

N-acetylcysteine inhibits chromium hypersensitivity in coadjuvant chromium-sensitized albino guinea pigs by suppressing the effects of reactive oxygen species

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Abstract

Background: Chromium hypersensitivity is an important issue in occupational skin disease. When hexavalent chromium enters the cell, it can be reduced to trivalent chromium, resulting in the formation of reactive oxygen species (ROS). ROS are considered to play an important role in the progression of allergic contact dermatitis. N-acetylcysteine (NAC) could increase glutathione levels in the skin and act as an antioxidant.

Aims: We attempted to demonstrate that NAC could inhibit chromium hypersensitivity in a coadjuvant chromium-sensitized albino guinea pig model by counteracting the formation of ROS.

Methods: We utilized a coadjuvant chromium-sensitized albino guinea pig model to evaluate both the severity of the skin reaction by intradermal and epicutaneous elicitation tests and the sensitization rate of chromium hypersensitivity in NAC-treated and NAC-untreated albino guinea pigs (GP). Furthermore, three

ROS parameters, including H₂O₂, malondialdehyde (MDA) levels in the skin and the oxygen radical absorbance capacity (ORAC) in plasma, were analyzed in NAC-treated and NAC-untreated coadjuvant chromium-sensitized albino GP.

Results: The severity of the skin reaction in the intradermal and epicutaneous elicitation test significantly diminished when the albino GP were treated with a dose of 1200 mg/kg/day of NAC. This dose also significantly decreased the sensitization rate of chromium hypersensitivity. In addition, treatment with 1200 mg/kg/day of NAC significantly reduced the H₂O₂ and MDA levels in the skin and significantly increased the ORAC in the plasma of albino GP. Therefore, NAC could be a potential chemopreventative agent to prevent the progression of chromium hypersensitivity.

Key words: cement workers – chromium – chromium hypersensitivity – N-acetylcysteine – reactive oxygen species

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Introduction

The element chromium was discovered by Vaquelin in 1798. It is ubiquitous in the environment and is widely used in the plating, leather tanning, pigmentation, dye production, metallurgy and chemical industries and in cement as an inadvertent impurity from the cement manufacturing process itself (1,2). Chromium exposure induces

many health hazards, including lung cancer (3), kidney, prostate and bladder cancer (4). In skin, chromium salts can induce both cutaneous irritation, which may progress to ulceration, and allergic contact dermatitis (ACD), because of chromium hypersensitivity (5). Chromium hypersensitivity is common in the general population, with a prevalence of around 0.5% for European populations (6,7). The prevalence of chromium hypersensitivity in cement workers in Europe is around 4–5% (1,6); in fact, chromium hypersensitivity is an important occupational skin disease among cement workers.

There are variable forms of the oxidation state of chromium, including Cr⁺², Cr⁺³, Cr⁺⁴, Cr⁺⁵ and Cr⁺⁶, but only

Abbreviations: ACD, allergic contact dermatitis; FCA, Freund's complete adjuvant; MDA, malondialdehyde; NAC, N-acetylcysteine; ORAC, oxygen radical absorbance capacity; ROS, reactive oxygen species.

Cr^{+3} and Cr^{+6} are stable. Cr^{+6} is soluble in aqueous mediums and can be absorbed through the skin. It is believed that the reduction of Cr^{+6} is the most important factor in the induction of chromium cytotoxicity. In contrast, most Cr^{+3} compounds are insoluble and therefore cannot easily enter cells. Cr^{+6} is allergenic and carcinogenic in nature (8). After entering the cell, Cr^{+6} can be reduced to Cr^{+5} , Cr^{+4} and Cr^{+3} by NADPH and cell reductants, such as ascorbate and glutathione; during the reduction process, many reactive oxygen species (ROS) are generated (9).

Reactive oxygen species are related to many diseases, such as atherosclerosis, neuro-degenerative diseases, osteoarthritis and cancer (10,11). In skin, ROS play an important role in the pathogenesis of psoriasis (12), atopic dermatitis (13) and contact dermatitis (14,15). ACD is a cell-mediated type IV hypersensitivity reaction. There are many cell types involved in this process, including dendritic cells, keratinocytes, T-lymphocytes and leucocytes, and all of these cells might be affected by ROS during the progression of contact hypersensitivity (10,16–22). In certain types of cells, ROS-regulated redox-sensitive protein kinases and transcription factors (for example NF- κ B, MAPK and Akt pathway) (23) might affect the release of cytokines, such as TNF- α and IL-1 (24–26). The release of these cytokines is suggested to be a central and early event in the progression of ACD (10,27,28). NF- κ B is an important transcriptional factor in the skin inflammatory process, and nickel-induced allergic responses are related to the activation of NF- κ B (9,29). Nickel and chromium are both producers of ROS and could regulate NF- κ B, although this is not true for lead (30). Hence, nickel and chromium are common agents that induce ACD, while lead is not (31).

When ROS are generated, antioxidants, including enzymatic antioxidant systems (superoxide dismutase, catalase and glutathione peroxidase) (32) and non-enzymatic antioxidant systems (ascorbate, Vitamin E, thioredoxin and glutathione) (33), will counteract the oxidative effects of these ROS to protect the body. There are many antioxidants, such as tea extracts, resveratrol and *N*-acetylcysteine (NAC), which can be used to protect the body from oxidative stress-induced damage and carcinogenesis (34–36). In the antioxidant system, glutathione plays a central role in the reduction of, and defense against, ROS. However, the availability of cysteine is a critical factor for glutathione synthesis. NAC, a thiol and mucolytic agent, is an effective precursor of cysteine that has been used for research on the role of ROS in many disease processes (36). For instance, when NAC was fed to Sprague-Dawley rats, it was proved to effectively increase the level of glutathione in the liver, kidney and skin (37).

Clinically, it usually takes years for cement workers to become sensitized to hexavalent chromium (38). The refractory phase of chromium hypersensitivity is long, and

this is the reason why researchers find it difficult to observe the effects of preventative agents that might benefit cement workers in a short period of time. Fortunately, in an animal model of a coadjuvant chromium-sensitized albino guinea pig chromium hypersensitivity can effectively be induced within 3 weeks, with a mixture of Freund's complete adjuvant (FCA) and chromium (39).

We hypothesized that the antioxidant, NAC, might inhibit chromium hypersensitivity in the coadjuvant chromium-sensitized albino GP by counteracting the formation and effects of ROS. To our knowledge, this is the first study to successfully demonstrate that NAC can prevent the progression of chromium hypersensitivity and inhibit the formation of ROS *in vivo*.

Materials and methods

Animals

Female albino GP of the Hartley Strain from the Animal Center of National Taiwan University, were used in this study. The animals were housed in the animal center of the National Cheng-Kung medical center in standard macrolon cages with free access to water and pellet food. Based on the recommendations outlined in van Hoogstraten et al. (39), the albino GP were acclimated for 2 weeks and weighed approximately 400 g before the experiment. All of the procedures involving animals were approved by the Animal Experiment Committee of National Cheng-Kung University, in accordance with the policies of the Animal Protection Law in Taiwan.

Reagents

Potassium dichromate, dimethyl sulfoxide (DMSO), H_2O_2 and ammonium sulphate were all obtained from Merck Chemical Co. (Darmstadt, FRG). FCA containing *Mycobacterium butyricum*, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH), β -phycoerythrin (β -PE), horseradish peroxidase (HRP) and butylated hydroxyl toluene (BHT) were obtained from Sigma Chemical Co. (St Louis, MO, USA). The depilatory cream was obtained from Hor-Chen Chemical Co. (Taoyuan, Taiwan). 1,1,3,3-Tetraethoxy propane (TEP) and trichloroacetic acid (TCA) were obtained from Acros Organics (Geel, Belgium). Phenol red was obtained from ICN Biomedica Inc. (Aurora, OH, USA). 2-thiobarbituric acid (TBA) was obtained from MP Biomedicals, Inc. (Aurora, OH, USA). Ketamine was obtained from Pfizer (Taipei, Taiwan). Rompun was obtained from Bayer Health Care AG (Leverkusen, Germany).

For the GP anaesthesia, 1 $\mu\text{L/g}$ body weight of a 4:3 mixture of ketamine and Rompun was injected intramuscularly.

Administration of NAC and the coadjuvant chromium sensitization method

Our study had two major focuses. One section dealt with the effects of NAC upon intradermal and epicutaneous elicitation test results using a coadjuvant chromium-sensitized GP; the other dealt with effects of NAC on three ROS parameters [H_2O_2 , malondialdehyde (MDA) and oxygen radical absorbance capacity (ORAC)] generated after chromium/FCA–chromium emulsion injection. In order to detect the effects of NAC on the intradermal and epicutaneous elicitation test outcomes, three groups of GP (A, B and C; $n = 10/\text{group}$) were used. Group A GP were fed only ordinary food, while groups B and C GP were fed ordinary food and 600 and 1200 mg/kg/day of NAC, respectively. Each GP was weighed daily, and the daily NAC dosage was calculated and administered according to their body weight (Table 1). After a period of 2 weeks, all three of the GP groups were injected with 0.3 ml of a solution containing 4 mg/ml potassium dichromate (dissolved in saline and then emulsified with an equal volume of FCA). Briefly, anaesthetized GP received 0.2 ml of this emulsion into the shaved sites of their dorsal skin on each thigh (0.1 ml/thigh) and 0.05 ml into the pinna of each ear by intradermal injections. This process followed the coadjuvant chromium sensitization method, as described by van Hoogstraten et al. (39). The feeding of these three groups (in the manner described above) continued for 3 weeks after sensitization. On Days 14 and 21 postsensitization, GP in groups A, B and C each underwent intradermal and epicutaneous elicitation tests. For the portion of our study examining the effects of NAC on three ROS parameters, e.g. H_2O_2 , MDA and ORAC, seven groups of GP (groups D–J; $n = 8/\text{group}$) were used. Groups D–F and group I were all fed only ordinary food. Group G was fed ordinary food and 600 mg/kg/day of NAC, and groups H and J were fed ordinary food and 1200 mg/kg/day of NAC (Table 2). After feeding in this manner for a period of 2 weeks, GP in group D were injected with saline only; group E received FCA–saline emulsion; groups F–H were

injected with 0.3 ml of the potassium dichromate mixture described above; and groups I, J were injected with a chromium–saline solution (2 mg/ml potassium dichromate in saline). Forty-eight hours after these injections, skin from each GP was harvested for use in determination of H_2O_2 and MDA levels, and their plasma was used to assess ORAC levels.

Intradermal and epicutaneous elicitation test

On Days 14 and 21 after sensitization, GP in groups A, B and C underwent the intradermal and epicutaneous elicitation tests. On day 14 after sensitization, the intradermal elicitation test was performed by intradermal injection of 25 μg of potassium dichromate (in 0.1 ml saline) into the clipped and freshly depilated dorsal midflank skin, as suggested by Polak (40). On Day 21 after sensitization, an epicutaneous elicitation test with 25 μl of 0.5% potassium dichromate per 40% DMSO was painted onto the skin in a circular area with a 1-inch diameter, and was dried with an electric hair drier. In each case, the reactions were then evaluated 24, 48, 72 and 96 h later, and were recorded by two independent observers who were blinded to each test group. The result of the intradermal elicitation test was expressed as the diameter of erythaema multiplied by the intensity of the erythaema; the result of the epicutaneous elicitation test was expressed as the intensity of the erythaema. The diameter of the erythaema (mm) was determined by measuring the vertical and horizontal diameter through the centre of the reaction (41). The intensity of erythaema was graded according to the following scale: equivocal or no reaction = 0, pink spots = 0.5, red and confluent = 1, intense red or swollen = 2. The GP were considered to be successfully sensitized by the coadjuvant chromium sensitization method if they showed 'swollen' or infiltration in the skin at 72 h, which is the same as the standard patch test reading method (31). In these cases, the GP were therefore recognized as having chromium hypersensitivity resulting from the coadjuvant chromium sensitization method.

Table 1. The feeding regimen, NAC administration and application procedure of albino GP groups for the intradermal and epicutaneous test

Group	Feeding regimen and NAC administration	Application procedure	Intradermal test	Epicutaneous test
A (Control)	Normal diet	Injected with chromium in FCA emulsion	14 days after injection treatment	21 days after injection treatment
B (Semi NAC)	NAC 600 mg/kg/day for 2 weeks, and then fed with the same dosage for a further 3 weeks after injection treatment	Injected with chromium in FCA emulsion	14 days after injection treatment	21 days after injection treatment
C (Full NAC)	NAC 1200 mg/kg/day for 2 weeks, and then fed with the same dosage for a further 3 weeks after injection treatment	Injected with chromium in FCA emulsion	14 days after injection treatment	21 days after injection treatment

Table 2. The feeding regimen, NAC administration and application procedure of albino GP groups for the determination of H₂O₂, MDA and ORAC

Group	Feeding regimen and NAC administration	Application procedure	Test for H ₂ O ₂ , MDA, ORAC
D	Normal diet for 14 days	Saline injection	Skin biopsy and blood sample 2 days after injection treatment
E	Normal diet for 14 days	Injected with FCA-saline emulsion	Skin biopsy and blood sample 2 days after injection treatment
F	Normal diet for 14 days	Injected with chromium in FCA emulsion	Skin biopsy and blood sample 2 days after injection treatment
G	NAC 600 mg/kg/day for 14 days	Injected with chromium in FCA emulsion	Skin biopsy and blood sample 2 days after injection treatment
H	NAC 1200 mg/kg/day for 14 days	Injected with chromium in FCA emulsion	Skin biopsy and blood sample 2 days after injection treatment
I	Normal diet for 14 days	Injected with chromium-saline solution	Skin biopsy and blood sample 2 days after injection treatment
J	NAC 1200 mg/kg/day for 14 days	Injected with chromium-saline solution	Skin biopsy and blood sample 2 days after injection treatment

Skin specimens and plasma for the determination of H₂O₂, MDA and ORAC

Two skin excisions (1 × 1 cm² area) were performed 48 h after sensitization on each previously injected dorsal thigh site of the GP in groups D–J while under anaesthesia. The excised skin specimen was carefully separated from the subcutaneous tissue. The left dorsal thigh skin specimen was used to test H₂O₂ levels, and the right dorsal thigh skin specimen was used to test MDA levels. In order to assess ORAC levels, the heart was punctured by a 27 gauge needle and blood (non-terminal) was collected into a heparin-coated tube. The blood was then centrifuged at 4°C at 3000 rpm for 15 min, to separate out the plasma. All skin specimens and plasma were stored at -80°C until analysis.

Determination of H₂O₂ levels in skin of GP from groups D–J

With the modified phenol red–HRP method of Pick and Keisari (42), the H₂O₂ levels in GP skin were measured. Briefly, the skin (1 × 1 cm²) sample from each GP was minced with scissors and homogenized with a polytron homogenizer with 2 ml of buffer (50 mM KH₂PO₄ with 10 mM sodium azide, pH 7.0) and centrifuged at 1200 rpm for 20 min. A 0.5-ml aliquot of the supernatant was removed and then mixed with 0.5 ml of phenol red/HRP solution (25 µg phenol red/ml and 50 µg HRP/ml). After the samples were incubated for 10 min, the reaction was terminated by the addition of 10 µl of 1 N NaOH. The solution was transferred to a cuvette and measured by a spectrometer (Beckman, DU640B, San Diego, CA, USA) at 610 nm. The final results were represented as equivalents of H₂O₂ (nmol/cm² of skin) based on a standard curve of H₂O₂–oxidized phenol red.

Determination of MDA levels in skin of GP from groups D–J

Malondialdehyde in the skin was determined by the modified method of Tian, (43). Briefly, the specimen (1 × 1 cm² skin) from each GP was minced with scissors and homogenized using a polytron homogenizer with 1.8 ml of 10% TCA and 0.2 ml of BHT in methanol (0.5 g/l). The homogenate was heated for 30 min at 100°C in a water bath and then cooled to room temperature. After centrifugation at 5000 rpm for 5 min, 0.5 ml of the generated supernatant was removed and added to 0.5 ml of 0.67% 2-TBA and heated at 100°C for 30 min. The mixture was then centrifuged at 5000 rpm for 5 min. The absorbance at 532 nm (Beckman, DU640B) was used to calculate the concentration of MDA. The result was represented as µmoles of MDA per cm² of skin based on the standard curve of TBARS formation using authentic MDA.

Determination of ORAC in the plasma of GP from groups D–J

There are several different methods for assessing the total antioxidant capacity. These include the Randox Trolox equivalent antioxidant capacity, the ferric reducing ability of plasma (FRAP) and the ORAC assay. Of these, ORAC is proved to be a simple, sensitive and reliable method for measuring the total antioxidant capacity (44). Therefore, the modified method of Cao et al. (45) was used to assess the ORAC in the plasma from each GP. In this assay, β-PE was used as an indicator protein, AAPH was used as a peroxyl radical generator and the water-soluble Vitamin E analogue, Trolox, was used as a control standard. For each assay, the plasma was first treated with ammonium sulphate to remove the albumin, by mixing 400 µl of 100% saturated ammonium sulphate with 50 µl plasma for 30 min (placed in ice water), and then centrifuged at 4°C

at 12 000 rpm for 10 min. The supernatant was carefully separated. From this processed supernatant, three aliquots of 50 μ l each were placed into dedicated wells of a 96-well plate; 50 μ l of Trolox standard, 50 μ l β -PE and 50 μ l AAPH were added to the samples in each well. Fluorescence in each well was measured every 5 min thereafter for a total of 90 min, at an emission of 590 ± 35 nm and an excitation of 530 ± 25 nm, using a microplate fluorescence reader (FLx 800, Bio-Tek instrument INC, Mississauga, ON, Canada) until the background fluorescence decreased to the same level as the phosphate-buffered saline (PBS) control. The ORAC value (units) of a sample was calculated on the basis of a Trolox standard curve. The results were reported in μ mol Trolox equivalent/l.

Histological examinations of the GP skin

Skin biopsies were performed 48 hr after the epicutaneous elicitation test on GP from groups A, B and C. In order to differentiate whether the epicutaneous elicitation test utilizing 0.5% potassium dichromate/40% DMSO might produce an irritant reaction, skin biopsies were performed on another group of GP (group K, $n = 6$) that were injected with FCA-saline and then received an application of chromium in DMSO. All of the biopsy specimens were fixed in 10% formaldehyde and embedded in paraffin. Five-micrometre sections were stained with haematoxylin-eosin and examined by light microscopy.

Statistical analyses

Statistica Software release 6 (StatSoft, Tulsa, OK, USA), was used to compare the two independent groups by the non-parametric Mann-Whitney *U*-test, and the Fisher's exact test was utilized to evaluate the significant differences in the sensitization rate of chromium hypersensitivity in NAC-treated and NAC-untreated groups of coadjuvant chromium-sensitized GP.

Results

NAC administration diminished the severity of skin reaction in the intradermal elicitation test

Fourteen days after the GP were sensitized by the coadjuvant chromium sensitization method, an intradermal injection with 0.1 cc of 25 μ g $K_2Cr_2O_7$ was administered to the left dorsal midflank skin. Thereafter, the skin reaction was evaluated at 24, 48, 72 and 96 hr. For this particular study, group A is hereafter known as the Control group, group B as the Semi-NAC group and group C as the Full NAC group. At 48 h, the skin in the Control group showed marked erythaema with infiltration at the injection site (Fig. 1a). In the Full NAC group, the skin showed no erythaema or infiltration at the injection site at 48 h (Fig. 1b). Figure 1c shows the effect of NAC in the intradermal test

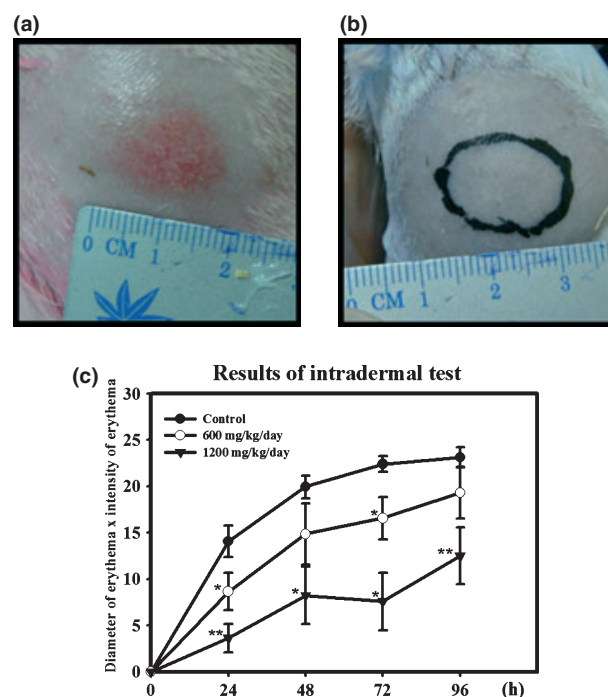


Figure 1. Results of the intradermal test: the skin reaction was evaluated at 24, 48, 72 and 96 h after intradermal injection with 0.1 cc of 25 μ g $K_2Cr_2O_7$ on GP sensitized by the coadjuvant chromium sensitization method. (a) In the Control group A, the skin showed marked erythaema and infiltration at the injection site. (b) In the Full NAC group C, the skin showed no erythaema or infiltration at the injection site. (c) The effect of NAC in the intradermal test. The values are the mean $\pm$ SEM. The Full NAC group C developed a significant decrease in the severity of skin reaction (diameter of erythema $\times$ intensity of erythema) compared with the Control group A at 24, 48, 72 and 96 h. The Semi-NAC group B also showed a significant decrease in the severity of skin reaction compared with the Control group A, but only at 24 and 72 h. *Significant versus Control group A, $P < 0.05$; **Significant versus Control group A, $P < 0.01$.

on the coadjuvant chromium-sensitized GP. The results are reported as the diameter of erythaema multiplied by its intensity. The values are mean $\pm$ SEM. The Semi-NAC group showed a significant decrease in the severity of skin reaction when compared with the Control group, but only at the 24- and 72-h time points. However, the Full NAC group had a significant decrease in the severity of skin reaction of more than twofold, when compared with the Control group at the 24-, 48-, 72- and 96-h time points.

NAC administration diminished the severity of skin reaction in the epicutaneous elicitation test and reduced the sensitization rate of chromium hypersensitivity

Twenty-one days after the GP were sensitized by the coadjuvant chromium sensitization method, an epicutaneous elicitation test was performed by painting the right dorsal

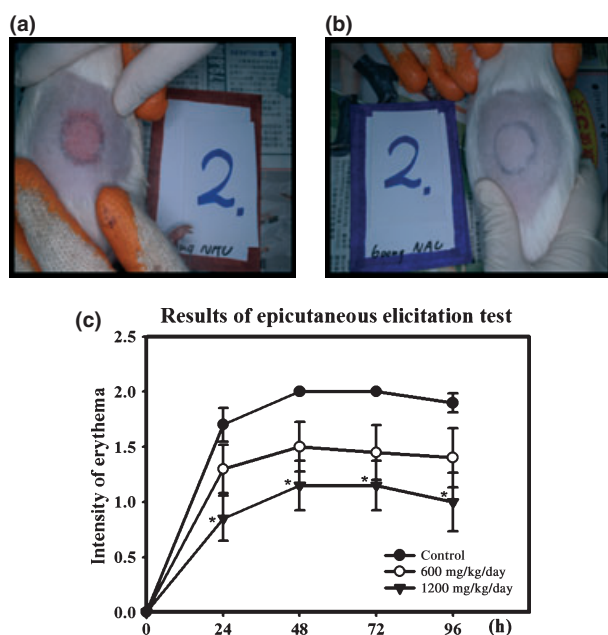


Figure 2. Results of the epicutaneous test: the skin reaction was evaluated at 24, 48, 72, and 96 h after painting 0.02 cc of 0.5% $K_2Cr_2O_7$ /40% DMSO on GP sensitized by the coadjuvant chromium sensitization method. (a) In the Control group A, the skin showed marked erythema with infiltration at the painted site. (b) In the Semi-NAC group B, the skin showed no erythema or infiltration at the painted site. (c) The effect of NAC in the epicutaneous test: the values are mean $\pm$ SEM. The Full NAC group C had a significant decrease in the severity of skin reaction (intensity of erythema) compared with the Control group A at 24, 48, 72 and 96 h. The Semi-NAC group B also had a decrease in the severity of skin reaction compared with the Control group, although this was not statistically significant. *Significant versus Control group A, $P < 0.05$.

midflank skin with 0.02 cc of 0.5% $K_2Cr_2O_7$ /40% DMSO. Thereafter, the skin reaction was evaluated at 24, 48, 72 and 96 h. In the Control group, the skin showed marked erythema with infiltration at the painted site at 48 h (Fig. 2a). In the Full NAC group, the skin showed no erythema or infiltration at the painted site at 48 hr (Fig. 2b). Figure 2c shows the effect of NAC in the epicutaneous test on the coadjuvant chromium-sensitized GP. The Full NAC group reflected a significant decrease ($P < 0.05$) in the severity of the skin reaction (intensity of erythema) when compared with the Control group at 24, 48, 72 and 96 h. The Semi-NAC group also showed a decrease in the severity of skin reaction when compared with the Control group, although it was not statistically significant.

This epicutaneous elicitation test also clearly illustrated the successful sensitization to chromium in all 10 GP within the Control group. Notably, the Full NAC group showed a statistically significant decrease in the sensitization rate ($P < 0.01$) when compared with the Control group, where only 4 out of 10 GP in the Full NAC group

manifested chromium hypersensitivity. The Semi-NAC group also showed a decrease in the sensitization rate ($P = 0.11$) when compared with the Control group, where 7 out of 10 GP in the Semi-NAC group manifested chromium hypersensitivity (data not shown).

NAC decreased H_2O_2 levels in the skin of chromium and/or FCA injected GP

The H_2O_2 levels in the skin from groups D–J were assayed by the phenol red–HRP method, which revealed very low H_2O_2 levels in group D (0.87 ± 0.44). Forty-eight hours after treatment of group E with FCA–saline emulsion, the H_2O_2 levels were significantly elevated (7.97 ± 2.82 ; $P < 0.01$). Similarly, the H_2O_2 levels were significantly elevated (13.87 ± 2.54 ; $P < 0.01$) in group I, 48 h after treatment with chromium–saline solution. Furthermore, the H_2O_2 levels were elevated to an even greater degree (26.95 ± 8.06 ; $P < 0.001$) after treatment with chromium emulsified in FCA to group F. This finding clearly indicates that both chromium and FCA were responsible for significantly increasing the levels of H_2O_2 in skin. Moreover, we found that pretreatment of group G with 600 mg/kg/day dose of NAC for 2 weeks, could effectively and significantly decrease the H_2O_2 levels (6.69 ± 2.31) ($P < 0.05$). Pretreatment with 1200 mg/kg/day dose of NAC to group H reduced the H_2O_2 levels to near background levels (1.22 ± 0.30), and reflected a significant decrease in the H_2O_2 levels ($P < 0.05$) compared with group G. For the chromium–saline solution injected groups I–J, pretreatment with 1200 mg/kg/day dose of NAC to group J reduced the H_2O_2 levels to near background levels (0.88 ± 0.66), and reflected a significant decrease in H_2O_2 levels ($P < 0.01$) when compared with group I (Fig. 3a).

NAC decreased MDA levels in the skin of chromium- and/or FCA-injected GP

The MDA levels in the skin from groups D–J were assayed by the modified method of Tian, and revealed very low MDA levels in group D (2.935 ± 0.25). Forty-eight hours after group E was treated with FCA–saline emulsion, and group I was treated with chromium–saline solution, the MDA levels were significantly increased (9.01 ± 1.63 and 7.11 ± 0.61 ; $P < 0.01$). Furthermore, 48 h after group F was treated with chromium emulsified in FCA, the MDA levels were significantly increased (9.73 ± 1.53 ; $P < 0.01$). Similarly (following the same trend as the H_2O_2 level results), pretreatment of groups G and H with 600 and 1200 mg/kg/day dose of NAC, respectively, significantly decreased the MDA levels (6.06 ± 0.66 and 4.11 ± 1.16 ; $P < 0.05$ and $P < 0.01$). In addition, for the chromium–saline solution injected groups I–J, pretreatment with 1200 mg/kg/day dose of NAC to group J reduced the MDA levels (3.88 ± 0.59), and reflected a significant decrease in MDA levels ($P < 0.01$) when compared with group I (Fig. 3b).

NAC increased ORAC levels in the plasma of chromium and/or FCA injected GP

The ORAC levels in the plasma from groups D–J were assayed by the modified method of Cao (45), revealing high ORAC levels in group D (267.80 ± 36.91). Forty-eight hours after an injection of FCA–saline emulsion to group E, and an injection of chromium–saline solution to group I, the ORAC levels were significantly decreased (108.86 ± 17.93 and 98.97 ± 35.5 ; $P < 0.01$). Furthermore, the ORAC levels also significantly decreased after group F received an injection of

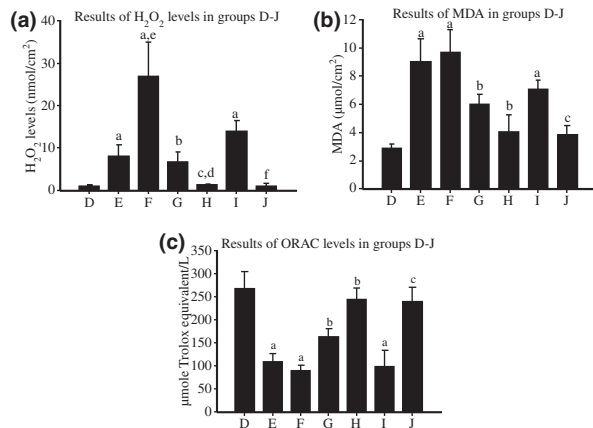


Figure 3. Effects of NAC on H₂O₂, MDA and ORAC production in coadjuvant chromium-sensitized GP: the seven groups, D–J, were treated as described in Table 2. The H₂O₂, MDA and ORAC levels were evaluated 48 h after application injection. The values are mean $\pm$ SEM. (a) H₂O₂ production significantly increased in group E, I and F compared with group D. Pretreatment with NAC (600, 1200 mg/kg/day) in groups G–H significantly reduced H₂O₂ production compared with group F. Notably, group F also showed a significant increase in H₂O₂ production compared with group E. For the two chromium–saline injection groups, pretreatment with a dose of 1200 mg/kg/day NAC to group J significantly reduced the H₂O₂ production when compared with group I. ^aSignificant versus group D, $P < 0.01$; ^bSignificant versus group F, $P < 0.05$; ^cSignificant versus group F, $P < 0.05$; ^dSignificant versus group G, $P < 0.01$; ^eSignificant versus group E, $P < 0.05$; ^fSignificant versus group I, $P < 0.01$. (b) MDA production significantly increased in groups E, I and F compared with group D. Pretreatment with NAC (600, 1200 mg/kg/day) in groups G–H significantly reduced MDA production compared with group F. For the two chromium–saline injection groups, pretreatment with a dose of 1200 mg/kg/day NAC to group J significantly reduced the MDA production when compared with group I. ^aSignificant versus group D, $P < 0.01$; ^bSignificant versus group F, $P < 0.05$; ^cSignificant versus group F, $P < 0.01$; ^dSignificant versus group I, $P < 0.01$. (c) ORAC levels were significantly decreased in groups E, I and F compared with group D. Pretreatment with NAC (600, 1200 mg/kg/day) in groups G–H significantly increased the ORAC levels compared with group F. For the two chromium–saline injection groups, pretreatment with a dose of 1200 mg/kg/day NAC to group J significantly increased ORAC when compared with group I. ^aSignificant versus group D, $P < 0.01$; ^bSignificant versus group F, $P < 0.01$; ^cSignificant versus group I, $P < 0.05$.

chromium emulsified in FCA (89.94 ± 11.77 ; $P < 0.01$). Similarly, pretreatment with 600 mg/kg/day dose of NAC to group G, or 1200 mg/kg/day dose of NAC to group H, significantly increased the ORAC levels (163.98 ± 16.55 and 243.81 ± 24.93 ; $P < 0.01$). For the chromium–saline solution injected groups I–J, pretreatment with 1200 mg/kg/day dose of NAC to group J increased the ORAC levels to near control levels (240.40 ± 30.19), and reflected a significant increase in ORAC levels ($P < 0.05$) when compared with group I (Fig. 3c).

Histological examination showed that NAC could inhibit the eczematous reaction in coadjuvant chromium-sensitized GP

Forty-eight hours after the epicutaneous elicitation test, the skin specimen in the Control group A showed parakeratosis with spongiotic changes in the epidermis and skin appendages. There was a marked lymphocyte infiltration in the dermis (Fig. 4a). In the Semi-NAC group B, the histological examination also showed spongiosis in the epidermis, but lymphocyte infiltration was to a lesser extent in the dermis when compared with Control group A (Fig. 4c). In the Full NAC group C, the histological examination showed no spongiotic changes in the epidermis, and only a scarce cellular infiltration in the dermis (Fig. 4d).

In order to differentiate whether the procedure of the epicutaneous elicitation test might produce an irritant reaction, group K was injected with FCA–saline (not the

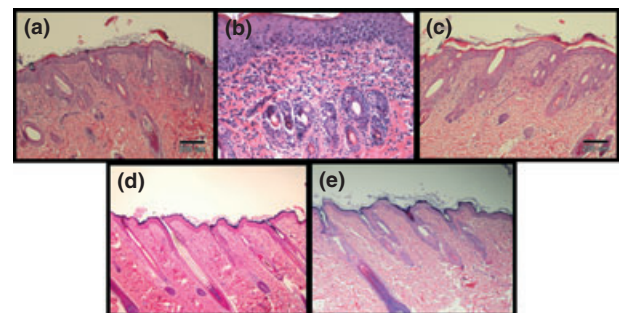


Figure 4. Histological images of GP skin sensitized by the coadjuvant chromium sensitization method: skin biopsies were performed 48 h after epicutaneous painting with 0.02 cc of 0.5% K₂Cr₂O₇/40% DMSO. (a) In the Control group A, the histological examination showed parakeratosis with spongiotic changes in the epidermis and skin appendages. Marked lymphocyte infiltration was seen in the dermis (40 $\times$). (b) Close-up view of (a) (100 $\times$). (c) In the Semi-NAC group B, the histological examination showed parakeratosis and less lymphocyte infiltration in the dermis compared with Control group A (40 $\times$). (d) In the Full NAC group C, the histological examination showed only scarce cellular infiltration in the dermis (40 $\times$). (e) In another group (group K) of GP, which were injected with FCA–saline and received an application of chromium in DMSO, the histological examination showed no spongiotic changes in the epidermis, and only a scarce cellular infiltration in the dermis (40 $\times$).

chromium–FCA emulsion). The results revealed no erythema or skin infiltration at the application site after 48 h. Skin biopsies from these hosts also provided no evidence of spongiotic changes in the epidermis, and only a scarce cellular infiltration in the dermis (Fig. 4e). This indicates that the skin reaction in the epicutaneous elicitation test in coadjuvant chromium-sensitized GP is allergic in nature, and not because of the presence of an irritant.

Discussion

Chromium hypersensitivity is a major occupational skin disease among cement workers. The factors contributing to the development of chromium hypersensitivity presumably include the following: (i) the inherent immunogenicity of hexavalent chromium (46); (ii) the parameters affecting the percutaneous absorption of an adequate concentration of hexavalent chromium per unit area of skin (47), such as skin exposure area, skin barrier (state of stratum corneum), skin pH level, duration of exposure, occlusion, ambient temperature, ambient humidity and sunlight exposure (6); (iii) appropriate epidermal cytokines, such as TNF- α and IL-1, that are generated after skin trauma or inflammation (48–50); and (iv) genetic background (51). As mentioned earlier, when hexavalent chromium enters the cells, many free radicals, including H₂O₂, are generated. These ROS might regulate redox-sensitive protein kinases and transcription factors (23), and affect the release of cytokines such as TNF- α and IL-1 (24–26). The release of these cytokines is suggested to be a central and early event in the progression of ACD (chromium hypersensitivity) (10,27,28). The findings of our study show that an injection of hexavalent chromium into the skin of GP from group I could significantly increase H₂O₂ and MDA levels, as well as significantly decrease the ORAC levels in their plasma (Fig. 3). This result suggests that hexavalent chromium serves as both an antigen and a ROS producer to induce chromium hypersensitivity within this animal model.

Another important finding was that an injection of FCA–saline emulsion could induce marked H₂O₂ and MDA production in the skin, and decrease the total body antioxidant content. The use of FCA is an important method in the evaluation of immunogenicity of chemical agents, especially in the FCA test (52). FCA is paraffin oil with the addition of a water-in-oil emulsifier (incomplete Freund's adjuvant) and heat-killed *Mycobacterium butyricum*. It was found that sensitization to contact allergens could be intensified by an intradermal injection of FCA, and the application of a sensitizer to the injected skin site. Therefore, FCA is usually used as an immunopotentiator for contact allergens (53). The reasons that FCA is a strong immunopotentiator are not fully understood. The effects of the adjuvant are mainly because of two reactions: the

concentration of the antigen at the site (the depot effect), and the induction of cytokines that regulate lymphocyte functions (54). The side-effects of an injection with FCA include the induction of a chronic cutaneous ulcer, the production of a migratory granuloma and adjuvant arthritis (55,56). The pathogenesis of adjuvant arthritis is related to the production of ROS and could be prevented by the use of antioxidants. It was demonstrated that antioxidants, such as α -lipoic acid, can reverse the effects of FCA-induced adjuvant arthritis by decreasing MDA and TNF- α production and by increasing both glutathione levels and the enzymatic activities of superoxide dismutase and glutathione peroxidase (57). Our finding, that the FCA–saline emulsion could significantly increase the H₂O₂ levels in GP skin 48 h after injection, indicates that FCA may facilitate the progression of chromium hypersensitivity via the mechanism of ROS production.

Many strategies have been proposed to prevent chromium hypersensitivity in cement workers, including the use of protective gloves to prevent direct hand contact with cement (58), the addition of ferrous sulphate to cement (59) and the use of protective barrier cream, such as ascorbic acid cream (60). The addition of ferrous sulphate to cement was proved to be an effective way to reduce chromium hypersensitivity (61). Antioxidative agents that could reduce the formation of ROS might be another potential approach to prevent chromium hypersensitivity by, for example, interfering with the effects of ROS on the migration of dendritic cells (62) and/or on the production of cytokines (e.g. TNF- α , IL-1) by keratinocytes and the expression/placement of adhesion molecules (e.g. ICAM-1, VCAM-1) on endothelial cells (14,24). As mentioned earlier, glutathione plays a central role in antioxidant defense against ROS. The availability of cysteine is generally a critical factor in the synthesis of glutathione, and NAC is one of the effective precursors of cysteine (63). NAC is one of the most common antioxidants used to investigate the effects of ROS in the pathogenesis of many oxidative stress-related diseases (10,36).

We used NAC to evaluate its effects on coadjuvant chromium-sensitized GP, and found that NAC significantly diminished the severity of skin reactions in the intradermal and epicutaneous elicitation test, for both the Semi-NAC group B and the Full NAC group C (Figs 1 and 2). Most importantly, the sensitization rate of chromium hypersensitivity was significantly decreased for the Full NAC group C. We also evaluated the ability of NAC to reduce the formation and effects of ROS on three levels: (i) the formation of stable ROS, such as H₂O₂; (ii) MDA, which is the result of ROS-damaged cells by lipid peroxidation; and (iii) ORAC, which is the final response in GP to correct the effects of abnormal and excessive ROS. Our results showed that pretreatment with 600 and 1200 mg/kg/day dose of

NAC could effectively reduce the H_2O_2 and MDA levels in the skin, and could increase the total antioxidant capacity in the plasma, of the coadjuvant chromium sensitized GP (Fig. 3). The data of these three levels are consistent and reflect a dose-related effect. ROS are known to play an important role in the progression of contact hypersensitivity, and our data has demonstrated that NAC could significantly diminish the severity of skin reaction and the sensitization rate of chromium hypersensitivity, as well as decrease the ROS formation in the skin of coadjuvant chromium-sensitized GP. The above observations indicate that the use of NAC could effectively prevent the progression of chromium hypersensitivity in coadjuvant chromium-sensitized GP by interfering with the formation and/or effects of ROS.

N-acetylcysteine has been used in clinics for more than 50 years. At the molecular level, NAC has been shown to inhibit the activation of C Jun N-terminal kinase, p38 MAP kinase, redox-sensitive activating protein-1 (AP-1) and the NF- κ B transcriptional factor, thereby resulting in the suppression of numerous genes that regulate the expression of many cytokines, such as TNF- α and IL-1 (23,24,36,64,65). In addition, NAC was shown to stimulate glutathione-S-transferase transcription (66), to inhibit prostaglandin synthesis and IL-8 expression (67,68), and to block binding of TNF- α to its receptor (69). Although NAC has many different effects, most research has focused on its antioxidant and direct ROS-scavenging ability. A previous study showed that when NAC was taken orally, it underwent extensive first-pass metabolism that, in turn, resulted in low plasma and tissue availability. Such scenarios, therefore, should call into question the ability of NAC to act as a direct ROS scavenger (70). However, NAC has been shown to increase the glutathione level in skin after 30 days of oral ingestion of high doses (37).

Previous studies did show, however, that NAC is a safe and well-tolerated agent (71). Furthermore, in our study, no major side-effects of NAC were found. The GP which were administered with 600 and 1200 mg/kg/day dose of NAC were healthy and no significant body weight loss was noted. Using the FDA guidelines (72) to translate these above doses to the equivalent human doses, the doses would be 130 and 260 mg/kg, respectively. Currently, in the treatment of acetaminophen intoxication, NAC is usually administered orally to humans, at a loading dose of 140 mg/kg, and 17 subsequent doses of 70 mg/kg every 4 h, without significant side-effects (71). It should be noted, however, that in reality, cement workers are rarely exposed to the same high doses of hexavalent chromium that the GP were subjected to in this study. In other words, it is likely that lower doses of NAC used here (if extrapolated) would prove effective in the prevention of chromium hypersensitivity. While NAC has a good efficacy in preventing the

progression of chromium hypersensitivity in this GP model, many factors, including its compliance, the cost of NAC and its long-term usage, might compromise its efficiency in clinics. If these limitations could be overcome, NAC has the potential to be used as a chemopreventative agent to prevent the progression of chromium hypersensitivity.

In conclusion, we clearly demonstrate that the use of NAC, in a coadjuvant chromium-sensitized GP model, could significantly reduce the severity of skin reaction and, most importantly, significantly decrease the sensitization rate of chromium hypersensitivity. NAC also effectively reduced H_2O_2 and MDA levels in the skin, and increased the ORAC in the plasma. Hence, NAC could be a potential agent to prevent the progression of chromium hypersensitivity in those who are unavoidably exposed to work-related chromium.

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