



Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Contact sensitizing potential of pyrogallol and 5-amino-*o*-cresol in female BALB/c mice

T.L. Guo^{a,*}, D.R. Germolec^b, Ling X. Zhang^a, W. Auttachoat^a, M.J. Smith^a, K.L. White Jr.^a^a Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298-0613, United States^b Division of the National Toxicology Program, NIEHS, RTP, NC 27709, United States

ARTICLE INFO

Article history:

Received 17 June 2013

Received in revised form 14 August 2013

Accepted 18 October 2013

Available online xxx

Keywords:

Hair dyes

Pyrogallol

5-Amino-*o*-cresol

Chemical hypersensitivity

Lymph node subpopulations

ABSTRACT

Hair dye components such as pyrogallol and cresol have been shown previously to promote allergic reactions such as rashes, dermal inflammation, irritation and dermatitis. The objective of this study was to determine the contact sensitization potential of pyrogallol (PYR) and 5-amino-*o*-cresol (AOC) when applied dermally to female BALB/c mice. Measurement of the contact hypersensitivity response was initially accomplished using the local lymph node assay. For PYR, significant increases in the proliferation of lymph node cells were observed at concentrations of 0.5% (w/v) and higher. For AOC, borderline increases, albeit significant, in auricular lymph node cell proliferation were observed at 5% and 10%. Results from the irritancy assay suggested that PYR, but not AOC, was an irritant. To further delineate whether PYR was primarily an irritant or a contact sensitizer, the mouse ear swelling test (MEST) was conducted. A significant increase in mouse ear thickness was observed at 72 h following challenge with 0.5% PYR in mice that had been sensitized with 5% PYR. In contrast, no effects were observed in the MEST in mice sensitized and challenged with the highest achievable concentration of AOC (10%). Additional studies examining lymph node subpopulations and CD86 (B7.2) expression by B cells further support the indication that PYR was a sensitizer in BALB/c mice. The results demonstrate that PYR is both a sensitizer and an irritant in female BALB/c mice. However, the contact sensitization potential of AOC is minimal in this strain of mouse.

© 2013 Published by Elsevier Ireland Ltd.

1. Introduction

It is estimated that one in three women above the age of 18 and one in ten men above the age of 40 use some type of hair coloring. Permanent coloring accounts for approximately 70% of the dollar volume of the retail market and 85% of the professional market (Corbett, 1988) for hair care products. The use of synthetic colors in hair dyes has been brought to the attention of the public because some ingredients can induce rash, dermal inflammation, irritation, and dermatitis, or even promote cancer (Shvedova et al., 2000; Ferrari et al., 2005; Khumalo et al., 2006; O'Connell et al., 2010).

Pyrogallol (PYR), or pyrogalllic acid (1,2,3-trihydroxybenzene), colorless crystals or laminae when freshly prepared, rapidly assume a darker color upon oxidation. Both PYR and its oxidative product, purpurogallin, are mutagenic (Ben-Gurion, 1979; Takemura et al., 2010). Although the European Community has PYR on the list of

banned substances as ingredients for cosmetics, it is still being used in many other countries (Takemura et al., 2010; ANVISA, 2013), and positive responses to PYR continue to be reported in patients who were suspected to have allergic contact dermatitis following exposure to hairdressing chemicals (Basketter and English, 2009; Wang et al., 2011). In addition, PYR has found extensive use in industrial and commercial processes, including uses in photograph developing, in the manufacture of dyes, in fruit storage, as a component of hair and fur dye, as a tanning agent for leather, and in the production of a novel class of carbamate insecticides (Windholtz, 1976; Dresslerr and Holter, 1982; Mercado-Feliciano et al., 2012; Jing et al., 2013). PYR concentrations in hair dyes and colors typically range from 0.25 to 0.38% by weight (Clayton and Clayton, 1981), and the maximum concentration allowed is 5% (ANVISA, 2013). Moreover, PYR was used as a topical therapy for chronic plaque psoriasis, and it is still used in some places to treat treatment resistant psoriasis (van de Kerkhof, 1986; Gruner et al., 1992; Upadhyay et al., 2010).

5-Amino-*o*-cresol (AOC) is an oxidative intermediate used in hydrogen peroxide-induced coupling reactions in permanent hair dye formulations (Akerson et al., 1994). According to the Food and Drug Administration (FDA) product formulation data, AOC was used in 149 out of 960 hair dye formulations in 1986, and it is still

* Corresponding author at: Department of Veterinary Biosciences and Diagnostic Imaging, College of Veterinary Medicine, University of Georgia, Athens, GA 30602-7382, United States. Tel.: +1 706 542 1358; fax: +1 706 542 0051.

E-mail address: tlguo1@uga.edu (T.L. Guo).

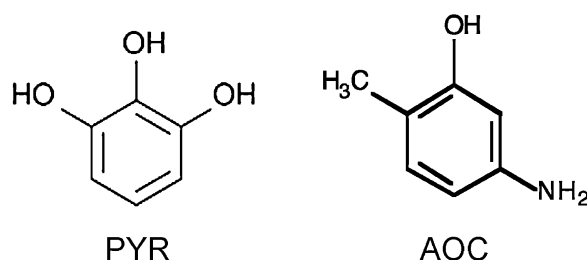


Fig. 1. The structures of pyrogallol and 5-amino-o-cresol.

being used worldwide (Chemland, 2013). AOC concentrations in hair dyes range between 0 and 5%. According to a study by Beck et al. (1994), both pig skin (in vitro) and rat skin (in vivo) are permeable to AOC. In the report from the Scientific Committee on Consumer Products; Opinion on: 4-Amino-2-hydroxytoluene (Opinions SCCP, 2006), two murine studies regarding the skin sensitization potential of AOC have been cited; however, only one mouse strain (CBA/Ca01aHsd) was used. Thus, further evaluation of AOC sensitizing potential in a different mouse strain was useful, especially when only 4 of the 10 female albino Hartley guinea pigs showed evidence of a skin sensitization in another study cited in the same report.

The objective of the present study was to evaluate the contact sensitization potential of both PYR and AOC when applied dermally to female BALB/c mice using the well-established local lymph node assay (LLNA), the irritancy assay (IRR), and the mouse ear swelling test (MEST). In addition to the LLNA and MEST, additional information on the sensitization potential of a compound can be obtained from flow cytometric analysis of the draining lymph nodes (Sikorski et al., 1996; Manetz and Meade, 1999; Gerberick et al., 2002; Auttachoat et al., 2011). Thus, a detailed evaluation of lymph node subpopulations and CD86 (B7.2) expression was also conducted for PYR. The results demonstrate that PYR is both a sensitizer and an irritant in female BALB/c mice. In contrast, data from the LLNA, IRR and MEST suggest that the contact sensitization potential of AOC is minimal.

2. Methods

2.1. Chemicals

PYR, a white odorless crystal (FW 126.11, CAS No. 87-66-1) and AOC, a light brown powder (FW 123.17, CAS No. 2835-95-2), were purchased from Sigma (St. Louis, MO). The structures of PYR and AOC are shown in Fig. 1. Test solutions of both PYR and AOC were prepared daily in the vehicle 4:1, acetone:olive oil (AOO) as recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM; Dean et al., 2001). Initial studies on the solubility of PYR demonstrated that the compound was soluble at concentrations of 75% (w/v) and below in AOO, and AOC at concentrations of 10% (w/v) and below. The positive control, 0.15% (v/v) 1-fluoro-2,4-dinitrobenzene (DNFB, Sigma) was prepared in AOO. α -Hexylcinnamaldehyde (HCA; 85% pure, Sigma) is a mild/moderate skin sensitizer and was used as a calibrant for the LLNA assay, as recommended (Dearman et al., 2001). HCA was prepared at 25% (v/v) in AOO.

2.2. Animals

Female BALB/c mice, 6–8 wks old, were obtained from Charles River Laboratories. The BALB/c mouse is the National Toxicology Program (NTP) strain of choice for conducting contact hypersensitivity testing in rodents. The mice were determined to be hepatitis and Sendai virus free by serology testing. The animals were quarantined for at least 7 days prior to being placed on study, housed and maintained at 21–24°C with 40–70% relative humidity, with a 12-h light/dark cycle. Food (Harlan Teklad laboratory diets NIH-07) and tap water were provided ad libitum in all experiments. Mice were housed four animals per cage in plastic shoebox cages with hardwood bedding. The mice (17–20 g) were randomized prior to the study, and eight mice were assigned for each dose group. Animals were observed for signs of moribundity daily, weighed on the first and last days of each study, and euthanized by CO₂ inhalation. All animal procedures were conducted under an animal protocol approved by the VCU Institutional Animal Care and Use Committee (IACUC).

2.3. Local lymph node assay

Chemical sensitization was initially evaluated using the LLNA (Kimber et al., 1989). On days 1, 2, and 3, mice were treated with test chemical at various concentrations or vehicle by applying a total of 25 μ l to the dorsum of each ear. Three days after the last treatment (day 6), all animals were injected with 0.25 ml (20 μ Ci) ³H-thymidine by lateral tail vein (i.v.). Five hours later, mice were sacrificed, and both the right and left auricular lymph nodes from each mouse were excised and placed into cold phosphate buffered saline (PBS). All lymph nodes were dissociated by mashing between two microscope slides. Lymph node cells were washed twice in PBS, and then resuspended in 3 ml of 5% trichloroacetic acid (TCA, w/v in distilled H₂O), and allowed to incubate for 18–24 h at approximately 4°C. Cell suspensions were then centrifuged and resuspended in 1 ml of 5% TCA and then added to 5 ml of scintillation cocktail. ³H-thymidine incorporation was determined using a Beta Counter (1214 Rackbeta LKB Wallac, Perkin Elmer Instruments, MA, USA). Disintegrations per minute (DPM) were calculated as (counts per minute (CPM) – background count)/(the efficiency of the Beta Counter, which was 71%). Based on the ICCVAM-recommended criteria, a chemical would be classified as positive if at least one concentration reached a stimulation index (SI) of ≥ 3 with the results being statistically significant and dose-responsive. The SI was defined as the total DPM per mouse divided by vehicle DPM per mouse.

2.4. Irritancy assay (IRR)

An irritancy assay was conducted, using a modification of the method described by Gerberick and Ryan (1989), in order to establish the minimal irritating concentration (i.e. the lowest concentration of chemical to elicit a significantly greater ear swelling response than that observed in the vehicle control group) and the maximal non-irritating concentration (the highest concentration producing ear swelling that is not significantly different from the vehicle control). These concentrations were then used to establish treatment levels for the mouse ear-swelling test (MEST) described below. Prior to treatment on day 1, duplicate measurements of ear thickness were obtained for each ear (pre-measurement) using a modified Mitutoyo micrometer (Mitutoyo Corporation, Japan). Each mouse received 12.5 μ l of either test chemical or vehicle on both sides of each ear (Karrow et al., 2002) for four consecutive days. Naïve animals were left untreated. At 24 (± 2) h after the final treatment, duplicate measurements of ear thickness were obtained (post-measurement). The percentage of ear swelling was calculated for each mouse as the [(mean thickness of the post-treated ear/mean thickness of the pre-treated ear) $\times 100$] – 100.

2.5. Mouse ear swelling test

The potential for induction of hypersensitivity responses following treatment with PYR and AOC was also evaluated using the MEST (Gad et al., 1986; Auttachoat et al., 2011), with slight modifications. Experiments were conducted using exposure levels based on the findings in the LLNA and IRR. For sensitization, 50 μ l of PYR or AOC, DNFB, or vehicle was applied directly to the shaved dorsal lumbar surface of each animal on days 1, 2 and 3. The thickness of the right ear of each mouse was measured 5 days following the last exposure (day 8). For the challenge phase, mice were exposed on the dorsal side of the right ear pinna with a total of 25 μ l of vehicle, PYR, AOC or DNFB. Measurements of ear thickness were then made 24 (± 2) h and 48 (± 2) h after challenge in the same manner as the pre-treatment measurements. In some experiments with PYR, a measurement was also taken at 72 (± 2) h after challenge when the difference between the groups of positive control and positive control challenge only (PCCO) did not reach the level of statistical significance at either 24 or 48 h time point. The percent ear swelling at different time points was calculated as described above. The sensitization and challenge concentrations are shown in Table 1.

2.6. Immunofluorescent staining of lymph node cells

Mice were exposed to 0.125–10% PYR using the treatment regimen for the LLNA as described above and were sacrificed at 24, 72 and 96 h after the last PYR treatment. Mice were euthanized with CO₂ inhalation, and the auricular lymph nodes were removed and dissociated as described above. Cells (1.0×10^6) were labeled in a 96-well culture plate with 0.10 ml of the appropriate anti-mouse monoclonal antibodies diluted in PBS (pH 7.4, 1% BSA + 0.1% sodium azide). All antibodies were purchased from BD PharMingen (San Diego, CA). Anti-mouse CD3 ϵ (145-2C11) conjugated to fluorescein isothiocyanate (FITC; 1:80) was used to label T cells. B cells were stained with FITC conjugated affinity purified anti-mouse immunoglobulin (1:80, Becton Dickinson) and phycoerythrin (PE) conjugated antibody for CD86 (B7.2). In some experiments, T cell subpopulations were dual-stained with a PE conjugated anti-mouse CD4⁺ monoclonal antibody (1:80) and a FITC conjugated anti-mouse CD3 monoclonal antibody (1:80). The appropriate isotype controls were included in the analyses. All cell-staining combinations were incubated on ice in the dark for at least 30 min, washed, and then resuspended in 0.10 ml of a 1 μ g/ml solution of propidium iodide (PI) for 5 min prior to flow cytometric enumeration (Becton Dickinson FACScan). The data were analyzed using the CellQuest software (Becton Dickinson, San Jose, CA). Five thousand cells were counted for each sample. Dead cells were

Table 1
Experimental design for MEST with PYR and AOC.^a

Group	Sensitization phase	Challenge phase	Designation group
1	AOO	AOO	Vehicle (VH)
2	AOO	PYR	Vehicle irritancy control (VHIC)
		AOC	
3	PYR	PYR	Treatment groups
	AOC	AOC	
4	AOO	0.15% DNFB	Positive control challenge only (PCCO)
5	0.15% DNFB	0.15% DNFB	Positive Control (PC)

^a Because the test compound might have the potential to induce an irritant effect, which could confound the measurement of mouse ear swelling, both VH and VHIC were included.

eliminated from the analysis by gating out the PI-stained cells, and the forward scatter threshold was set to eliminate red blood cells (Auttachoat et al., 2011).

2.6.1. Data handling and statistical analysis

The data obtained in this study were first tested for homogeneity of variances using Bartlett's Chi Square Test. Homogeneous data were evaluated by a parametric one-way analysis of variance. When significant differences occurred, treatment groups were compared to the appropriate control using Dunnett's Test. If data were non-homogeneous, they were evaluated using a non-parametric analysis of variance. When significant differences occurred, treatment groups were compared to the appropriate control using the Wilcoxon Rank Test. For the Irritancy and LLNA results, treatment groups were compared to vehicle control group. The positive control and naïve animals were compared to the vehicle control using the Student's *t* Test. For the MEST, the vehicle control group was compared to the vehicle irritancy control (VHIC) using the Student's *t* Test, and all treatment groups were compared to VHIC using Dunnett's Test. The positive control was compared to the PCCO using the Student's *t* Test. The level of statistical significance was set at $p \leq 0.05$. All values are presented as mean $\pm$ standard error of mean.

3. Results

3.1. Both PYR and AOC induced a positive response in the LLNA

Based on the ICCVAM-recommended protocol, the highest exposure level of PYR selected for the LLNA was 50% in the first study (NIH, 1999; Dean et al., 2001). Four additional exposure levels of PYR (2.5%, 5%, 10%, 25%, and 50%) were also tested. PYR induced a significant increase in the proliferation of lymph node cells ($SI > 3$) at all concentrations (Fig. 2A). The concentrations of PYR used in the second experiment were 0.5%, 1%, and 2.5%. PYR induced significant increases in the proliferation of lymph node cells at each of these concentrations as well, although the low concentration group (0.5%) did not reach a $SI > 3$ (Fig. 2B). In the third experiment, mice were sensitized with 0.25%, 0.5%, 1%, 2.5%, 5%, and 10% PYR. All groups except 0.25% PYR exhibited a significant increase ($SI > 3$) in the proliferation of lymph node cells when compared to VH controls (Fig. 2C).

The highest exposure level of AOC selected for the LLNA was 10%. As shown in Fig. 2D, exposure to AOC at both 5% and 10% significantly increased the proliferation of the draining lymph node cells, with the increase at 10% exceeding a SI of 3. AOC at 2.5% did not induce significant cell proliferation when compared to the vehicle control. In addition, the borderline positive response in the LLNA at 5–10% was not reproduced in a repeat study (data not shown). The positive controls, 0.15% DNFB and 25% HCA, both produced significant increases in the proliferation of lymph node cells when compared to the vehicle control (Fig. 2).

3.2. PYR, but not AOC, induced a positive response in the IRR assay

It has been noted that some irritants can also induce proliferation in the draining lymph nodes, thereby manifesting as false positives in the LLNA (Montelius et al., 1994; Basketter et al., 1998). Thus, it was important to determine whether irritation contributed to the positive responses observed in the LLNA. As shown in Fig. 3A,

PYR induced a positive response in the IRR at concentrations as low as 0.125%. In contrast, there were no statistically significant changes in percent ear swelling in animals that were exposed to AOC when compared to the vehicle group (Fig. 3B). There were no significant differences in percent swelling between the naïve animals and the vehicle control groups in either study. As expected, significant increases in percent ear swelling were observed in mice treated with 25% HCA and 0.15% DNFB.

3.3. PYR, but not AOC, induced a positive response in the MEST

To further distinguish between the irritant and sensitizing effects of PYR, the MEST was conducted using concentrations based on the results of the LLNA and the IRR studies. In the first MEST, mice were sensitized with PYR at concentrations of 0.25%, 1%, and 5%, and challenged with 0.25% PYR. A challenge concentration of 0.25% PYR was selected as this concentration produced a positive response in the IRR but not in the LLNA. Surprisingly, and in contrast to the strong positive response in the LLNA, there were no significant differences when the naïve, vehicle, or three PYR dose groups were compared to the VHIC group at either 24- or 48-h post-challenge (data not shown).

Because of the lack of agreement between the LLNA and the first MEST study, a second MEST was conducted, using a higher concentration of PYR for the challenge dose. Mice were sensitized with PYR at concentrations of 1% and 5% and were subsequently challenged with 0.5% PYR. Measurements of ear thickness were obtained at 24, 48 and 72 h following the challenge. A significant increase in percent ear swelling was observed at 72 h after challenge in mice that had been sensitized with 5% PYR when compared to VHIC (Fig. 4). The positive control group also exhibited significantly increased percent ear swelling when compared to the PCCO group. No significant changes were observed at either 24 or 48 h time point (data not shown).

Treatment with 5% and 10% AOC for the sensitization phase, and 10% for the challenge phase did not affect ear swelling in the MEST at any concentration or time point evaluated (data not shown).

3.4. PYR-induced changes in lymphocytes of the draining lymph nodes

Although contact sensitization is generally considered to be a T cell-mediated immune response, mice exposed to contact sensitizers may exhibit increases in the percentage of B cells in draining lymph nodes (Manetz and Meade, 1999; Sikorski et al., 1996; Auttachoat et al., 2011). Furthermore, there is evidence that contact sensitizers and irritants can be discriminated using the ratio of %B cells in treated animals to %B cells in vehicle control animals ("B ratio"), with a cutoff value of 1.25 separating irritants (%B ratio < 1.25) and sensitizers (%B ratio ≥ 1.25) (Gerberick et al., 2002). Therefore, a time-course and dose-response study was conducted to determine if changes in lymph node cell populations in

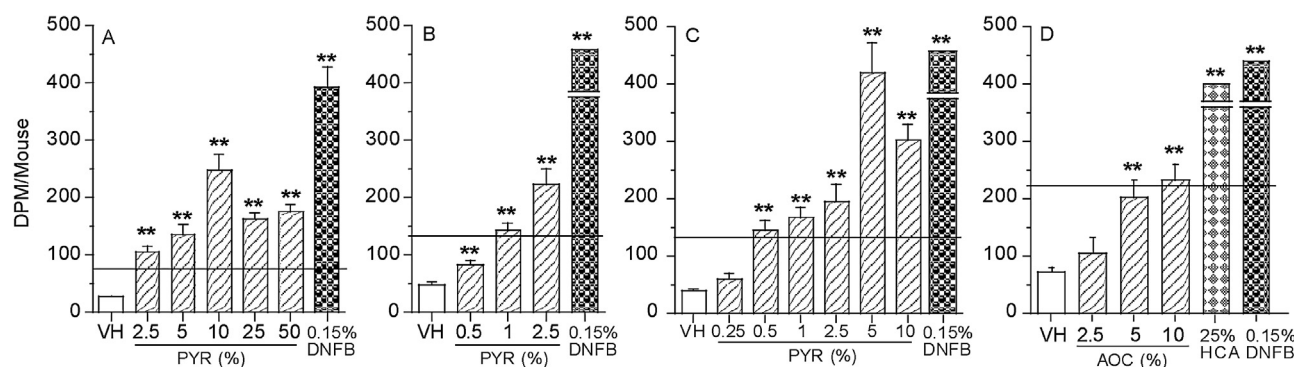


Fig. 2. Pyrogallol (PYR) induced positive contact sensitization responses in three separate experiments using 2.5–50% PYR (A), 0.5–2.5% PYR (B) and 0.25–10% PYR (C) in female BALB/c mice as determined by the local lymph node assay, and a borderline positive response (D) was induced by 5-amino-*o*-cresol (AOC). Results are expressed as the mean DPM $\pm$ SE ($N=7-8$ mice). Positive controls were 0.15% 2,4-dinitrofluorobenzene (DNFB) and 25% hexyl cinnamic aldehyde (HCA). ** $p \leq 0.01$ as compared to the vehicle control group. The solid horizontal line across the graph indicates a stimulation index (SI) = 3.

the draining lymph nodes could differentiate between the irritant and sensitizing effects of PYR. Mice were exposed to 0.125–10% PYR using the same treatment regimen as the LLNA and were sacrificed at 24, 72 and 96 h after the last PYR treatment. As shown in Fig. 5, 24 h after the last exposure to PYR, the %B ratios exceeded 1.25 for the 5% and 10% PYR treatment groups, when compared to the VH control group (Fig. 5A). Consistent with the increase in %B cells, %T cells were decreased at these same PYR treatment levels (Fig. 5C). CD86 (B7.2) is a selective marker for modulation of B cell activation, and its expression in murine draining lymph node cells has been demonstrated following sensitizer, but not irritant, treatment (Gerberick et al., 1999). A significant increase in the expression (the mean fluorescence intensity) of CD86 was observed for B cells at 5% and 10% PYR (Fig. 5B). This was accompanied by a significant increase in the number of total lymph node cells (Fig. 5D). Similar results were observed for the number of total lymph node cells, %B cells, %T cells, and CD86 expression by B cells at 72 and 96 h after the last treatment of PYR, in addition to a 10% decrease in %T cells at the 1% PYR treatment level at the 96 h time point (data not shown). Based on these data, PYR would be classified as a sensitizer at 5% and above according to the criteria previously described (Gerberick et al., 1999, 2002). The positive control DNFB (0.15%)

also decreased %T cells and increased the number of total lymph node cells, %B cells, and CD86 expression (Fig. 5).

In order to determine whether the PYR-induced changes in the draining lymph node cells was dependent on the dosing paradigms utilized, flow cytometric analyses of the percentages of B cells and T cells and the expression of CD86 by B cells was also conducted using the treatment regimen of the MEST. Seventy-two hours after challenge with PYR (0.5%), %B ratios of 1.22 and 1.63 were observed in animals sensitized with 1% PYR and 5% PYR, respectively (Fig. 6A). The %T cells was slightly, albeit significantly, decreased in the 5% PYR group (Fig. 6B). Interestingly, greater decreases were observed for CD4⁺ T cells than total T cells, and a significant decrease was produced in both PYR (1% and 5%)-sensitized groups (Fig. 6C). The positive control DNFB also produced an increase in %B cells and decreases in both %T cells and %CD4⁺ T cells. The expression of CD86 by B cells was not affected by either PYR or DNFB in this study (data not shown).

4. Discussion

In these studies, dermal exposure to PYR produced a positive response in the LLNA at concentrations as low as 0.5%. Furthermore,

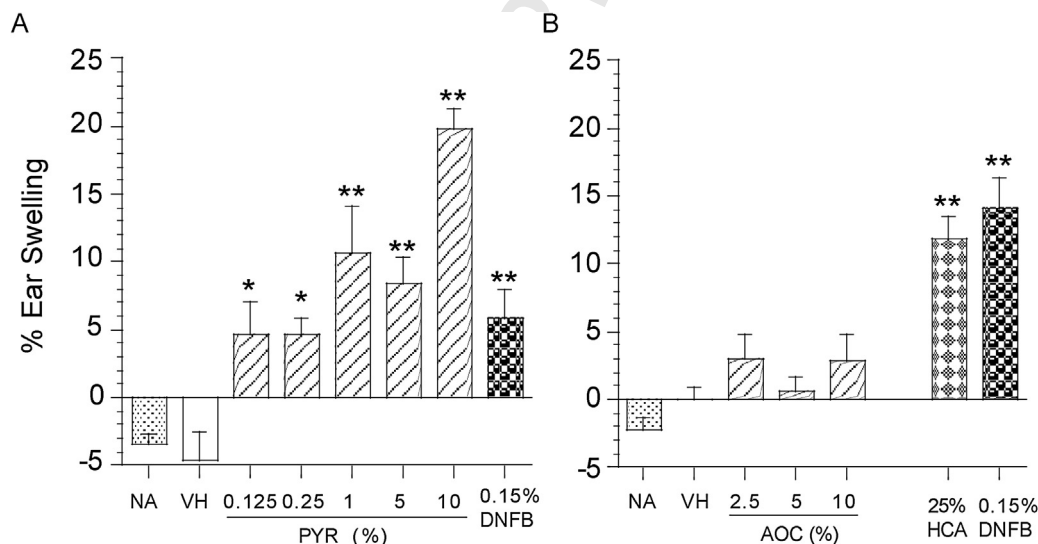


Fig. 3. Irritancy response induced by pyrogallol (A) and 5-amino-*o*-cresol (B) in female BALB/c mice. BALB/c mice were treated with pyrogallol (PYR) and 5-amino-*o*-cresol (AOC) at varied concentrations as described. NA = naïve mice. Vehicle control (VH) was acetone: olive oil (AOO). Positive controls were 0.15% 2,4-dinitrofluorobenzene (DNFB) and 25% hexyl cinnamic aldehyde (HCA). Ear thickness was measured before the treatment and again 24-h after the last treatment in order to calculate the percent change in ear swelling. Results are expressed as the mean $\pm$ SE for each treatment group (6–8 mice). * $p \leq 0.05$; ** $p \leq 0.01$ as compared to the vehicle control group.

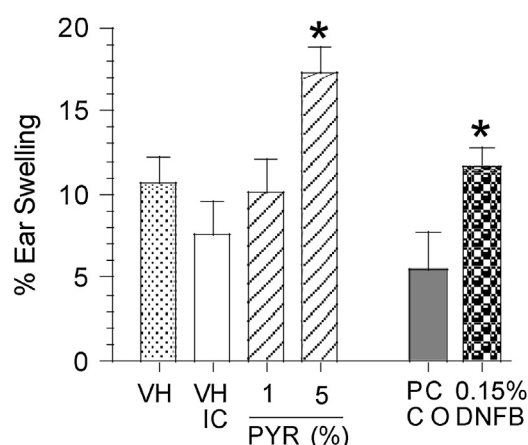


Fig. 4. Contact sensitization response to pyrogallol (PYR) in female BALB/c mice evaluated by the mouse ear swelling test. The data represent the percent ear swelling at 72-h after PYR challenge (0.5%) in BALB/c mice that had been sensitized with PYR at 1% and 5%. The data were expressed as the means $\pm$ SE for each group ($N=8$). Vehicle (VH) = acetone: olive oil (AOO). VHIC (vehicle, irritancy control) = animals sensitized with AOO, and challenged with PYR at 0.5%. PCCO (positive control, challenge only) = animals sensitized with AOO and challenged with 0.15% of 2,4-dinitrofluorobenzene (DNFB). * $p \leq 0.05$ when compared to the VHIC.

an increase in the contact sensitization response was also observed in the MEST. The results from the flow cytometric analysis on the draining lymph node lymphocyte populations also supported the classification of PYR as a sensitizer. These findings are in agreement with the report that 7.4–9.1% of patients who were suspected of having allergic contact dermatitis from hairdressing chemicals were found to have positive allergic reactions to PYR in a patch test (Basketter and English, 2009; Wang et al., 2011).

Because PYR also induced a positive irritant response, it should be classified as an irritant with sensitization potential (Berg et al.,

2012). We have demonstrated that PYR is irritating to the skin at concentrations as low as 0.125%. Skin irritation is defined as a locally arising and non-immunogenic inflammatory reaction, which appears shortly after stimulation and usually disappears in a few days (Harvell et al., 1995). As an acid, PYR has been shown to cause irritation of mucosal membranes in the gastrointestinal tract (Meiser et al., 2000; Gosselin et al., 1981). Therefore, the acidic nature of PYR may be responsible for its irritant effects. Additionally, these effects may be related to PYR's superoxide-generating and apoptosis-inducing activity (Saeki et al., 2000; Kim et al., 2008; Han et al., 2008). Resorcinol, another hair dye ingredient with a similar structure to PYR, has also been shown to be either a weak or moderate sensitizer (Basketter et al., 2007; Goebel et al., 2012).

In the IRR assay for PYR, mice received four doses of PYR at the same site, therefore, the cumulative exposure for the 0.125% dose group was 0.5%. The lack of irritant effects at the concentration of 0.5% in the MEST (e.g. the percent ear swelling was not significantly different between the VHIC and VH groups) suggests that PYR causes a cumulative skin irritation instead of acute skin irritation (Welss et al., 2004). Cumulative skin irritation is the most common type of skin irritation and typically arises after repetitive exposures to mild irritants (Schwindt et al., 1998).

Although the magnitude of proliferation in the draining lymph node cells has been shown to closely correlate with chemical sensitization (Dearman et al., 1999), it has been noted that some irritants induce proliferation in the draining lymph nodes, which are regarded as false positives of the LLNA (Basketter et al., 1998; Gerberick et al., 1992; Montelius et al., 1994). For PYR, a positive response in the LLNA ($SI > 3$) was observed at concentrations as low as 0.5% (an EC₃ value between 0.5 and 1% after combining all three LLNAs). However, PYR was also an irritant at these concentrations. The MEST suggested that PYR was indeed a sensitizer at higher concentrations. Therefore, correct identification of the sensitization potential of a compound demands a combined analysis of data from the LLNA, IRR and MEST studies.

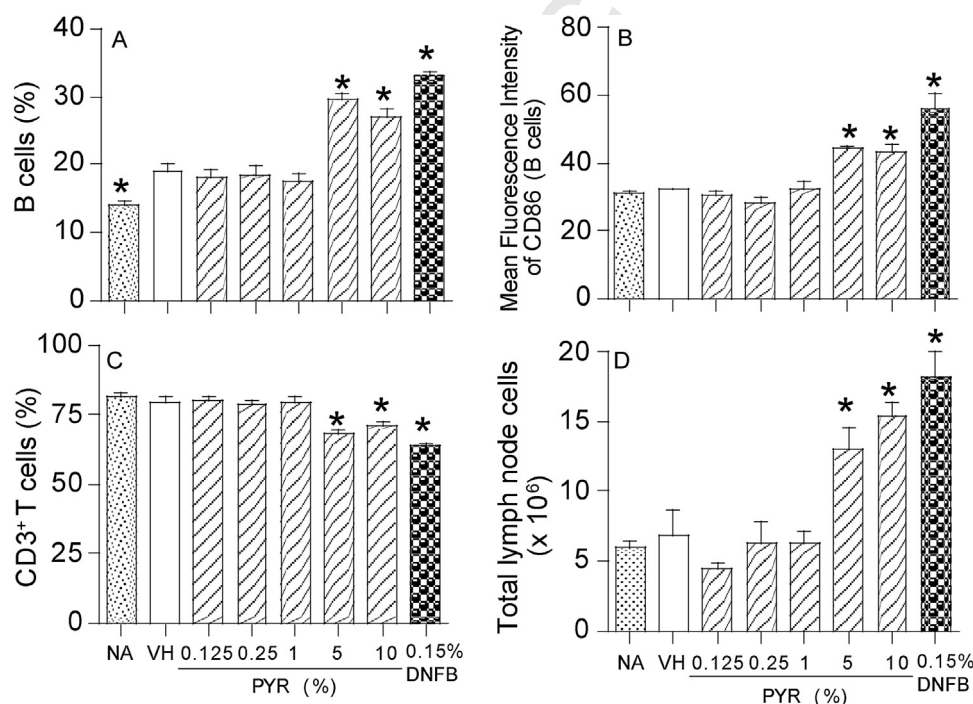


Fig. 5. Flow cytometric analysis of lymph node subpopulations in female BALB/c mice following exposure to pyrogallol (PYR) at varied concentrations using the LLNA treatment regimen. Mice were sacrificed at 24 h after the last PYR treatment. (A) The percentage of B cells, (B) the mean fluorescence intensity (MFI) of CD86 expression by B cells, (C) the percentage of CD3⁺ T cells, and (D) total number of lymph node cells. Results are expressed as the mean $\pm$ SE for each treatment group (6–8 mice). NA = Naïve. Positive control (PC) was 0.15% of 2,4-dinitrofluorobenzene. * $p \leq 0.05$ when compared to vehicle (VH).

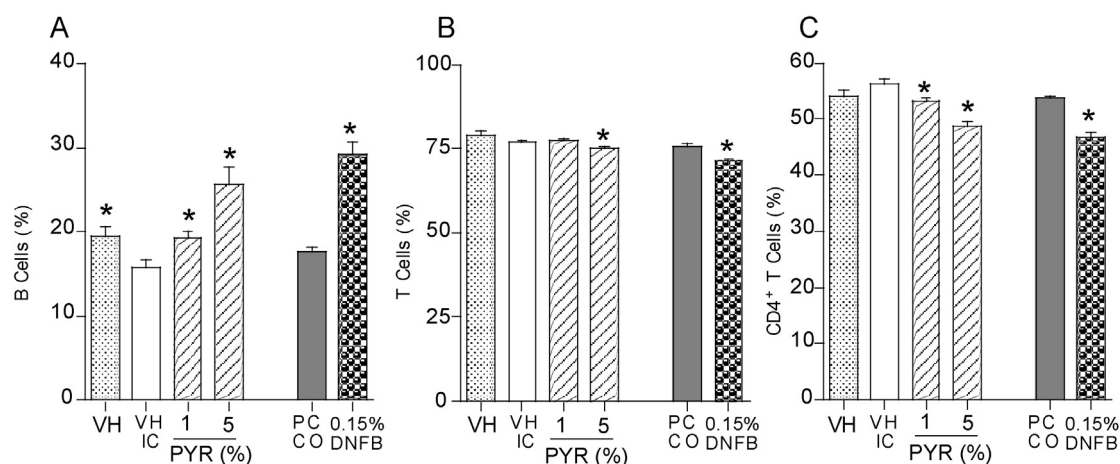


Fig. 6. Flow cytometric analysis of lymph node subpopulations in female BALB/c mice following exposure to pyrogallol (PYR) using the MEST treatment regimen. BALB/c mice were sensitized with 1%, and 5% and challenged with 0.5% PYR. The %B cells (A), %CD3⁺ T cells (B) and %CD4⁺ T cells (C) were examined at 72 h after the PYR challenge. The data represent the means $\pm$ SE for each group ($N=8$). Vehicle (VH) = acetone: olive oil (AOO); VHIC (vehicle, irritancy control) = animals sensitized with AOO, and challenged with PYR at 0.5%; PC (positive control, challenge only) = animals sensitized with AOO and challenged with 0.15% of 2,4-dinitrofluorobenzene (DNFB). * $p \leq 0.05$ when compared to VHIC for VH and PYR groups, and to PC for positive control (PC).

Additional evidence supporting the classification of PYR as a contact sensitizer arises from the examination of lymphocyte subpopulations in the auricular lymph nodes. It has been proposed that allergens and irritants can be distinguished using the %B cell test: vehicle ratio cutoff of 1.25 in the LLNA (Gerberick et al., 2002). Using the same treatment regimen as the LLNA, PYR at 5–10% produced an increase in the %B cells when compared to the vehicle that exceeds the 1.25 cutoff ratio. We also measured the %B cells in mice treated with PYR using the MEST dosing regimen. The %B ratio at 5% PYR also exceeded the 1.25 ratio, while this ratio was just below the cutoff (1.22 vs. 1.25) in animals sensitized with 1% PYR using the dosing regimen of the MEST. Thus, it does not appear that the dosing paradigm impacted the increase in %B cells in the draining lymph nodes.

The effects of PYR on the percentages of CD3⁺ T cells and CD4⁺ T cells in the draining lymph node were also determined using different treatment regimens. The decreases in the %CD3⁺ and %CD4⁺ T cells inversely associated with the sensitization potential of PYR, with a stronger association observed for the %CD4⁺. As for the expression of CD86 by B cells, there were no consistent changes using the different treatment regimens. Although the increases in CD86 expression appeared to correlate with the sensitization potential of PYR using the LLNA dosing regimen, exposure to PYR did not affect the expression of CD86 on B cells in the MEST. The reason for this discrepancy is unclear, and it is possibly related to the timing of the assessments following initial chemical application on the ears (i.e. days 4–7 in the LLNA vs. day 3 in the MEST) and/or the numbers of times the chemical was applied to the ears (i.e. 3 $\times$ in the LLNA vs. 1 $\times$ in the MEST) in these different assays.

In this study, AOC induced a borderline positive response in only one of two LLNA studies conducted, and it failed to induce a positive MEST response. Thus, the contact sensitizing potential of AOC at concentrations up to 10% is considered to be minimal. In the report from the Scientific Committee on Consumer Products; Opinion on: 4-Amino-2-hydroxytoluene (Opinions SCCP, 2006), two murine studies using strain CBA/Ca01aHsd have been included regarding the skin sensitization potential of AOC. In the first study, an EC3 of 0.44% was obtained when aqua/acetone (1:1) mixed with olive oil at a ratio of 4:1 was used as the vehicle. In the second murine study, an EC3 value of 3.4% was obtained when DMSO was used as vehicle. In our studies, an approximate EC3 value of 9% for AOC was obtained when the positive LLNA study was used for extrapolation. The vehicle, acetone:olive oil (4:1), might contribute

partially to the discrepancy; however, the strain of mice used might play a major role because BALB/c is Th2-biased while CBA is Th1-biased (De Vooght et al., 2010). Both CBA and BALB/c mice have been used extensively in the LLNA (ICCVAM, 1999; Woolhiser et al., 1998, 2000; Ullmann et al., 2003; Auttachoat et al., 2011).

In summary, PYR, but not AOC, elicited dermal sensitization in female BALB/c mice, as demonstrated using the LLNA and the MEST. Increases in auricular lymph node cellularity and in the percentage of B cells also support classifying PYR as a contact sensitizer. These findings have significant implications given that 2–10% PYR has been utilized in ointments for use as a topical antipsoriatic (Upadhyay et al., 2010). These data can also be used to estimate the human health risks associated with the use of hair coloring dyes.

Conflict of interest

None.

Uncited references

Luster et al. (1988) and van der Veen et al. (2004).

Acknowledgments

This study was supported by the NIEHS contract N01-ES-55538. The authors would like to thank Dr. Steven D. Holladay at University of Georgia for his critical and editorial review, and D.L. Musgrove and R.D. Brown at Virginia Commonwealth University for their technical assistance. This article may be the work product of an employee or group of employees of the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), however, the statements, opinions or conclusions contained therein do not necessarily represent the statements, opinions or conclusions of NIEHS, NIH or the United States government.

References

- Akerson, J., Pohl, S., Varco, J., Wallace, P., Wolfram, L.J., 1994. Hair preparations. In: Kroschwitz, J.I., Howe-Grant, M. (Eds.), Kirk-Othmer Encyclopedia of Chemical Technology, vol. 12, 4th ed. John Wiley & Sons, Inc., New York, pp. 881–918.
- ANVISA, 2013. The National Health Surveillance Agency (ANVISA). <http://www.isaude.net/en/noticia/34276/public-health/anvisa-authorizing-use-of-lead-acetate-06-in-hair-dye>

- Auttachoat, W., Germolec, D.R., Smith, M.J., White Jr., K.L., Guo, T.L., 2011. Contact sensitizing potential of annatto extract and its two primary color components, cis-bixin and norbixin, in female BALB/c mice. *Food Chem. Toxicol.* 49, 2638–2644.
- Basketter, D.A., Gerberick, G.F., Kimber, I., 1998. Strategies for identifying false positive responses in predictive skin sensitization tests. *Food Chem. Toxicol.* 36, 327–333.
- Basketter, D.A., Sanders, D., Jowsey, I.R., 2007. The skin sensitization potential of resorcinol: experience with the local lymph node assay. *Contact Dermatitis* 56, 196–200.
- Basketter, D.A., English, J., 2009. Cross-reactions among hair dye allergens. *Cutan. Ocul. Toxicol.* 28, 104–106.
- Beck, H., Bracher, M., Bartnik, F.G., 1994. Percutaneous absorption of hair dyes: an interlaboratory comparison of *in vivo* and *in vitro* data with rat and pig. *In Vitro Toxicol.* 7, 305–312.
- Ben-Gurion, R., 1979. Mutagenic and colicine-inducing activity of two antioxidants: pyrogallol and purpurogallin. *Mutat. Res.* 68, 201–205.
- Berg, J.E., Bowman, J.P., Saenz, A.B., 2012. Cumulative irritation potential and contact sensitization potential of tazarotene foam 0.1% in 2 phase 1 patch studies. *Cutis* 90, 206–211.
- Chemland, 2013. <http://chemland.net/05-5-Amino-o-cresol.htm>.
- Clayton, G.D., Clayton, F.E., 1981. *Patty's Industrial Hygiene and Toxicology*, vol. IIA., 3rd ed., rev. John Wiley and Sons, New York, pp. 2594.
- Corbett, J.F., 1988. Hair coloring. *Clin. Dermatol.* 6, 93–101.
- Dean, J.H., Twerdok, L.E., Tice, R.R., Sailstad, D.M., Hattan, D.G., Stokes, W.S., 2001. ICCVAM evaluation of the murine local lymph node assay. Conclusions and recommendations of an independent scientific peer review panel. *Regul. Toxicol. Pharm.* 34, 258–273.
- Dearman, R.J., Basketter, D.A., Kimber, I., 1999. Local lymph nose assay: use in hazard and risk assessment. *J. Appl. Toxicol.* 19, 299–306.
- Dearman, R.J., Wright, Z.M., Basketter, D.A., Ryan, C.A., Gerberick, G.F., Kimber, I., 2001. The suitability of hexyl cinnamic aldehyde as a calibrant for the murine local lymph node assay. *Contact Dermatitis* 44, 357–361.
- De Vooght, V., Vanoirbeek, J.A., Luyts, K., Haenen, S., Nemery, B., Hoet, P.H., 2010. Choice of mouse strain influences the outcome in a mouse model of chemical-induced asthma. *PLoS ONE* 5, e12581.
- Dresslerr, H., Holter, S.N., 1982. Polyhydroxybenzenes. In: Mark, H.F., Other, D.F., Overberger, C.G., Seaborg, G.T. (Eds.), *Kirk-Othmer's Encyclopedia of Chemical Technology*, vol. 18, 3rd ed. Wiley and Sons, New York, p. 670.
- Ferrari, M., Moscato, G., Imbriani, M., 2005. Allergic cutaneous diseases in hairdressers. *Med. Lav.* 96, 102–118.
- Gad, S.C., Dunn, B.J., Dobbs, D.W., Reilly, C., Walsh, D.L., 1986. Development and validation of an alternative dermal sensitization test: the mouse ear swelling test (MEST). *Toxicol. Appl. Pharmacol.* 84, 93–114.
- Gerberick, G.F., Ryan, C.A., 1989. A predictive mouse ear-swelling model for investigating topical phototoxicity. *Food Chem. Toxicol.* 12, 813–819.
- Gerberick, G.F., House, R.V., Fletcher, E.R., Ryan, C.A., 1992. Examination of the local lymph node assay for use in contact sensitization risk assessment. *Fundam. Appl. Toxicol.* 19, 438–445.
- Gerberick, G.F., Cruse, L.W., Miller, C.M., Ridder, G.M., 1999. Selective modulation of B cell activation markers CD86 and I-Ak on murine draining lymph node cells following allergen or irritant treatment. *Toxicol. Appl. Pharmacol.* 159, 142–151.
- Gerberick, G.F., Cruse, L.W., Ryan, C.A., Hulette, B.C., Chaney, J.G., Skinner, R.A., Dearman, R.J., Kimber, I., 2002. Use of a B cell marker (B220) to discriminate between allergens and irritants in the local lymph node assay. *Toxicol. Sci.* 68, 420–428.
- Goebel, C., Diepgen, T.L., Krasteva, M., Schlatter, H., Nicolas, J.F., Blömeke, B., Coenraads, P.J., Schnuch, A., Taylor, J.S., Pungier, J., Fautz, R., Fuchs, A., Schuh, W., Gerberick, G.F., Kimber, I., 2012. Quantitative risk assessment for skin sensitization: consideration of a simplified approach for hair dye ingredients. *Regul. Toxicol. Pharm.* 64, 459–465.
- Gosselin, et al. (Eds.), 1981. *Clinical Toxicology of Commercial Products.*, 5th ed. Williams & Wilkins, Baltimore, p. 190 (Section II).
- Gruner, S., Zwirner, A., Strunk, D., Sönnichsen, N., 1992. The influence of topical dermatological treatment modalities on epidermal Langerhans cells and contact sensitization in mice. *Contact Dermatitis* 26, 241–247.
- Han, Y.H., Kim, S.Z., Kim, S.H., Park, W.H., 2008. Pyrogallol as a glutathione depletor induces apoptosis in HeLa cells. *Int. J. Mol. Med.* 21, 721–730.
- Harvell, J.D., Lammintausta, K., Maibach, H.I., 1995. Irritant contact dermatitis. In: Guin, J.D. (Ed.), *Practical Contact Dermatitis*. McGraw-Hill, New York, pp. 7–18.
- Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), 1999. The Murine Local Lymph Node Assay: A test method for assessing the allergic contact dermatitis potential of chemical/compounds. NIH Pub No. 99-4494.
- Jing, G., Huang, H., Yang, B., Li, J., Zheng, X., Jiang, Y., 2013. Effect of pyrogallol on the physiology and biochemistry of litchi fruit during storage. *Chem. Cent. J.* 7, 19.
- Karrow, N.A., Guo, T.L., Leffel, E.K., Zhang, L.X., McCay, J.A., Germolec, D.R., White Jr., K.L., 2002. Sodium metasilicate hypersensitivity in BALB/c mice. *Am. J. Contact Dermat.* 13, 133–139.
- Kim, S.W., Han, Y.W., Lee, S.T., Jeong, H.J., Kim, S.H., Kim, I.H., Lee, S.O., Kim, D.G., Kim, S.H., Kim, S.Z., Park, W.H., 2008. A superoxide anion generator, pyrogallol, inhibits the growth of HeLa cells via cell cycle arrest and apoptosis. *Mol. Carcinog.* 47, 114–125.
- Kimber, I.A., Hilton, J., Weisenberger, C., 1989. The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of *in situ* measurement of lymphocyte proliferation. *Contact Dermatitis* 21, 215–220.
- Khumalo, N.P., Jessop, S., Ehrlich, R., 2006. Prevalence of cutaneous adverse effects of hairdressing: a systematic review. *Arch. Dermatol.* 142, 377–383.
- Luster, M.I., Munson, A.E., Thomas, P.T., Holsapple, M.P., Fenters, J.D., White, K.L., Lauer, L.D., Germolec, D.R., Rosenthal, G.J., Dean, J.H., 1988. Development of a testing battery to assess chemical-induced immunotoxicity: National Toxicology Program's guidelines for immunotoxicity evaluations in mice. *Fundam. Appl. Toxicol.* 10, 2–19.
- Manetz, T.S., Meade, B.J., 1999. Development of a flow cytometry assay for the identification and differentiation of chemicals with the potential to elicit irritation, IgE-mediated, or T cell-mediated hypersensitivity responses. *Toxicol. Sci.* 48, 206–217.
- Mercado-Feliciano, M., Herbert, R.A., Wyde, M.E., Gerken, D.K., Hejtmancik, M.R., Hooth, M.J., 2012. Pyrogallol-associated dermal toxicity and carcinogenicity in F344/N rats and B6C3F1/N mice. *Cutan. Ocul. Toxicol.* PubMed PMID: 23231012, PubMed Central PMCID: PMC3637858 (Epub ahead of print).
- Meiser, H., Hagedorn, H.W., Schulz, R., 2000. Pyrogallol poisoning of pigeons caused by acorns. *Avian Dis.* 44, 205–209.
- Montelius, J., Wahlkvist, H., Boman, A., Fernstrom, P., Grabergs, L., Wahlberg, J.E., 1994. Experience with the murine local lymph node assay: inability to discriminate between allergens and irritants. *Acta Derm. Venerol. (Stockh.)* 74, 22–27.
- NIH, 1999. The murine local lymph node assay: a test method for assessing the allergic contact dermatitis potential of chemicals/compounds. The results of an independent peer review evaluation coordinated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICEATM). NIH Publication No. 99-4494.
- O'Connell, R.L., White, I.R., Mc Fadden, J.P., White, J.M., 2010. Hairdressers with dermatitis should always be patch tested regardless of atopy status. *Contact Dermatitis* 62, 177–181.
- Opinions SCCP, 2006. Scientific Committee on Consumer Products opinion on: 4-Amino-2-hydroxytoluene COLIPA no. A27 (191KB) Adopted by the SCCP during the 9th plenary meeting of 10 October 2006.
- Saeki, K., Hayakawa, S., Isemura, M., Miyase, T., 2000. Importance of a pyrogallol-type structure in catechin compounds for apoptosis-inducing activity. *Phytochemistry* 53, 391–394.
- Schwindt, D.A., Wilhelm, K.P., Miller, D.L., Maibach, H.I., 1998. Cumulative irritation in older and younger skin: a comparison. *Acta Derm. Venerol.* 78, 279–283.
- Shvedova, A.A., Kommineni, C., Jeffries, B.A., Castranova, V., Tyurina, Y.Y., Tyurin, V.A., Serbinova, E.A., Fabisak, J.P., Kagan, V.E., 2000. Redox cycling of phenol induced oxidative stress in human epidermal keratinocytes. *J. Invest. Dermatol.* 114, 354–364.
- Sikorski, E.E., Gerberick, G.F., Ryan, C.A., Miller, C.M., Ridder, G.M., 1996. Phenotypic analysis of lymphocyte subpopulations in lymph nodes draining the ear following exposure to contact allergens and irritants. *Fundam. Appl. Toxicol.* 34, 25–35.
- Takemura, Y., Wang, D.H., Sauriasari, R., Horita, M., Tsutsui, K., Sano, K., Masuoka, N., Takigawa, T., Takaki, J., Ogino, K., 2010. Evaluation of pyrogallol-induced cytotoxicity in catalase-mutant *Escherichia coli* and mutagenicity in *Salmonella typhimurium*. *Bull. Environ. Contam. Toxicol.* 84, 347–350.
- Ullmann, L.G., Corney, S.J., Arcelin, G., Wang-Fan, W., 2003. Comparison of the allergenic potency of alpha-hexylcinnamaldehyde (HCA) and 2-mercaptobenzothiazole (MBT) in six strains mice in murine local lymph node assay (LLNA). *Toxicologist* 72, 1835.
- Upadhyay, G., Gupta, S.P., Prakash, O., Singh, M.P., 2010. Pyrogallol-mediated toxicity and natural antioxidants: triumphs and pitfalls of preclinical findings and their translational limitations. *Chem. Biol. Interact.* 183, 333–340.
- van der Veen, R.C., Dietlin, T.A., Karapetian, A., Holland, S.M., Hofman, F.M., 2004. Extra-cellular superoxide promotes T cell expansion through inactivation of nitric oxide. *J. Neuroimmunol.* 153, 183–189.
- Wang, M.Z., Farmer, S.A., Richardson, D.M., Davis, M.D., 2011. Patch-testing with hairdressing chemicals. *Dermatitis* 22, 16–26.
- Welss, T., Basketter, D.A., Schroder, K.R., 2004. *In vitro* skin irritation: facts and future. State of the art review of mechanisms and models. *Toxicol. In Vitro* 18, 231–243.
- Windholtz, M. (Ed.), 1976. *Merk Index.*, 9th ed. Merck and Co., Inc., Rahway, NJ, p. 1038.
- Woolhiser, M.R., Hayes, B.B., Meade, B.J., 1998. A combined murine local lymph node and irritancy assay to predict sensitization and irritancy potential of chemicals. *Toxicol. Methods* 8, 245–256.
- Woolhiser, M.R., Munson, A.E., Meade, B.J., 2000. Comparison of mouse strains using the local lymph node assay. *Toxicology* 146, 221–227.