

The role of the antioxidant ascorbic acid in the elicitation of contact allergic reactions to *p*-phenylenediamine

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Summary

Background. An allergic contact reaction is accompanied by high oxidative stress in the skin. Pretreatment of the skin with antioxidative substances could reduce the elicitation reaction.

Objectives. To investigate, in a proof-of-principle study, whether pretreatment of the skin with the antioxidant ascorbic acid reduces the elicitation reaction to a *p*-phenylenediamine (PPD)-containing hair dye in sensitized subjects.

Methods. Twelve subjects with contact allergy to PPD, a documented skin reaction to a hair dye simulation exposure model and a history of hair dye-related skin complaints were included in this study. Skin areas on the forearms were, in a left versus right design, exposed to an emulsion with ascorbic acid and an emulsion without ascorbic acid, and then to a 2% PPD-containing hair dye testing formulation. In addition, control areas were exposed to the emulsions and to the PPD-containing hair dye formulation without pretreatment. Skin reactions were graded on day (D)2 and D3.

Results. Pretreatment with ascorbic acid emulsion resulted in a reduction in the elicitation reaction in 7 of 12 subjects at D3 ($p = 0.046$). No statistically significant difference was observed at D2.

Conclusions. Pretreatment of the skin with the antioxidant ascorbic acid had an attenuating effect on the elicitation reaction to PPD in sensitized individuals.

Key words: antioxidant; ascorbic acid; contact allergy; hair dye; *p*-phenylenediamine; CAS no. 106-50-3.

For years, it has been suggested that reactive oxygen species (ROS) play an important role in the stimulation and maintenance of both irritant and allergic contact

dermatitis (1, 2). As a result of exposure of the skin to different irritants and allergens, ROS are upregulated and can disrupt the epidermal barrier (3, 4). ROS can activate several cellular signalling pathways, cause the secretion of various cytokines, and upregulate cellular surface molecules (5–7).

In order to limit damage to the tissue, ROS can be scavenged not only by antioxidative enzymes, such as certain glutathione-*S*-transferases, but also by many non-enzymatic antioxidants, such as water-soluble ascorbic acid (vitamin C) and lipid-soluble α -tocopherol (vitamin E) (8). Both non-enzymatic antioxidants belong to the group of chain-breaking antioxidants, which scavenge radicals by the termination of free

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radical reactions and prevention of chain propagation reactions. Moreover, they are well known for their immunomodulatory properties. The effect of antioxidants on the production of ROS after exposure to the contact allergens 2,4,6-trinitrobenzene sulfonic acid, 1-fluoro-2,4-dinitrobenzene and 4-ethoxymethylene-2-phenyloxazol-5-one has been studied in mice and cultured mice keratinocytes (2). Ascorbic acid contributes to the thickening and improvement of the epidermal barrier in an *in vitro* skin model, and to the reduction of cellular damage caused by oxidative stress in human dermal fibroblasts (9, 10).

The aim of this study was to investigate whether pretreatment of the skin with the antioxidant ascorbic acid reduces the elicitation of a contact allergic reaction. It was prompted by the findings in persons allergic to the hair dye component *p*-phenylenediamine (PPD, 1,4-diaminobenzene, CAS no. 106-50-3), in whom this pretreatment appeared to have an attenuating effect on a patch test with PPD or a PPD-containing hair dye (D A Basketter *et al.*, unpublished data on file). For this study, we chose a group of subjects who were well characterized with respect to their contact allergic reaction to PPD (history and patch test) and to PPD-containing hair dye in a hair-dyeing simulation model (11).

Methods

This study was conducted from April 2014 to July 2014. The study was approved by the Medical Ethical Committee of the University Medical Centre Groningen.

Subjects

The subjects for this study were recruited from a panel that had been well-characterized with respect to their hair dye contact allergy (11). Of this original cohort, 12 subjects were available for this study. In brief, all subjects had a relevant PPD contact allergy, that is, a positive patch test reaction to PPD in combination with a positive history of skin complaints after hair dye use. They also had a positive reaction to a 30-min application of a 2% PPD-containing hair dye formulation on the forearms, mimicking a hair-dyeing procedure (11).

Test materials and procedure

The following test materials, provided by the Procter & Gamble Company, were used in the present study:

- (1) Blank hair dye formulation (Koleston Perfect Basic Chassis[®]); a hair dye cream without PPD and couplers.
- (2) Colour hair dye formulation (Koleston Perfect[®]); a hair dye cream containing 4% PPD and the couplers 1.9% 2-methyl-5-hydroxyethylaminophenol and 3.6% 2-methylresorcinol. The couplers were selected on the basis of their negligible sensitization potency (11–13).
- (3) Emulsion (Magic Off Liquid[®]), containing aq., citric acid, ketoglutaric acid, PEG-12 dimethicone, sodium hydroxide, PEG-35 castor oil, sodium formate, and parfum.
- (4) Powder containing ascorbic acid (Magic Off Powder[®]), containing ascorbic acid (vitamin C), cysteine, methyl hydroxyethyl cellulose, and magnesium sulfate.
- (5) Powder without ascorbic acid (Magic Off Powder[®] without ascorbic acid), containing cysteine, methyl hydroxyethyl cellulose, and magnesium sulfate.

Blank and colour hair dye formulation were mixed in a 1:1 ratio with hydrogen peroxide [6% (wt/wt) Welloxon] before application, resulting in a PPD-free and a PPD-containing hair dye cream, respectively.

The emulsion (# 3) was mixed with either 9.5 g of powder with ascorbic acid or 9.5 g of powder without ascorbic acid to create 'antioxidant emulsion' or 'antioxidant-free emulsion', respectively.

The emulsions with or without ascorbic acid (0.3 ml) were applied in an open test to both forearms on six different 3.8-cm² test areas on both forearms. Hair dye cream (0.1 ml) with either no PPD or 2% PPD was applied to a 0.79-cm² area in the centre of these areas. In addition, on two 0.79-cm² areas, a hair dye cream containing 2% PPD and a hair dye cream without PPD, without pretreatment with the emulsion, were applied (Fig. 1):

- A: PPD-free hair dye cream for 30 min
- B: PPD-containing hair dye cream for 30 min
- C: Antioxidant emulsion for 10 min followed by PPD-free hair dye cream for 30 min
- D: Antioxidant emulsion for 10 min followed by PPD-containing hair dye cream for 30 min
- E: Antioxidant-free emulsion for 10 min followed by PPD-free hair dye cream for 30 min
- F: Antioxidant-free emulsion for 10 min followed by PPD-containing hair dye cream for 30 min
- G: Antioxidant emulsion for 40 min
- H: Antioxidant-free emulsion for 40 min

Readings were performed on day(D)2 and D3, according to ICDRG criteria.

The primary comparison was made between the reaction on the skin area pretreated with an emulsion

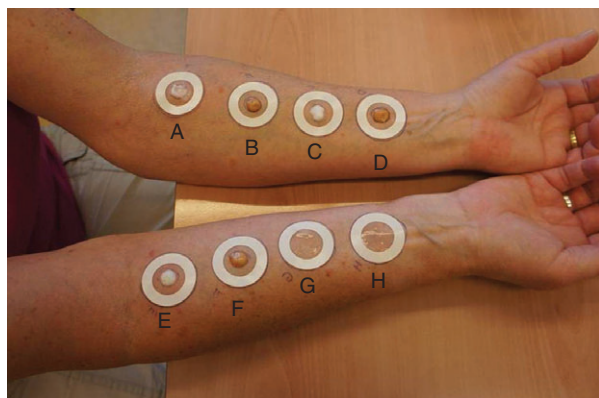


Fig. 1. Test setting. On the left arm from proximal to distal: A, *p*-phenylenediamine (PPD)-free hair dye cream for 30 min; B, PPD-containing hair dye cream for 30 min; C, antioxidant emulsion for 10 min followed by PPD-free hair dye cream for 30 min; D, antioxidant emulsion for 10 min followed by PPD-containing hair dye cream for 30 min. On the right arm from proximal to distal: E, antioxidant-free emulsion for 10 min followed by PPD-free hair dye cream for 30 min; F, antioxidant-free emulsion for 10 min followed by PPD-containing hair dye cream for 30 min; G, antioxidant emulsion for 40 min; H, antioxidant-free emulsion for 40 min.

containing ascorbic acid (area D) and the reaction on the skin area pretreated with an ascorbic acid-free emulsion (area F) at D3. The remaining areas were used as control areas to discern allergic or irritant reactions to ingredients of the antioxidant emulsion, antioxidant-free emulsion, and combinations.

Data analysis and statistics

Data were analysed with IBM™ SPSS™ STATISTICS version 22 for Windows. Testing for differences between patch test grading of the different areas at D2 and D3 was performed with the non-parametric Wilcoxon signed-rank test. Outcomes with a *p*-value of <0.05 were considered to be statistically significant differences.

Results

The results are summarized in Table 1. A total of 12 subjects participated in the study (2 males; 10 females; age 18–68 years). Atopic symptoms were reported in 6 subjects, 1 of whom had a history of atopic dermatitis. None of them reported current symptoms of atopic dermatitis.

Two subjects reported hairdressing as their occupation.

Original patch test reactions (Table 1, column 2) were weakly positive (*n* = 4), strongly positive (*n* = 7), and extremely strongly (*n* = 1) positive. Column 3 (Table 1) shows the results of hair dye use condition testing in

the past, that is, reactions of test subjects to a 30-min application of a PPD-containing hair dye formulation on the forearms > 5 years ago (11).

None of the subjects presented with any reactions on the control areas A, C, E, G, and H.

Pretreatment with ascorbic acid

Elicitation reactions on the areas that were pretreated with the ascorbic acid emulsion are shown in columns 6 (D2) and 9 (D3) of Table 1. A comparison of columns 5 and 8 (pretreatment with an emulsion without ascorbic acid) shows that ascorbic acid reduced the grade of the elicitation reaction to the PPD-containing hair dye product in 7 of 12 subjects on D3 (an impression of the reduced grade is shown in Fig. 2), which is a statistically significant difference (*p* = 0.046). Also on D2, 3 of 12 subjects showed a reduced reaction (*p* = 0.41). Two of these subjects had reduced reactions on both day (D)2 and D3. On D2, an increase in the reaction was seen in 2 subjects, followed by a reduction in the reaction on D3.

Discussion

The aim of this pilot study was to investigate whether pretreatment of the skin with the antioxidant ascorbic acid could reduce the elicitation reaction to PPD in allergic subjects. We chose this contact allergen as a model because a well-characterized panel of subjects was available to us. For this study under hair-dyeing conditions, it was essential that all subjects had, on a previous occasion, shown a positive skin reaction to a PPD-containing hair dye under a hair dye-mimicking exposure. It is remarkable to note that the grades of the current reactions to a hair dye simulation exposure (Table 1, columns 4 and 7) are similar to those after a similar exposure in the previous study a few years ago (Table 1, column 3). This shows that the immune status is maintained over time without boosting (14).

The main finding of this study was the reduced elicitation reaction to a PPD-containing hair dye when the skin was pretreated with the antioxidant ascorbic acid as compared with pretreatment with the vehicle alone (*p* = 0.046) when the reaction was examined according to the conventional ICDRG criteria. As the number of individuals included in this study is low, and the effect of the antioxidant is not large, we consider that, in a similar study to the present one, a more expanded reading protocol should be used. This protocol should also focus on the size of the erythematous area, the number of papules, and the number of vesicles. We suggest a protocol similar to that used in the repeated open application test (15).

Table 1. Results of the open-use test at day (D)2 and D3

Subject number	Original patch test	Previous use test ^a	4	5	6	7	8	9
			D2			D3		
			PPDh	AOX-free + PPDh	AOX + PPDh	PPDh	AOX-free + PPDh	AOX + PPDh
1	+++	+++	+++	+++	+++	+++	+++	+++
2	++	++	++	++	+++	+++	+++	++
3	++	+++	+++	++	++	+++	++	++
4	+	++	++	++	+++	+++	+++	++
5	+	++	++	+++	++	+++	++	++
6	++	+++	++	+++	++	+++	++	++
7	++	N/A	+	++	++	++	++	++
8	+	+	+	++	++	+	++	++
9	++	+	++	++	+	++	++	+
10	++	N/A	++	+	+	++	+	+
11	++	++	++	++	++	+	+	+
12	+	+++	+	++	+	+	++	+

Pretreatment with an ascorbic acid-containing emulsion resulted in a reduction in the elicitation reaction to a hair dye product containing 2% *p*-phenylenediamine (PPD) as compared with pretreatment with emulsion without ascorbic acid, at D3 (Wilcoxon signed-rank test, $p = 0.046$). At D2, no difference was seen between the strengths of the reaction in the areas pretreated with the ascorbic acid-containing emulsion and the areas pretreated with the emulsion without ascorbic acid (Wilcoxon signed-rank test, $p = 0.41$). PPDh: reaction on open use test with hair dye cream containing 2% PPD. AOX-free + PPDh: reaction on open use test with emulsion without ascorbic acid, followed by exposure to hair dye cream containing 2% PPD. AOX+ PPDh: reaction on open use test with pretreatment with antioxidant emulsion containing ascorbic acid, followed by exposure to hair dye cream containing 2% PPD. N/A: not applicable.

^aHair dye use test: positive reaction to a 30-min application of a 2% PPD-containing hair dye formulation on the forearms.

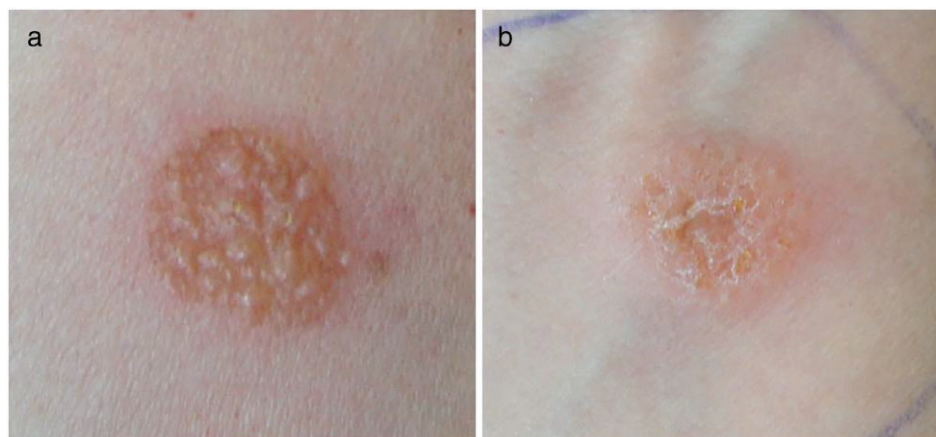


Fig. 2. An impression of the reduced elicitation reaction after pretreatment with ascorbic acid. (a) Area B: the elicitation reaction as a result of exposure to a 2% *p*-phenylenediamine (PPD)-containing hair dye product, showing erythema and coalescing vesicles. (b) Area D: the elicitation reaction as a result of exposure to a 2% PPD-containing hair dye product after pretreatment of the skin with the ascorbic acid-containing emulsion, showing erythema, papules, and probably some vesicles.

It is tempting to also examine the reduction in the elicitation reaction when the skin is not pretreated with anything versus when it is subjected to antioxidant pretreatment. However, this comparison encompasses the vehicle effect of the ascorbic acid emulsion as an additional variable. Nevertheless, this comparison also showed a statistically significant reduction on the antioxidant emulsion-treated skin as compared with the skin that had been solely exposed to 2% PPD-containing hair dye

($p = 0.035$) at D3 (Table 1, column 7 versus column 9). No difference could be observed at D2.

ROS and PPD

There is some evidence of ROS generation during the oxidation of PPD. The oxidative stress that is produced during contact with PPD has been considered to be a reason for its toxicity, although PPD-induced ROS are hardly

detected (16–19). One could assume that ROS originate both from oxidation of PPD and from the contact allergic reaction itself, resulting in a higher ROS concentration, but, as far as we know, this has not been investigated up to now. Nevertheless, this justifies the assumption that antioxidants can play a role in the reduction in the elicitation reaction to PPD.

The role of antioxidants in the reduction in the allergic response to the oxidizing chemical limonene-2-hydroperoxide was investigated by Gäfvert et al. They concluded that antioxidants were able to reduce the allergic reaction to the subgroup of allergens that bind proteins through a radical-mediated pathway (20). Esser et al. showed that, in a mouse model of contact hypersensitivity (to 2,4,6-trinitrobenzene sulfonic acid, 1-fluoro-2,4-dinitrobenzene, oxazolone, and 2,4,6-trinitrochlorobenzene), pretreatment with topical antioxidants (*N*-acetylcysteine, *Reseda luteola* extract, Trolox, tocopherol, and luteoline) could inhibit a contact allergic reaction, even when the antioxidant had been applied up to 180 min after exposure to the allergen. In this study, antioxidants effectively inhibited contact allergic responses in both the sensitization phase and the elicitation phase (2). Ascorbic acid was effective in decreasing the ROS level during exposure to arylhydroxylamine metabolites (21). In canine kidney cells, ascorbic acid was efficient in lowering the ROS level, and remained efficient after longer exposure to PPD (22).

One of the characteristics of ascorbic acid is its instability and its rapid degradation during contact with visible light, and when it is present in an aqueous formulation or in a solution with a high pH (23). In

the present study, these characteristics had a negligible influence, as the antioxidant mixture was prepared immediately before use. Another potential concern with ascorbic acid is its penetration through the stratum corneum. Pinnell et al. showed that ascorbic acid, when applied to porcine skin, easily penetrates when the pH is < 3.5. As the pH of our ascorbic acid-containing emulsion is ~ 2.5, sufficient penetration can be assumed (24, 25).

When the formation of the final colouring molecules is completed, it has been shown that up to 1.1% of unconsumed PPD remains (26), and a limited proportion is known to be able to enter the skin. As sufficient penetration of ascorbic acid can be assumed, we consider it to be very likely that the pretreatment may interfere with the oxidation of this unconsumed PPD while it diffuses through the stratum corneum to the living epidermis. Oxidation of PPD in the absence of couplers was shown to lead to PPD self-coupling products (such as Bandrowski's base), which have been reported to be relevant for contact allergic responses in both the sensitization phase and the elicitation phase (27). Correspondingly, it has been shown *in vitro* that treatment with the antioxidant glutathione (GSH) can prevent the oxidation of PPD (leading to reduced binding to cysteine residues of peptides) and can prevent the formation of self-coupling products (28). In line with the antioxidant effects of GSH, pretreatment with ascorbic acid is likely to reduce the formation of allergenic determinants related to PPD oxidation.

In conclusion, the results of this, albeit small, study are indicative of a reduction in the allergic response of the skin upon pretreatment with the antioxidant ascorbic acid.

References

- Fuchs J, Zollner T M, Kaufmann R, Podda M. Redox-modulated pathways in inflammatory skin diseases. *Free Radic Biol Med* 2001; **30**: 337–353.
- Esser P R, Wolffe U, Durr C. Contact sensitizers induce skin inflammation via ROS production and hyaluronic acid degradation. *PLoS One* 2012; **7**: e41340.
- Byamba D, Kim T G, Kim D H et al. The roles of reactive oxygen species produced by contact allergens and irritants in monocyte-derived dendritic cells. *Ann Dermatol* 2010; **22**: 269–278.
- Kim D H, Byamba D, Wu W H et al. Different characteristics of reactive oxygen species production by human keratinocyte cell line cells in response to allergens and irritants. *Exp Dermatol* 2012; **21**: 99–103.
- Corsini E, Galbiati V, Nikitovic D, Tsatsakis A M. Role of oxidative stress in chemical allergens induced skin cells activation. *Food Chem Toxicol* 2013; **61**: 74–81.
- Larbi A, Kempf J, Pawelec G. Oxidative stress modulation and T cell activation. *Exp Gerontol* 2007; **42**: 852–858.
- Martin S F. New concepts in cutaneous allergy. *Contact Dermatitis* 2015; **72**: 2–10.
- Blokhina O, Virolainen E, Fagerstedt K V. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann Bot* 2003; **91**: 179–194.
- Boyce S T, Supp A P, Swope V B, Warden G D. Vitamin C regulates keratinocyte viability, epidermal barrier, and basement membrane *in vitro*, and reduces wound contraction after grafting of cultured skin substitutes. *J Invest Dermatol* 2002; **118**: 565–572.
- Taniguchi M, Arai N, Kohno K et al. Anti-oxidative and anti-aging activities of 2-O-alpha-glucopyranosyl-L-ascorbic acid on human dermal fibroblasts. *Eur J Pharmacol* 2012; **674**: 126–131.
- Goebel C, Coenraads P J, Rothe H. Elicitation of the immune response to p-phenylenediamine in allergic patients: the role of dose and exposure time. *Br J Dermatol* 2010; **163**: 1205–1211.
- Scientific Committee on Consumer Products. *Opinion on: 2-Methylresorcinol COLIPA No. A44 Adopted by the SCCP During the 18th Plenary Meeting on 16 December 2008*; Brussels, European Commission, 2008.
- Scientific Committee on Consumer Products. *Opinion on: 2-Methyl-5-Hydroxyethylaminophenol COLIPA No. A31 Adopted by the SCCP During the 7th Plenary Meeting on 28 March*

- 2006: Brussels, European Commission, 2006.
- 14 Ho S G, Basketter D A, Jefferies D et al. Analysis of para-phenylenediamine allergic patients in relation to strength of patch test reaction. *Br J Dermatol* 2005; **153**: 364–367.
 - 15 Yazar K, Lundov M D, Faurschou A et al. Methylisothiazolinone in rinse-off products causes allergic contact dermatitis: a repeated open-application study. *Br J Dermatol* 2015; **173**: 115–122.
 - 16 Picardo M, Zompetta C, Marchese C et al. Paraphenylenediamine, a contact allergen, induces oxidative stress and ICAM-1 expression in human keratinocytes. *Br J Dermatol* 1992; **126**: 450–455.
 - 17 Brans R, Dickel H, Bruckner T et al. MnSOD polymorphisms in sensitized patients with delayed-type hypersensitivity reactions to the chemical allergen para-phenylene diamine: a case-control study. *Toxicology* 2005; **212**: 148–154.
 - 18 Viswanathan P N, Gupta V, Misra V. Studies on the dermal toxicity of p-phenylene diamine. *Int J Cosmet Sci* 1985; **7**: 213–218.
 - 19 Mathur A K, Gupta B N, Narang S et al. Biochemical and histopathological changes following dermal exposure to paraphenylene diamine in guinea pigs. *J Appl Toxicol* 1990; **10**: 383–386.
 - 20 Gäfvert E, Nilsson J L, Hagelthorn G, Karlberg A T. Free radicals in antigen formation: reduction of contact allergic response to hydroperoxides by epidermal treatment with antioxidants. *Br J Dermatol* 2002; **146**: 649–656.
 - 21 Vyas P M, Roychowdhury S, Woster P M, Svensson C K. Reactive oxygen species generation and its role in the differential cytotoxicity of the arylhydroxylamine metabolites of sulfamethoxazole and dapsone in normal human epidermal keratinocytes. *Biochem Pharmacol* 2005; **70**: 275–286.
 - 22 Chen S C, Chen C H, Tioh Y L et al. Para-phenylenediamine induced DNA damage and apoptosis through oxidative stress and enhanced caspase-8 and -9 activities in Mardin-Darby canine kidney cells. *Toxicol In Vitro* 2010; **24**: 1197–1202.
 - 23 Ahmad I, Sheraz M A, Ahmed S. Photostability and interaction of ascorbic acid in cream formulations. *AAPS PharmSciTech* 2011; **12**: 917–923.
 - 24 Pinnell S R, Yang H, Omar M et al. Topical L-ascorbic acid: percutaneous absorption studies. *Dermatol Surg* 2001; **27**: 137–142.
 - 25 Stamford N P. Stability, transdermal penetration, and cutaneous effects of ascorbic acid and its derivatives. *J Cosmet Dermatol* 2012; **11**: 310–317.
 - 26 Rastogi S C, Søsted H, Johansen J D et al. Unconsumed precursors and couplers after formation of oxidative hair dyes. *Contact Dermatitis* 2006; **55**: 95–100.
 - 27 Aebly P, Sieber T, Beck H et al. Skin sensitization to p-phenylenediamine: the diverging roles of oxidation and N-acetylation for dendritic cell activation and the immune response. *J Invest Dermatol* 2009; **129**: 99–109.
 - 28 Jenkinson C, Jenkins R E, Maggs J L et al. A mechanistic investigation into the irreversible protein binding and antigenicity of p-phenylenediamine. *Chem Res Toxicol* 2009; **22**: 1172–1180.