antimouse tumour necrosis factor (TNF)- α , or rat antimouse IL-12 antibodies (Biosource International, Inc, Camarillo, CA, U.S.A.) in 1% bovine serum albumin in phosphate buffered saline. Incubation with secondary antibodies, streptavidin/biotin, and chromogenic detection of peroxidase was performed according to the manufacturer's protocol. Sections were counterstained, dehydrated, mounted and examined. The intensity of the immunoperoxidase staining was graded as described previously.¹⁹ The expression of cytokines was graded as: 0, no staining in the section; 1, <25% of the section were positive; 2, 25–50% of the section; 3, 50–75% of the section; 4, >75% of the section with significant staining.

RNA extraction and reverse transcriptase polymerase chain reaction

The primers and probes for interleukin (IL)-12B were designed by the use of Primer Express Design

Software (Applied Biosystem Inc, Foster City, CA, U.S.A.). They were synthesized by Biosource International: mIL-12B forward primer TAGCAGTCCCCTGACTCTCG; reverse primer TCCAGTGTGACCTTCTCTGCA and probe FAM-CAGTGACATGTGGAATGGCGTCTCTGT. The primers and probes for mIL-12A and TNF- α and the m18S rRNA were purchased from Qiagen (Valencia, CA, U.S.A.) as ready to use in QuantiTect Gene Expression assays.

Total RNA from the ear of each mouse was extracted using RNeasy kit, as per the instruction of the manufacture (Qiagen Inc.); 150 ng of total RNA was used per well. Each sample was run in duplicate. The RNAs were tested for the level of expression of the genes of interest in monoplex and multiplex reactions. The one-step reverse transcriptase-polymerase chain reaction (RT-PCR) runs were carried out according to the protocol provided by the enzyme master-mix supplier (Qiagen). All reactions were tested with the Applied Biosystems Prism 7700 Sequence Detector. The thermal cycler conditions for these experiments were: reverse transcription for 30 min at 50 °C, initial activation step for 15 min at 95 °C, followed by three-step cycling repeated 40 times; denaturation for 15 s at 94 °C, annealing for 30 s at 56 °C and extension for 30 s at 76 °C.

Upon completion of the RT-PCR runs, the fluorescence data were analysed with SDS 1.7 software (Applied Biosystems). The corresponding Ct (Threshold cycle) values of each well were collected. The calculation of the gene expression was done according to the TaqMan protocol (Applied Biosystems Inc, TaqMan cytokine gene expression Plate 1 protocol).

Measurement of apoptosis using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labelled staining

Paraffin-embedded sections of mouse ear ($n = 15$) were stained using the Mebstat apoptosis kit (MBL LTD, Watertown, MA, U.S.A.) to visualize apoptotic cells with terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labelling (TUNEL). This kit uses an apoptosis detection technique based on the TUNEL method. The main advantage of this rapid and simple procedure is the use of fluorescein-dUTP to label DNA strand breaks, which allows the direct detection of DNA fragmentation. In brief, the slides were deparaffinized, and hydrated, then DNA nick end labelling was done using fluorescein isothiocyanate (FITC)-dUTP and terminal deoxynucleotidyl transferase (TdT). Then the slides were washed with phosphate buffer solution (PBS) and counter-stained with propidium iodide. Slides were mounted with 90% glycerin and 10% PBS. Five different microscopic fields at 400 $\times$ magnifications were examined under a fluorescence microscope and the apoptotic cells were counted.

Statistics

The statistical significance of the difference in mean ear erythema, thickness, histopathological changes, number of apoptotic cells and the expression of cytokines were determined

using Tukey-Kramer's multiple comparison tests following one-way analysis of variance. Data were expressed as mean $\pm$ SE. $P \leq 0.05$ was considered statistically significant.

Results

Effects of nanocrystalline silver on dinitrochlorobenzene induced allergic contact dermatitis in mice

Within 24 h of re-challenge with DNFB, all mice developed clinical signs of erythema and oedema. The topical treatments of 1% nanocrystalline silver significantly reduced ear thickness after 1 day and erythema after 3 days of treatment (Fig. 1A,B). Tacrolimus significantly reduced ear thickness after 2 days and erythema after 3 days of treatment. High potency steroid significantly reduced the ear thickness after 1 day and the erythema after 2 days of treatment. (Fig. 1A,B). After 4 days of treatment no significant differences among these three active treatments were found. Significant reduction of body weight after three days of treatment was observed in the group of animals treated with high potency steroid compared with the other treatment groups, so that by day 4 average loss in this group was about 13% of body weight and mice showed piloerection, lethargy and a clearly abnormal appearance.

The anti-inflammatory effect of nanocrystalline silver, tacrolimus and high potency steroid was further investigated

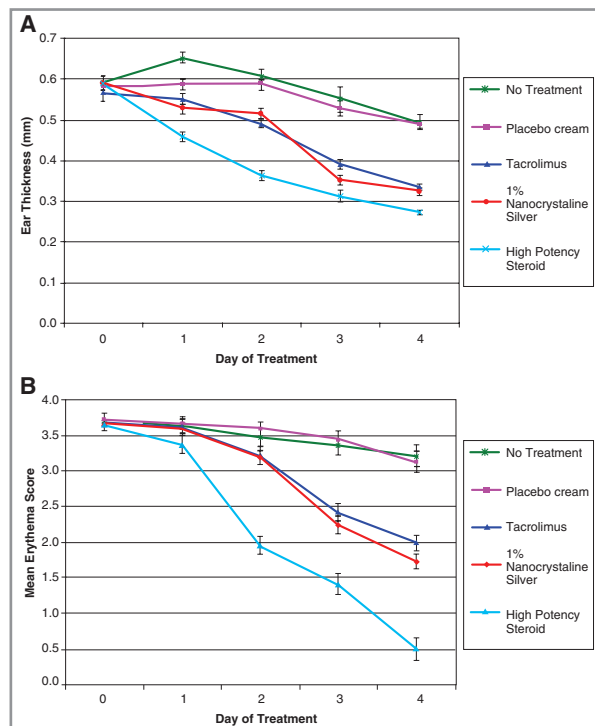


Fig 1. (A) Ear thickness scores as a function of treatment in mice with allergic contact dermatitis. (B) Erythema scores as a function of treatment in mice with allergic contact dermatitis. Values are mean $\pm$ SE, $n = 15$ –30.

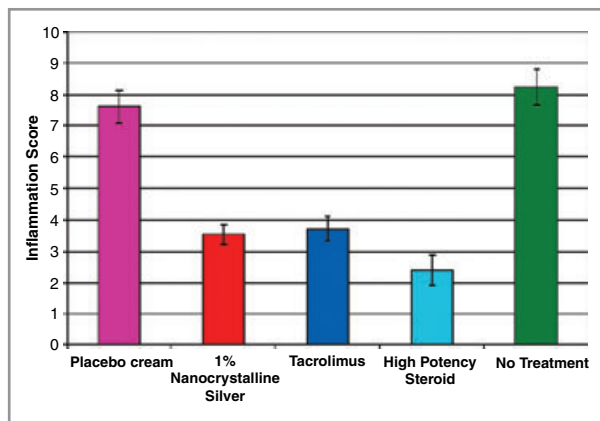


Fig 2. Histopathological inflammation scores of allergic contact dermatitis in mice after 4 days of treatment (Mean $\pm$ SE), $n = 15$.

histopathologically. The reductions in inflammation in the groups treated with nanocrystalline silver, tacrolimus and high potency steroid were significant compared with the placebo

and no-treatment groups (Fig. 2). No differences among the effective treatments were observed. After 4 days of treatment most of the sections from the active treatment groups showed normal epidermis and significantly reduced inflammatory cells, whereas severe oedema and marked inflammatory cell infiltration were seen in the sections from ears treated with vehicle or untreated (Fig. 3).

Effects of nanocrystalline silver on cytokines protein expression determined by immunohistochemistry

Both IL-12 and TNF- α were upregulated after re-challenge with DNFB. As seen in Figs 4 and 5, the positive staining for both IL-12 and TNF- α were markedly reduced in the groups treated with 1% nanocrystalline silver cream, tacrolimus and high potency steroid after 4 days of treatment. When the grading of the intensity of the staining for IL-12 and TNF- α , was analysed, nanocrystalline silver, tacrolimus and high potency steroid significantly reduced the protein expression of IL-12 and TNF- α (Fig. 6A,B) without any significant differences among them.

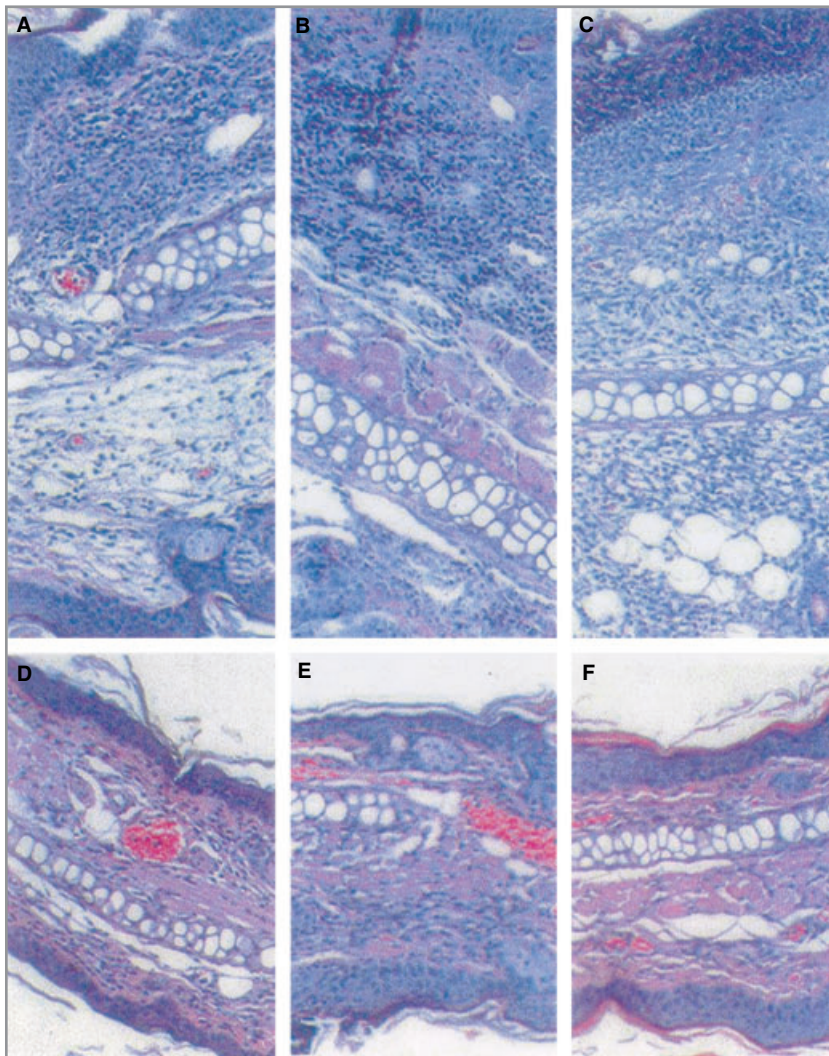


Fig 3. Nanocrystalline silver reduces inflammation and inflammatory infiltration in the allergic contact dermatitis in mice ears. Haematoxylin and eosin-stained sections of ears after 4 days of treatment. (A, B, C) Ears treated with placebo cream, placebo ointment and no treatment, respectively. (D, E, F) Ears treated with 1% nanocrystalline silver, tacrolimus and high potency steroid, respectively. The sections in A, B, C, illustrate epidermal ulceration, pronounced intracellular oedema, marked inflammatory infiltration. The sections in D, E, F, show normal epidermis, decreased oedema and no or few inflammatory cells.

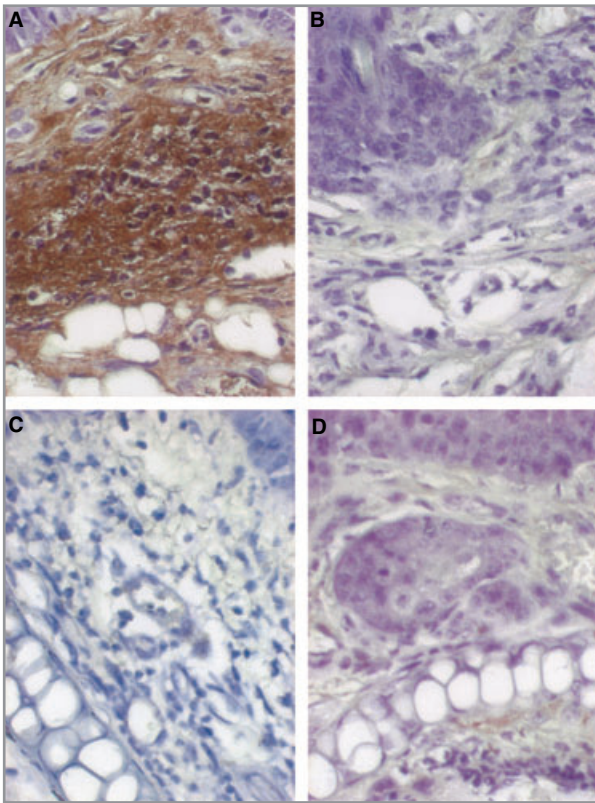


Fig 4. Nanocrystalline silver reduces protein expression of IL-12. Immunohistochemistry of representative sections of mice ears stained with antibodies to interleukin (IL)-12. Note the increased expression of IL-12 after 4 days treatment with placebo cream (A) and decreased expression of IL-12 after treatment with 1% nanocrystalline silver (B), tacrolimus (C), and high potency steroid (D).

Effects of nanocrystalline silver on cytokine mRNA expression determined by RT-PCR

To confirm the expression of protein level of TNF- α and IL-12, we examined the effect of nanocrystalline silver on the mRNA expression of the two proinflammatory cytokines by RT-PCR after hapten re-challenge in the DNFB-induced ACD in mice. Both TNF- α and IL-12B mRNA were up-regulated after 24 h of re-challenge (data not shown). After 4 days, both TNF- α , and IL-12B mRNA expression were significantly increased in the DNFB challenged mice with no treatment, while nanocrystalline silver, tacrolimus and high potency steroid treatment significantly decreased mRNA expression for these cytokines (Fig. 7A,B). Expression of IL-12A was not detected in any of the samples (data not shown).

Effects of nanocrystalline silver on induction of apoptosis

TUNEL staining of the ear sections from the different groups demonstrated TUNEL-positive apoptotic cells (Fig. 8) after 4 days of treatment. A significant increase in numbers of

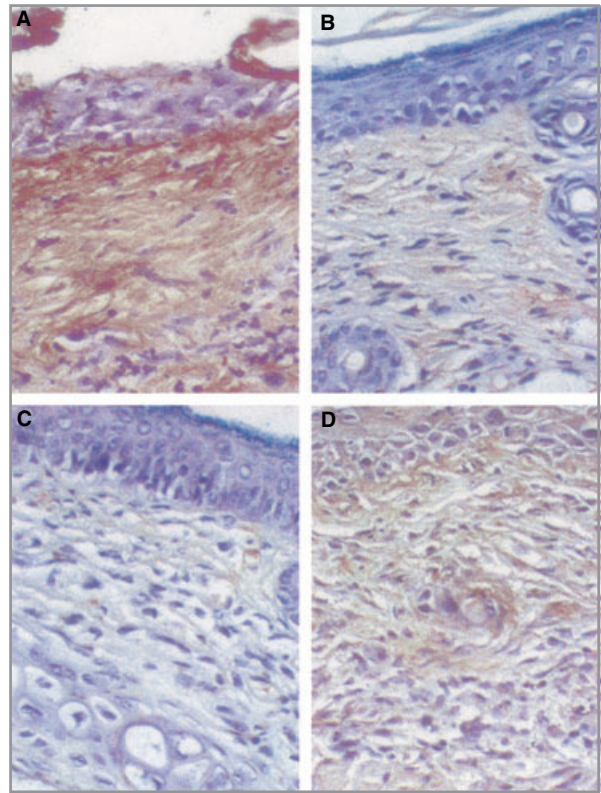


Fig 5. Nanocrystalline silver reduces protein expression of TNF- α . Immunohistochemistry of representative sections of mice ears stained with antibodies to tumour necrosis factor (TNF)- α . Note the increased expression of TNF- α after 4 days treatment with placebo cream (A) and decreased expression of TNF- α after treatment with 1% nanocrystalline silver (B), tacrolimus (C), and high potency steroid (D).

apoptotic cells was observed in the group treated with 1% nanocrystalline silver cream, compared with sections from other treatment groups (Fig. 9).

Discussion

The present study demonstrates that topically applied nanocrystalline silver inhibits ear swelling and erythema in DNFB-induced ACD in mice and significantly reduces the intracellular oedema and infiltration with inflammatory cells consisting of mononuclear cells and neutrophils. The anti-inflammatory activity of nanocrystalline silver was similar to that of tacrolimus and high potency steroid. In this model, high potency steroid demonstrated anti-inflammatory effects more rapidly than 1% nanocrystalline silver and tacrolimus. However, the animals treated with steroid showed a significant reduction of their body weight during treatment compared with the groups treated with 1% nanocrystalline silver, tacrolimus and placebo cream or ointment, indicating a clear toxic effect of steroid on mice at the doses used (100 mg on each ear per day for 4 days).

As an approach to investigate the mechanism of the inhibitory activity of nanocrystalline silver against ACD, we

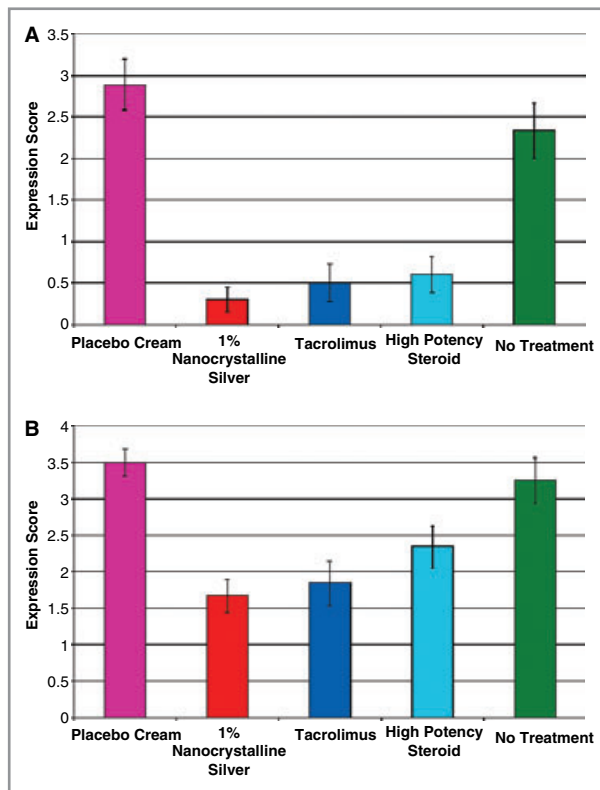


Fig 6. Nanocrystalline silver reduces expression of protein level of interleukin (IL)-12 (A) and tumour necrosis factor (TNF)- α (B). Semi-quantitative assessment of staining of IL-12 and TNF- α (Mean $\pm$ SE; $n = 15$).

examined the effect of topical nanocrystalline silver on the induction of apoptosis of inflammatory cells and the role of nanocrystalline silver on suppression of inflammatory cytokines by measuring the mRNA expression and protein expression of IL-12 and TNF- α . Test sites treated with nanocrystalline silver showed more extensive apoptosis of inflammatory cells than the test sites treated with placebo and other treatments. In addition, the expression of inflammatory cytokines (IL-12 and TNF- α) were significantly suppressed by nanocrystalline silver, tacrolimus and high potency steroid.

Several investigators have characterized the molecular events during the induction phase of contact hypersensitivity and demonstrated that inflammatory cytokines including TNF- α , IL-1, IFN- γ were up-regulated after elicitation with haptens.²⁰ TNF- α is a critical mediator of ACD in mice. It has been reported that TNF- α expression is increased after challenge with haptens and that administration of anti-TNF- α antibody abrogates ACD in mice.⁴ We speculate that the suppression of TNF- α may be one of the mechanisms underlying the inhibitory activity of anti-inflammatory compounds.

Previous reports indicate that IL-12, a heterodimeric cytokine which is composed of a constitutively expressed p35 (IL-12A) subunit and inducible p40 (IL-12B) subunit, plays

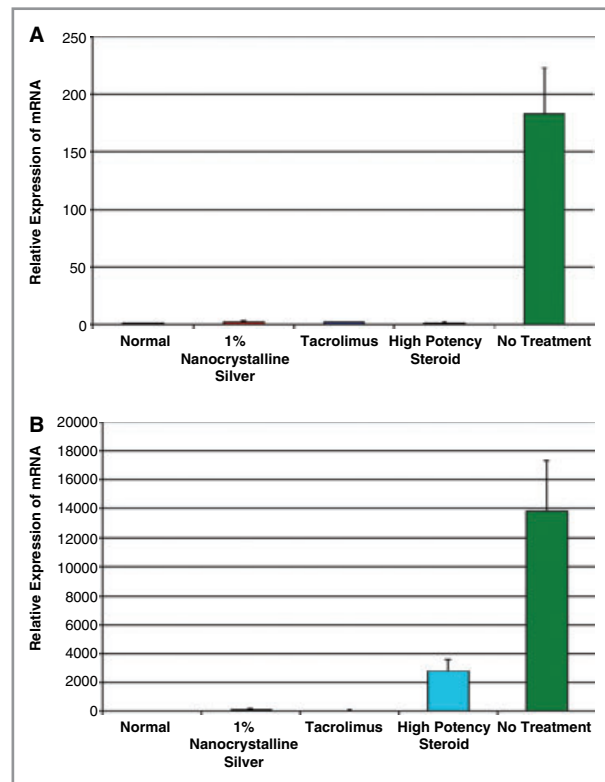


Fig 7. Nanocrystalline silver reduces mRNA expression of interleukin (IL)-12B (A) and tumour necrosis factor (TNF)- α (B) in allergic contact dermatitis in mice. Mean $\pm$ SE of the relative mRNA expression ($n = 5$).

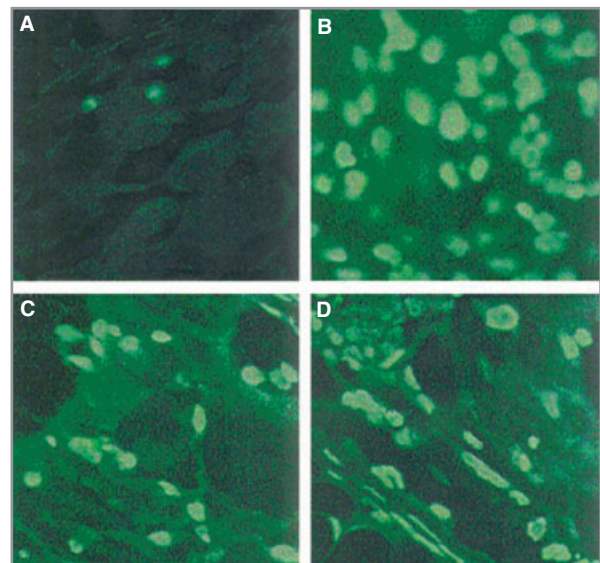


Fig 8. Nanocrystalline silver induces apoptosis of inflammatory cells in the ear sections of mice with allergic contact dermatitis. Paraffin-embedded sections of ear biopsies specimen were subjected to a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labelling (TUNEL) assay as outlined in the material methods to visualize apoptotic cells. A. normal mice, B, treated with 1% nanocrystalline silver cream; C, with tacrolimus and D with high potency steroid.

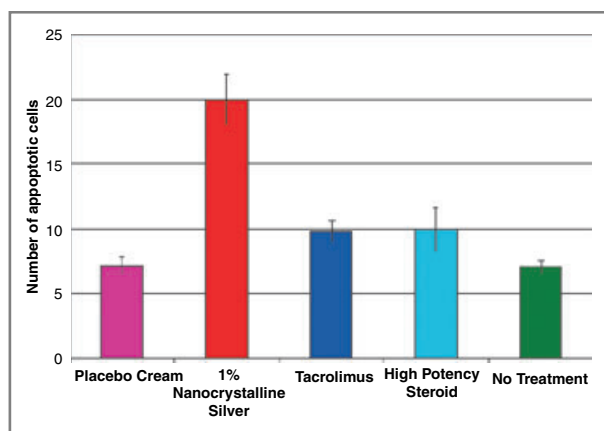


Fig 9. Histogram showing the mean $\pm$ SE of the number of apoptotic cells in the microscopic fields ($\times 400$) in the sections of mouse ear ($n = 15$).

a critical role in the pathogenesis of clinical allergic contact dermatitis^{5,21,22} atopic dermatitis^{23,24} and in psoriasis^{25,26} and in other non-dermatological inflammatory diseases in human and experimental animals.^{27–29} IL-12 substantially up-regulates the induction of sensitivity to the contact allergen DNFB in mice. Furthermore, neutralization of IL-12 by intraperitoneal injection of monoclonal antibody to recombinant mouse IL-12 prevents DNFB-induced ACD in mice.²¹ Down-regulation of IL-12 in ACD and atopic dermatitis by topical FK506 and topical corticosteroids has been reported.^{23,30} Several investigators have reported that IL-12B, but not IL-12A, is a critical element in the pathogenesis of inflammatory skin disease.^{23,25} Our RT-PCR results demonstrated that, in contrast to the IL-12A (p35) subunit, high expression of the IL-12B (p40) subunit only could be detected after hapten application. Our results confirm the up-regulation of TNF- α and IL-12B (p40 subunit) during the induction of contact hypersensitivity and interestingly, topical treatment of 1% nanocrystalline silver, tacrolimus and high potency steroid significantly suppressed their expression.

Programmed cell death, or apoptosis, occurs in various physiological and pathological conditions, exhibits a characteristic mechanism of intercellular sequential reaction, and may be involved in determining clinical outcome. Induction of apoptosis of T cells and inflammatory cells by glucocorticoids and other anti-inflammatory products has been reported.^{31–33} Apoptosis is thought to contribute to the elimination of inflammatory cells from the inflamed area. Hence, compounds which induce apoptosis may be beneficial in the treatment of inflammatory diseases. This study demonstrates that nanocrystalline silver significantly augments the induction of apoptosis of inflammatory cells. We did not observe any keratinocyte apoptosis in the nanocrystalline silver treatment groups, which suggests that nanocrystalline silver does not induce apoptosis of these cells. The induction of inflammatory cell apoptosis by nanocrystalline silver has been

shown previously in a porcine wound model,³⁴ and the present study confirms this activity.

In summary, the potential of nanocrystalline silver to modulate apoptosis of inflammatory cells and to inhibit the expression of two pro-inflammatory cytokines, IL-12 and TNF- α , may be responsible for the anti-inflammatory activity of nanocrystalline silver. On the basis of these results, nanocrystalline silver could be a potential therapeutic agent for treating inflammatory diseases of skin. The antimicrobial potency of nanocrystalline silver could serve as an added benefit in this regard.

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