

Serum levels of protein oxidation products in patients with nickel allergy

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ABSTRACT

Nickel sensitization can not only induce allergic contact dermatitis (ACD), but also can induce an overlapping disease referred to as "systemic nickel allergy syndrome" (SNAS), characterized by urticaria/angioedema and gastrointestinal symptoms correlated to the ingestion of nickel-containing foods. This study was designed to determine if oxidative stress occurs in patients with nickel allergy. Thirty-one female patients (mean age 31.26 ± 13.04 years, range 16–64 years) with confirmed nickel CD underwent oral nickel challenge because of clinically suspected SNAS; serum concentrations of protein carbonyl groups (PCGs) and nitrosylated proteins (NPs; biomarkers of oxidative stress) were measured before and after oral nickel challenge as well as in healthy female controls. Twenty-three of these 31 patients were diagnosed with SNAS because they had a positive reaction to the oral nickel challenge, and 8 patients had no reaction and therefore were classified as patients with contact nickel allergy only. Although both nickel-allergic patients and controls presented similar serum levels of PCGs, NP values in nickel-allergic patients appeared higher than in controls and tended to decrease after the challenge; furthermore, serum levels of NPs in patients affected by SNAS were higher (although not significantly) than in patients with nickel ACD only. The involvement of specific biomarkers of oxidative stress such as NPs and the lack of involvement of other biomarkers such as PCGs may help to better understand the alteration of the redox homeostasis occurring in nickel ACD and particularly in SNAS.

(Allergy Asthma Proc 30:552–557, 2009; doi: 10.2500/aap.2009.30.3275)

Key words: Biomarker, contact dermatitis, contact nickel allergy, nitrosylated proteins, oral challenge, oxidative stress, protein carbonyl groups, systemic nickel allergy syndrome

Nickel is a common cause of allergic contact dermatitis (ACD), more than all other metals combined.¹ It is present in most of the dietary items of humans and an average diet supplies 300–600 µg of nickel to the human body per day.² The presence of nickel in the diet of a nickel-sensitive subject can provoke/aggravate eczema and dermatitis³; in fact, there are flares of eczema and/or patch test sites after oral nickel challenge or dermatitis improvement after low nickel diet or treatment with oral disulfiram (which chelates nickel and increases its excretion).

The total dietary intake of nickel per day varies depending on the amount of plant and animal foods ingested. Because it is a ubiquitous trace metal, nickel can not be completely avoided from diet; however, the careful selection of foods with relatively low nickel concentrations can reduce the total dietary intake of

nickel per day and can influence the outcome of nickel dermatitis.⁴ It is well known that systemic exposure to haptens orally, transcutaneously, intravenously, or by inhalation can cause, in sensitized patients, the onset of a "systemic contact dermatitis" (CD) with the flare-up of previous dermatitis or *de novo* dermatitis similar to ACD.^{5–7}

Sensitization to nickel has also been reported to cause respiratory symptoms (rhinitis and asthma) and systemic CD as well as other systemic reactions such as urticaria, angioedema, and gastrointestinal symptoms (nausea, meteorism, abdominal pain, diarrhea, and/or constipation) correlated to the ingestion of foods and beverages containing nickel. This syndrome is referred to as "systemic nickel allergy syndrome" (SNAS).⁸

Although the immunologic mechanisms involved in the development of nickel ACD are well described in several studies on humans, few studies have attempted to describe the immunologic mechanisms underlying the cutaneous allergic reactions elicited by oral nickel exposure. The histopathology of the flare-up reactions in nickel-sensitive patients reacting to oral nickel challenge was found to be similar to that of ACD lesions; nevertheless, more than one type of immunologic reaction might be involved. In particular, Jensen and

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coworkers⁹ have shown that CD8⁺CD45RO⁺CLA⁺ T lymphocytes and T lymphocytes with a type 2 cytokine profile are involved in cutaneous reactions elicited by nickel intake. Furthermore, high serum levels of soluble intracellular adhesion molecule 1 were found in patients affected by SNAS in a phase of nonactivity of the disease,¹⁰ suggesting the presence of a minimal persistent inflammation.

It has been shown that many pathological conditions, including irritant and ACD, are characterized by an excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS); this causes an imbalance in the oxidative stressor/antioxidant system. ROS and RNS may damage all types of biological molecules as proteins that are the main target in cells. Because, at present, there are few data concerning the oxidative stress in nickel allergy, we performed a study on a group of 31 patients with a confirmed diagnosis of nickel ACD and undergone an oral nickel challenge due to SNAS clinical symptoms. To evaluate the occurrence of oxidative stress in these patients, we measured before and after oral nickel challenge the serum concentrations of protein carbonyl groups (PCGs) and of nitrosylated proteins (NP).

MATERIALS AND METHODS

Subjects

The study was performed on 31 female subjects (mean age, 31.26 ± 13.04 years; range, 16–64 years). They had a positive history of nickel ACD and presented the clinical signs of SNAS such as cutaneous manifestations (eczematous lesions at elbow and knee flexures, eyelids, sides of the neck and inner thighs, recurrent vesicular dermatitis of the palms, sides of the fingers and/or soles of the feet, symmetrical nummular eczema, anogenital eczema, urticarial rashes, and urticaria–angioedema), gastrointestinal symptoms (recurrent aphtous ulcers, abdominal bloating and distension, recurrent abdominal pain, diarrhea and/or constipation, nausea and/or vomiting, and, eventually, endoscopic findings of chronic gastroduodenitis), and other atypical systemic clinical manifestations (headache, chronic fatigue, postprandial dyspnea, cystitis and/or vulvovaginitis, acne, and iron deficiency anemia). Signs and symptoms were differently matched in each patient.

Tobacco smokers, alcohol abusers, and patients affected by severe acute or chronic pathological conditions, including dyslipidemia, obesity, and diabetes, were excluded from the study. The patients enrolled were instructed to completely avoid physical exercise and any kind of medications (including vitamins or antioxidant supplements) the week before the tests.

All of the patients included in the study were positive to a nickel patch test and had improved after a

month on a low nickel diet. To confirm the diagnosis of SNAS, all patients underwent an oral challenge with nickel sulfate by ingestion of a capsule containing talc (as placebo) and, after 1 hour, a capsule containing 1.25 mg of nickel sulfate. The oral challenge was performed in the morning and the patients had been fasting for 12 hours.

The result of the test was evaluated recording the status of the skin and the systemic symptoms. Positive reactions to the challenge were considered eruptions of previously unaffected skin; flare-up at previous sites of CD, including the flare-up reactions of previously positive nickel patch-test sites; urticaria; and systemic symptoms such as headache, marked fatigue, and gastrointestinal symptoms. Blood samples were obtained from peripheral blood of all patients before and 2 hours after oral exposure to nickel.

A group of 13 healthy nonsmoking age-matched women with a negative history of CD and allergic and chronic systemic diseases was included as control; the blood sample was withdrawn in the morning after 12 hours of fasting.

Written informed consent according to the Declaration of Helsinki was obtained from all subjects enrolled in the study. Sera were allowed to clot at room temperature for 2 hours and were separated by centrifugation at 1200 × g for 15 minutes and stored at –80°C until used.

Determination of the PCG

The serum content of the PCG was evaluated according to the Levine's method as previously described.¹¹ Briefly, 100 µL of serum was incubated with 100 µL of a 20-mM 2,4-dinitro-phenylhydrazin solution for 60 minutes. Then, the proteins were precipitated with the use of 20% trichloroacetate; the protein pellet was washed three times with ethanol and ethyl acetate and then resuspended in 1 mL of 6 M of guanidine at 37°C for 15 minutes. The carbonyl content was determined from the absorbance at 366 nm (molar absorption coefficient, 22,000 M^{–1} cm^{–1}). The serum concentration of the PCG was normalized to the total protein amount determined by the Bradford assay and expressed as nanograms of carbonyl groups for protein milligrams. Each sample was analyzed in triplicate.

Determination of NP

NPs in human sera were quantified by the Saville's method.¹² Briefly, 40 µL of serum were acidified by addition of 200 µL of 0.2 N H₂SO₄, and, after 2 minutes, added with 200 µL of a 0.5% solution of ammonium sulfamate. After 3 minutes, the mixture was supplemented with 160 µL of a solution of 0.25% HgCl₂ and 2.55% sulfanilamide in 0.4 N HCl and then with 160 µL of a solution of 0.38% *N*-1-naphthylethylenediamine in

Table 1 Patients' characteristics

Patient No.	Disease	Age (yr)	Race	Gender	Other Allergic Diseases
1	SNAS	33	White	Female	None
2	SNAS	43	White	Female	None
3	SNAS	25	White	Female	Drug hypersensitivity
4	SNAS	46	White	Female	None
5	SNAS	44	White	Female	None
6	SNAS	26	White	Female	None
7	SNAS	16	White	Female	Allergic rhinitis
8	SNAS	35	White	Female	None
9	SNAS	36	White	Female	None
10	SNAS	41	White	Female	None
11	SNAS	37	White	Female	None
12	SNAS	23	White	Female	None
13	SNAS	21	White	Female	None
14	SNAS	16	White	Female	None
15	SNAS	64	White	Female	None
16	SNAS	31	White	Female	None
17	SNAS	24	White	Female	None
18	SNAS	27	White	Female	None
19	SNAS	26	White	Female	None
20	SNAS	23	White	Female	None
21	SNAS	43	White	Female	None
22	SNAS	40	White	Female	Allergic rhinitis
23	SNAS	19	White	Female	None
24	ACD	24	White	Female	None
25	ACD	31	White	Female	None
26	ACD	38	White	Female	None
27	ACD	30	White	Female	None
28	ACD	27	White	Female	Allergic rhinitis
29	ACD	43	White	Female	Allergic rhinoconjunctivitis
30	ACD	19	White	Female	None
31	ACD	18	White	Female	None

ACD = allergic contact dermatitis; SNAS = systemic nickel allergy syndrome.

0.4 N HCl. After 5 minutes, absorbance at 540 nm was measured. NPs were quantified by extrapolation from a standard curve generated with *S*-nitrosoglutathione. The serum concentrations of NPs were normalized to the total protein amount determined by the Bradford assay and expressed as nanomoles of NP for protein milligram. Each sample was analyzed in triplicate.

Statistical Analysis

Data were analyzed with a statistical package (SPSS 13.0 for Windows, SPSS Italia s.r.l., Bologna, Italy), expressed as mean $\pm$ SD, and analyzed for statistical differences by the Wilcoxon signed-rank test and Mann Whitney *U* test one-way analysis of variance. Values of $p < 0.05$ were considered statistically significant.

RESULTS

The present study was performed with 13 healthy controls and 31 patients affected by ACD and who

had undergone oral nickel challenge to confirm a clinically suspected SNAS (Table 1). Eight patients were negative even at a further evaluation 24 hours after the challenge (and thus they were classified as with nickel ACD only) but 23 of the 31 patients with nickel ACD had a positive reaction to the provocation test, which confirmed the diagnosis of SNAS. Ten patients presented urticarial rashes and/or urticaria associated to dyspnea (one case), gastrointestinal symptoms (six cases), gastrointestinal symptoms and headache (two cases), and gastrointestinal symptoms and fatigue (one case); eight patients showed urticarial rashes and flare-up at previous sites of CD associated to gastrointestinal symptoms (two cases) and headache and fatigue (one case); five patients presented gastrointestinal symptoms associated with headache (two cases), fatigue (one case), headache and fatigue (one case), and postprandial dyspnea and fatigue (one case).

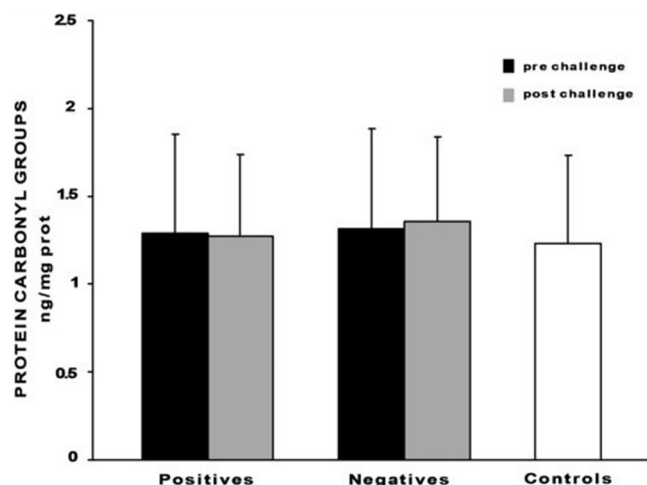


Figure 1. Serum levels of protein carbonyl groups in patients with a positive (positives) or a negative response (negatives) to the oral nickel challenge, as evaluated before and 2 hours after the challenge (prepost challenge), and in healthy subjects (controls). Data were expressed as mean + SD and were analyzed by using the Mann-Whitney U test and the Wilcoxon signed-rank test.

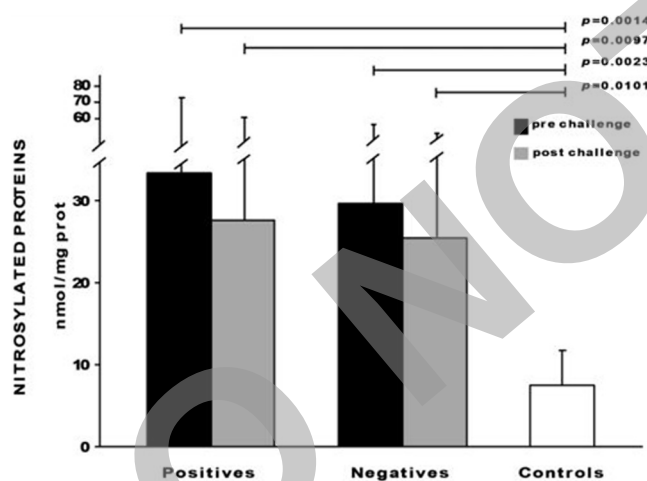


Figure 2. Serum levels of nitrosylated proteins in patients with a positive or a negative response to the oral nickel challenge, as evaluated before and 2 hours after the challenge (prepost challenge) and in healthy subjects (controls). Data were expressed as mean + SD and the statistical differences were calculated by using the Mann-Whitney U test (positives versus controls and negatives versus controls) and by using the Wilcoxon signed-rank test (pre-challenge versus postchallenge).

Controls and nickel-allergic patients had similar serum concentrations of PCG (positives; SNAS), 1.29 ± 0.56 ; negatives (ACD), 1.31 ± 0.57 ; controls, 1.23 ± 0.50 ; Fig. 1). On the contrary, nickel-allergic patients presented serum levels of NP higher than those measured in healthy subjects and also if the difference was statistically significant only for SNAS (SNAS, 33.36 ± 40.63 , versus controls, 7.48 ± 4.27 ; $p = 0.0014$; Fig. 2). In particular, serum levels of NP in patients with SNAS

were higher (even if not in a statistically significant way; $p = 0.513$, positives prechallenge, 33.36 ± 40.63 , versus negatives prechallenge, 29.69 ± 28.31 ; $p = 0.735$, positives postchallenge, 27.60 ± 33.91 , versus negatives postchallenge, 25.45 ± 26.73) than those measured in the patients affected by nickel ACD only (Fig. 2).

Finally, even if not in a statistically significant way ($p = 0.248$, positives prechallenge, 33.36 ± 40.63 , versus positives postchallenge, 27.60 ± 33.91 ; $p = 0.401$, negatives prechallenge, 29.69 ± 28.31 , versus negatives postchallenge, 25.45 ± 26.73), circulating levels of NP tended to decrease after the oral nickel challenge in patients with SNAS and, at a lower degree, also in patients with nickel ACD only (Fig. 2). Previously, we had observed no change in serum concentrations of NP in healthy subjects who had undergone oral nickel challenge (data not shown).

DISCUSSION

ROS and RNS are formed as by-products of normal cellular metabolism and in higher amounts under pathological conditions, including irritant and allergic dermatitis; they may damage all types of biological molecules. The most common protein modification provoked by free radicals and ROS are carbonyl groups, which can arise after oxidative attack on arginine, lysine, threonine, and proline residues. RNS provide another source of protein damage because peroxynitrite can modify aromatic amino acids producing nitrotyrosine, nitrophenylalanine, and nitrotryptophan.^{13–15}

Excessive levels of nitrated proteins, either systemic or local, have been detected in patients affected by several pathological conditions, including bronchopulmonary dysplasia, inflammatory lung disease, preeclampsia, tuberculosis, hypercholesterolemia, pneumonia, and stroke. In particular, elevated levels of nitrated plasma protein have been associated with development of lung injury and higher risk of coronary artery disease.¹⁶ Human diseases associated with PCG include acute respiratory distress syndrome, neurodegenerative diseases, cataractogenesis, chronic lung disease, chronic renal failure, diabetes, ischemia-reperfusion, preeclampsia, psoriasis, rheumatoid arthritis, and juvenile chronic arthritis.¹⁷

Proteins are, in general, key molecules in various structural and functional aspects of living organisms. Thus, while many other biomarkers, such as malondialdehyde (MDA) levels, show only the existence of an oxidative stress status, independently, on possible consequences on the organism, the observation of oxidative or nitrosative changes on proteins is suggestive for possible disturbances in physiological functioning of important biomolecules and, thus, may be, more efficiently than other biomarkers, used to monitor disease

progression and outcome.^{18,19} In fact, modification of conformation/structure of the polypeptide chain in conditions of oxidative/nitrosative stress can lead to dysfunction/function loss of proteins and inhibition of their degradation and can have also a wide range of downstream functional consequences, causing subsequent cellular dysfunctions and tissue damage.

Furthermore, protein oxidation may be induced either directly by ROS or indirectly by reaction with secondary by-products of oxidative stress; thus, protein carbonylation is the most widely used biomarker for oxidative damage to proteins also in pathological conditions, because it reflects cellular damage induced by a wide array of prooxidant agents, including H₂O₂, Fe²⁺, Cu¹⁺, HOCl, HOBr, ¹O₂, ONOO⁻, γ -irradiation in the presence of O₂, UV radiation, ozone, HNE (4-hydroxy-2,3-nonenal), MDA, xanthine oxidase, and myeloperoxidase. On the other hand, protein S-nitrosylation is emerging as a key mechanism by which nitric oxide (NO) regulates cell signaling and has a broad spectrum of effects in physiology; the occurrence of protein tyrosine nitration under disease conditions represents a shift from the signal transducing physiological actions of NO to oxidative and potentially pathogenic pathways.

Further advantages of PCG and NP as biomarkers of oxidative stress are the relative stability of modified proteins and the high protein concentration in blood. The findings of our biochemical analyses clearly contribute to a better definition of the oxidant/antioxidant network in ACD and in SNAS. In fact, similar serum concentrations of the PCG were observed in all subjects enrolled in our study (controls and nickel-allergic patients), but nickel-allergic patients (ACD only and SNAS) presented serum levels of NP higher than those measured in healthy subjects (Fig. 2). Thus, our findings show that allergy to nickel is associated to a systemic status of oxidative stress, characterized by a peculiar pattern of protein modifications. It has to be underlined that the blood samples used were collected in a period of clinical remission (*i.e.*, after a 1-month low nickel diet, which had improved the symptoms in all of the patients); thus, a persistent condition of nitrosative stress was evident in patients allergic to nickel, even during concomitant exacerbation periods of the underlying pathology. Furthermore, our data show that a similar, specific biochemical pathway leading to an increased protein nitrosylation (without changes in carbonylated proteins) is activated both in patients with ACD only (the 8 patients responsive only to nickel contact challenge) and in patients with SNAS (the 23 patients responsive also to the nickel oral challenge). However, serum levels of NP in patients with SNAS were higher than those measured in the patients affected by nickel ACD only (Fig. 2); thus, nickel-in-

duced oxidative stress could be stronger in patients with systemic signs and symptoms.

The involvement of ROS in the pathogenesis of CD is well described in the literature.²⁰ Consistent with our findings, ACD after exposure to nickel or colophony has been shown to be accompanied by severe abnormal changes in antioxidant activity of blood.²¹ In particular, there is evidence for the participation of NO in inflammatory autoimmune disorders of the skin such as pemphigus and lichen planus^{22,23}; furthermore, increased circulating levels of NO metabolites have been shown in children with atopic eczema/dermatitis syndrome.²⁴

Finally, our findings show that the circulating levels of NP tend to decrease after the oral nickel challenge in patients with SNAS and, at a lower degree, also in patients with nickel ACD only. To explain these findings, we could suppose that acute oral exposure to nickel can cause, in nickel-allergic subjects, a derangement in expression or activity of constitutive and/or inducible isoforms of NO synthase and thus in NO biochemical pathways, therefore justifying the decrease in circulating levels of NP after oral nickel challenge in all patients enrolled in this study.

Another suggestive hypothesis is the existence of a correlation between the cytokine network, involved in contact allergy, and the oxidative balance challenged by oral nickel intake, probably correlated to genetic polymorphisms.²⁵ Redox- and oxidant-mediated pathways positively and/or negatively regulate the expression, distribution, and functional properties of IL-10, which has recently emerged as an anti-inflammatory cytokine with antioxidant properties.²⁶ Several reports point to a possible role of IL-10 in the immunopathology of nickel-induced ACD. In particular, Minang *et al.*^{27,28} reported production of significant levels of IL-10 after nickel stimulation of peripheral blood mononuclear cells from nickel-allergic subjects. Furthermore, nickel sulfate can possess intrinsic capacities to polarize dendritic cells (DCs) toward both DC1 (TNF- α and CXCL10 production) and DC2 (decreased IL-12p70/IL-10 ratio) functions.²⁹ Thus, the oral challenge in nickel-allergic patients could induce the release of IL-10, which, in return, down-regulates the proteins. Consistent with this hypothesis, Borderie and coworkers³⁰ have shown that IL-10 can down-regulate the production of S-nitrosothiols by synovial cells from patients with rheumatoid arthritis; furthermore, the degree of oxidative and nitrosative damage was significantly higher in IL-10-deficient mice used in several models of oxidative stress and inflammation.^{31,32} Of course, these hypotheses are merely speculative at the present time and additional experiments are warranted to support them. In conclusion, this study can be considered the first *in vivo* report on circulating levels of NP (a biomarker of nitrosative stress) in patients with allergy to nickel; these findings may contribute to a better

understanding of the redox homeostasis dysregulation occurring in subjects with nickel ACD and SNAS.

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