

Skin irritation and sensitization potential of oxidative hair dye substances evaluated with *in vitro*, *in chemico* and *in silico* test methods

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ABSTRACT

Permanent oxidative hair dyes are widely used but their toxicity is not well-established. Here we aimed to evaluate the skin sensitization and irritation of nine hair dye substances (MAP, MRP-N, RS, PAOX, 2,4-DAPE, 2,6-PYR, PPD, Grey HED and PM) permitted for use in EU and Korea, using *in vitro* and *in chemico* and *in silico* test methods. Skin sensitization was evaluated by the KeratinoSens™ assay, Direct Peptide Reactivity Assay (DPRA) and DEREK. Six of nine dyes tested were determined as sensitizers in common. However, the decision for MAP, RS or PAOX was diverged across assays showing 2 positives and 1 negative. Skin irritation of hair dye substances was assessed with or without 6% H₂O₂ on a reconstructed human epidermis, Epiderm™, which demonstrated that H₂O₂ increased the skin irritation potential of some hair dyes. PPD and PM were determined to be irritants with H₂O₂. Epidermal damages by hair dye and H₂O₂ could be further confirmed through the histology of tissue remaining after MTT assay. Collectively, our study demonstrated that hair dyes possess potential skin sensitization and irritation issues which could be further aggravated by H₂O₂.

1. Introduction

The most commonly used permanent (oxidative) dyes contain a range of different components that react with each other to produce the desired end color. There are three main components in permanent hair dyes: primary intermediates, developer, and coupling agents. *p*-Phenylenediamine (PPD) and *p*-aminophenol (PAP) are the common examples of primary intermediates. They are oxidized by hydrogen peroxide (H₂O₂) to form reactive species, which then further react with coupling agents to produce the final colors (Seo et al., 2012). Developer includes H₂O₂ and ammonia or ethanolamine. H₂O₂ is the agent responsible for oxidation of primary intermediates, and also lightens the natural pigments, like melanin, eumelanin and pheomelanin, present in the hair. Ammonia or its milder alternative ethanolamine raises pH and

causes the hair cuticle to swell (Morel and Christie, 2011). Opened hair cuticle allows H₂O₂ and dye molecules to pass into the cortex of hair shaft. The last component, coupling agent, reacts with reactive forms of primary intermediates to produce coloration (Corbett, 1984) (Fig. 1).

Since numerous synthetic chemicals are included in hair dyes, there is a concern that hair dyes may cause adverse effects on the skin. One study showed that 89% (150/169 products) of commercial oxidative-type hair dye formulations are mutagenic (Ames et al., 1975). A higher risk ratio of the cancer development was also found among women who had used hair dyes than those who had never used them (Hennekens et al., 1979; Nohynek et al., 2004). Especially hair dressers are continually exposed to allergic substances in hair dye products, and they are at the risk of the skin sensitization and contact allergic dermatitis (Lind et al., 2005).

Abbreviations: MAP, 3-aminophenol; MRP-N, 2-methyl-1,3-benzenediol; RS, resorcinol; PAOX, 5-[(2-hydroxyethyl)amino]-2-methylphenol; 2,4-DAPE, 2-(2,4-Diaminophenoxy)ethanol dihydrochloride; 2,6-PYR, 2,6-pyridinediamine; PPD, 1,4-benzenediamine; Grey HED, 2,2'-[(4-aminophenyl)imino]diethanol sulfate (1:1); PM, 4-(methylamino)phenol sulfate (2:1)

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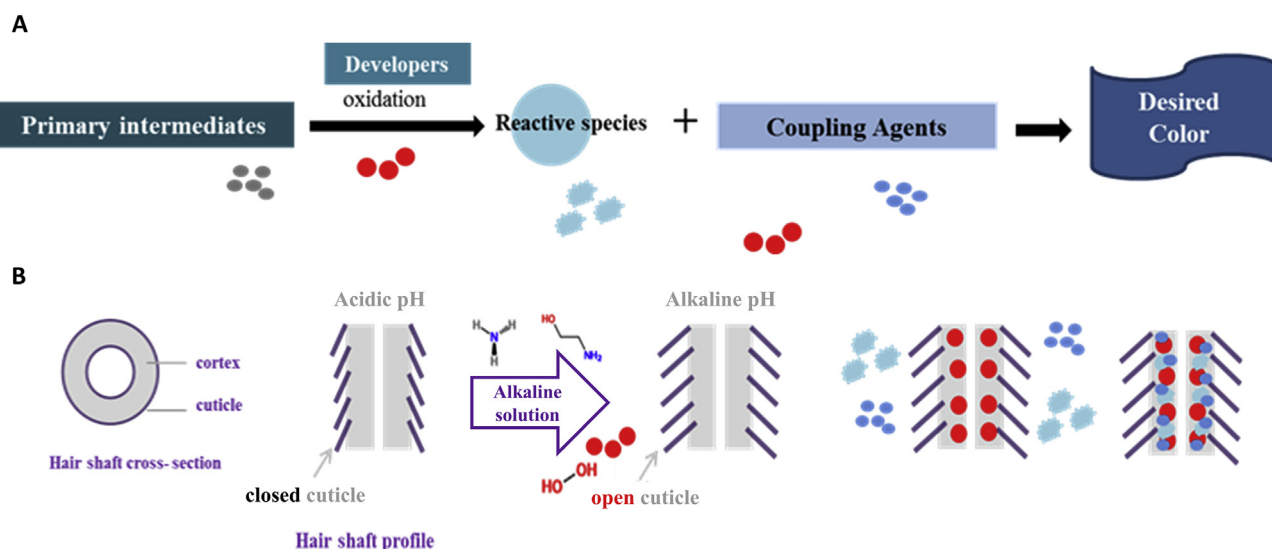


Fig. 1. A schematic view of permanent hair dye. (A) Chemistry of permanent hair dyeing, and (B) The process of hair dyeing.

Indeed, permanent hair dyes are well-known skin sensitizers. Consumers may frequently develop the sensitization reaction, allergic contact dermatitis (ACD) (Miller et al., 2005). While PPD is the most concerned allergen in permanent hair dyes, there are also several other hair dye substances that have shown skin sensitization potency. According to Schmidt et al., toluene-2,5-diamine (PTD), which is used more frequently in hair dye products than PPD in Scandinavian countries, induced the skin immune response at much lower concentration than PPD (Schmidt et al., 2014). A semi-permanent hair dye, Basic Red 51 also showed cytotoxicity effects on HaCaT cells and 3D culture (Zanoni et al., 2014). Considering the potential skin sensitization of hair dyes, it would be necessary to know the comprehensive skin sensitization potentials of hair dyes.

There are many alternative to animal test methods for the evaluation of the skin sensitization potency. Each test can only evaluate specific stages in the sensitization process, thus it is more accurate to integrate results from multiple assays when evaluating overall skin sensitization potency. Here we adopted three tests to evaluate skin sensitization potential of hair dyes. DEREK is a knowledge-based expert system designed to predict the toxicity of a chemical from its structure. Its skin sensitization predictions are the result of a reasoning process involving consideration of the presence of alerts, an estimation of skin permeability, and the existence of relevant toxicity data for the chemical of interest (Judson et al., 2003) which may broadly cover early phases of skin sensitization. DRPA assesses whether a chemical can bind with synthetic peptides to mimic haptenation process critical for T-cell recognition of the allergen (Gerberick et al., 2008) which would address the molecular initiation event of skin sensitization. On the other hand, KeratinoSens™ assay addresses keratinocyte activation with respect to the Keap1-Nrf2-ARE regulatory pathway leading to induction of luciferase under the control of the Antioxidant Response Element (Ball et al., 2011) which will address key event 1 of skin sensitization. Combining the results of these three assays will give more detailed information on the skin sensitization potential of hair dyes.

Skin irritation is a non-immunologic local inflammatory reaction caused by external stimuli. However, the differences between allergic and irritant reactions are rather conceptual. It is nearly impossible to discriminate the sensitization and irritation because some irritants can mimic weak allergens, and irritants also activate the similar profile of antigens as allergens (Fluhr et al., 2008). Therefore, both the skin sensitization and irritation reactions may co-exist during hair dying process. However, little information is available about the irritation

potency of hair dye substances. Especially there is no information on the skin irritation of the combination of hair dyes with H_2O_2 , the essential component of hair dye mixture, which reacts with other substances in the final mixture and can synergistically cause oxidative stress and irritation on skin (Seo et al., 2012). In the current study, we utilized 3D reconstituted human Epidermis equivalent, Epiderm™ for the skin irritancy testing of hair dyes with or without H_2O_2 .

Collectively, we aimed to provide a comprehensive evaluation of the skin sensitization and the skin irritation potential of 9 hair dye chemicals with or without H_2O_2 using *in vitro*, *in chemico* and *in silico* alternative animal tests. We believe that our study might provide a critical insight into developing safer hair dye products.

2. Materials and methods

2.1. Hair dye substances

Nine hair dye substances were selected through reviewing the previous report (Søstved et al., 2004; Regulation(EC), 2009), the availability in the hair dye product market of South Korea and solubility appropriate for *in vitro* tests. Maximum and final permitted concentration according to the Cosmetics Directed (76/768/EEC, consolidated version 2008-04-24) is given (Bølling et al., 2009). 9 hair dye compounds used in this study were provided in powder form by AMOREPACIFIC CO/R&D Center (Yongin-si, Republic of Korea) (Table 1).

2.2. DEREK Nexus

DEREK Nexus 2.2, an expert knowledge-based system, was purchased from Lhasa Limited (Leeds, UK) for use in predicting the skin sensitizing potential of chemicals (Langton et al., 2006). If the analysis outcome was defined as contradicted/certain/probable/plausible/equivocal, the test chemical was judged as a sensitizer. If defined as no report, the test chemical was judged as a non-sensitizer (Takenouchi et al., 2015).

2.3. Direct Peptide Reactivity Assay (DPRA)

The DPRA was conducted according to the method of Gerberick et al. (2007). Peptides, Ac-RFAAKAA-COOH (lysine peptide) and Ac-RFAACAA-COOH (cysteine peptide) were purchased from Peptron (Daejeon, Republic of Korea). Peptide stock solutions were prepared to a

Table 1
Hair dye substances.

Chemical	IUPAC name	CAS no.	Molecular Weight	Chemical structure	Maximum permitted concentration ^a	Final treatment concentration(%) ^b	Final treatment concentration(M)
MAP	3-Aminophenol	591-27-5	109.13		2.4%	1.2%	0.110
MRP-N	2-Methyl-1,3-benzenediol	608-25-3	124.14		1.8%	0.9%	0.072
RS	Resorcinol	108-46-3	110.10		2.5%	1.25%	0.114
PAOX	5-[(2-Hydroxyethyl)amino]-2-methylphenol	55302-96-0	167.21		2%	1%	0.060
2,4-DAPE	2-(2,4-Diaminophenoxy)ethanol dihydrochloride	66422-95-5	241.14		4%	2%	0.083
2,6-PYR	2,6-Pyridinediamine	141-86-6	109.13		0.3%	0.15%	0.078
PPD	1,4-Benzenediamine	106-50-3	108.14		4%	2%	0.185
Grey HED	2,2'-[(4-Aminophenyl)imino] diethanol sulfate (1:1)	54381-16-7	312.34		5%	2.5%	0.080
PM	4-(Methylamino)phenol sulfate (2:1)	55-55-0	344.38		1.35%	0.68%	0.020

^a Maximum concentration permitted according to the Cosmetics Directive (76/768/ECC, consolidated version 2008-04-24).

^b After mixing under oxidative conditions (6% of hydrogen peroxide solution), in ready product to apply to hair.

final concentration of 0.667 mM in 100 mM ammonium acetate buffer (pH of 10.2) for the Lys peptide and in 100 mM phosphate buffer (pH of 7.5) for the Cys peptide. Test chemical stock solutions were prepared to a concentration of 100 mM in acetonitrile. Each reaction was conducted in triplicate. Control samples were prepared by combining 750 µl of peptide solution with 250 µl of acetonitrile. The samples were mixed by inversion, and then incubate in the HPLC auto sampler (in the dark) at 25 °C for 24 h. Following incubation, the samples were analyzed by LC-MS for new adduct peaks (Ball et al., 2011).

2.4. KeratinoSens™ assay

The KeratinoSens™ cell line, a kind gift from Dr. Andreas Natsch, Head of *in vitro* Toxicology research at Givaudan Fragrances, is derived from the human keratinocyte cell line HaCaT. It contains a stable insertion of a luciferase gene under the control of the ARE-element of the gene AKR1C2. All tests were proceeded according to the previously published SOP (Emter et al., 2010). To summarize, cells cultured as described previously, were seeded to 96 well plates for 24 h. The medium was then replaced with medium containing the test substance and a final level of 1% of the solvent, DMSO. Each test substance was tested at 12 binary dilutions in the range from 0.98 to 2,000 µM. Two test plates contained 4 and 5 test chemicals respectively, 6 wells with the solvent control, 1 well with no cells for background value and 5 wells with the positive control, cinnamic aldehyde, in five different concentrations. In each repetition, three parallel replicate plates were prepared for cytotoxicity measurement using an MTT assay. Cells were incubated for 48 h with the test compounds, and then luciferase activity and cytotoxicity were determined. This full procedure was repeated at least two times for each chemical. For each chemical in each repetition and at each concentration, the gene induction compared to DMSO controls and the wells with statistically significant induction over the threshold of 1.5 and 3.0 were determined. Moreover the maximal fold-induction ($I_{\max}$) and the $EC_{1.5}$ and $EC_{3.0}$ value (concentration in µM for induction above the threshold) were calculated. Chemicals are rated as positive in the assay if the following three criteria are fulfilled (i) The $EC_{1.5}$ value is below 1,000 µM in all three repetitions or in at least 2 repetitions, (ii) at the lowest concentration with a gene induction above 1.5-fold (i.e. at the $EC_{1.5}$ determining concentration), the cellular viability is above 70% and (iii) there is an apparent overall dose-response for luciferase induction, which is similar between the repetitions (Andres et al., 2013).

2.5. *In vitro* skin irritation test (SIT) with 3D reconstructed human epidermis skin model

The skin model used in the skin irritation tests, Epiderm™ (MatTek, Ashland, MA, USA) is commercially available reconstructed human Epidermis models. Each chemical was first dissolved up to its maximum permitted concentration shown in Table 1 in 0.2% DMSO in DPBS, which is non-irritating to the tissue-samples. Groups containing H₂O₂ solution were mixed with 6% H₂O₂ in 0.2% DMSO in PBS into 1:1 ratio. Groups without 6% H₂O₂ solution were diluted with 0.2% DMSO in DPBS into 1:1 ratio.

Skin irritation test was conducted according to OECD TG 439 (OECD, 2015) and previous reports (Kim et al., 2016; Lee et al., 2017). After 18–24 h of pre-incubation, the tissue was removed from the incubator and 30 µL of the test chemicals were applied to the epidermal surface side at RT for 25 min followed by at 37 °C for 35 min. A nylon mesh was placed over the tissue on the surface of the insert with care not to damage tissue layer. After 60 min, the tissues were rinsed with DPBS. Tissues were post-incubated for 42 h and then the entire medium was removed. Tissues were blotted and transferred to a 24-well plate that pre-filled with 0.3 mL of MTT (1.0 mg/mL) and incubated for 3 h at 37 °C and 5% CO₂. Next, tissues were rinsed with DPBS again and transferred to a new 24-well plate, prefilled with 2 mL of isopropanol. Formazan extraction was performed at RT for at least 2 h with gentle shaking on a plate shaker and 200 µL of formazan extract per tissue was transferred to a 96-well plate. Optical density (OD) was measured at 570 nm using isopropanol as a blank.

2.6. Histology of Epiderm™ after MTT assay

For the histological examination, after MTT extraction, Epiderm™ samples were fixed in 10% neutral-buffered formalin. Preserved tissues from each group were paraffin wax-embedded, sectioned, stained with hematoxylin and eosin (H&E), and then examined microscopically under an Olympus DP71 microscope (Center Valley, PA, USA). All images of tissues were obtained using the virtual slide system (Aperio Scanscope XT, Vista, CA, USA).

2.7. Statistical analysis

Experimental values for DPRA and KeratinoSens™ assay have been represented as mean ± SD, and those for Epiderm™ as mean ± averaged difference (n = 3). Data were analyzed using analysis of variance (ANOVA) or student's *t*-test. *P*-values of 0.05 or less were considered significant.

Table 2
Assessment of skin sensitization potential of hair dye.

Chemical	DEREK	DPRA					KeratinoSens™				
	Derived LLNA EC ₃ (%)	Prediction (Alert) ^a	Cys (%)	Lys (%)	Average (%)	Reactivity class	I _{max}	EC _{1.5} (μM)	EC _{3.0} (μM)	Cell viability at EC _{1.5}	Result (Repetitions positive)
MAP	1.70 (Moderate)	Plausible (427)	3.0	0.1	1.6	Minimal	1.63	13.56	46.73	> 70%	+ (3/3)
MRP-N	50.00 (Weak)	Plausible (440)	4.6	13.7	9.1	Low	2.37	3.58	10.04	> 70%	+ (3/3)
RS	3.50 (Moderate)	Plausible (440)	7.9	11.4	9.7	Low	1.05	> 2000	> 2000	> 70%	- (0/3)
PAOX	Misclassified/ Unclassified (–)	Misclassified/ Unclassified (–)	50.5	22.2	36.3	Moderate	1.39	2.86	7.88	> 70%	+ (3/3)
2,4-DAPE	3.20 (Moderate)	Plausible (427)	86.5	5.5	46.0	High	6.99	1.69	4.05	> 70%	+ (3/3)
2,6-PYR	0.25 (Strong)	Equivocal (861)	100	7.2	53.6	High	1.32	15.42	38.85	> 70%	+ (3/3)
PPD	0.09 (Extreme)	Certain (837)	70.4	37.8	54.1	High	7.15	4.49	22.07	> 70%	+ (3/3)
Gray HED	1.00 (Moderate)	Plausible (837)	100	32.5	66.3	High	2.60	0.90	1.79	> 70%	+ (3/3)
PM	0.80 (Strong)	Plausible (837)	95.5	43.7	69.6	High	3.56	4.67	24.87	> 70%	+ (3/3)

^a Alert: 427, aromatic primary amine or secondary amine; 440, resorcinol or precursor; 837, ortho or para amino- or hydroxyl-aniline; 861, aminopyridine or derivative.

3. Results

3.1. Skin sensitization potential of 9 hair dyes in DEREK

Table 2 shows the data set of the results of the DEREK, DPRA and KeratinoSens™ assay for all 9 hair dye ingredients. The toxic predictions made by DEREK are the result of a two-part process (Sanderson and Earnshaw, 1991): (i) The program checks whether any alerts in the knowledge base are present in the query structure; (ii) The reasoning model is applied in order to determine the likelihood of toxicity. Each alert describes a structural feature or toxicophore associated with the occurrence of skin sensitization which has been derived by human expert interpretation of evidence including toxicity data for known compounds within the same chemical class and an understanding of the toxicological mechanism (Langton et al., 2006). Toxicophores associated with the skin sensitization were aromatic primary amine of MAP and 2,4-DAPE, resorcinol (RS) or its precursor, MRP-N, *p*-aminoaniline of PPD and Gray HED, *p*-hydroxyaniline of PM, and aminopyridine of 2,6-PYR. PAOX contains misclassified or unclassified features. The misclassified features have been observed in sensitizers that do not match skin sensitization structural alerts in DEREK, and the unclassified features were not found in the Lasha skin sensitization negative prediction data set. DEREK also generates an LLNA EC₃ predictions for skin sensitization. Based on the result of these reasoning processes, 8 of the 9 tested chemicals were determined as sensitizers (Table 2).

3.2. Skin sensitization potential of 9 hair dyes in Direct Peptide Reactivity Assay (DPRA)

Chemical-based peptide reactivity was used to measure cysteine and lysine peptide depletion. MAP showed the lowest average depletion of the cysteine and lysine peptides, and categorized as a non-sensitizer with the minimal sensitizing reactivity. Other 8 chemicals are all classified as sensitizers, though their sensitization reactivity potency levels are varying. MRP-N and RS depleted the peptides less than 10%, which is much lower than 22.62% of low reactivity threshold. Therefore, MRP-N and RS can be predicted as weak sensitizers. PAOX depleted 36.3% of cysteine and lysine peptides and assessed as the only moderate sensitizer. Other 5 chemicals were predicted as strong sensitizers with the mean % depletion of the peptides ranging from 46% to 66.3% (Table 2).

3.3. Skin sensitization potential of 9 hair dyes in KeratinoSens™ assay

Table 2 gives results of the standard parameters of the assay (I_{max} and EC_{1.5}) as well as the final rating of the preparation, i.e. positive (+)

or negative (–) and the number of independent repetitions positive/number of repetitions done are given (Andres et al., 2013). Chemicals that induce a dose-dependent luciferase induction at non-cytotoxic concentrations are classified as a sensitizer (Ball et al., 2011). 8 of 9 tested hair dye compounds exhibited dose-dependent increase in luciferase activity above the threshold of 1.5-fold at concentrations below 1000 μM and had IC₃₀ (concentration value for 30% reduction of cellular viability) values greater than the EC_{1.5} response. Only RS was predicted as a non-sensitizer because it did not induce luciferase activity (Settivari et al., 2015) (Table 2).

3.4. Skin irritation potential of 9 hair dyes with or without hydrogen peroxide evaluated by Epiderm™

To predict the skin irritation potential of 9 hair dye chemicals *in vitro*, they were tested in the Epiderm™ assay. The Epidermis viability was determined in Epiderm™ model using MTT assay. To compare the irritation effects on the tissue, 5% sodium dodecylsulfate, a known irritant, was used as a positive control. There were significant differences in the tissue viability in the groups treated with hair dye substances except RS. When H₂O₂ solution was added with PM, the tissue viability was the lowest (7%). 8 of 9 tested hair dye compounds except MAP reduced the viability of Epidermis by about 20–50% when mixed with 6% of H₂O₂ solution. According to the EU and GHS classification (R38/Category 2 or no label), an irritant is predicted if the mean relative tissue viability of three individual tissues exposed to the test substance is reduced to below 50% of the mean viability of the negative controls (OECD, 2004, 2015). When H₂O₂ solution was added with PPD and PM, the tissue viability was 40% and 7% respectively. Therefore, PPD and PM combined with 6% of H₂O₂ solution were categorized as irritants (Fig. 2A). When the Epiderm™ tissue remaining after MTT formazan extraction was processed and stained with hematoxylin and eosin, the MTT result could be further confirmed. The tissues treated with MRP-N, RS, 2,6-PYR, PPD and PM along with 6% of H₂O₂ solution showed decreased density of stratum corneum and vacuolization occurred in some parts of basal layer (Fig. 2B).

4. Discussion

There are many drawbacks in animal testing, such as poor reproducibility and limitations in recapitulating the actual human physiological conditions. More importantly, cosmetic testing on animals was completely banned in many countries since 2013 (Su et al., 2017). Numerous alternative test methods have been developed and validated by EU and OECD to assess the skin sensitization and irritation (Kreysa, 2008). Due to their similarities and differences, a comprehensive

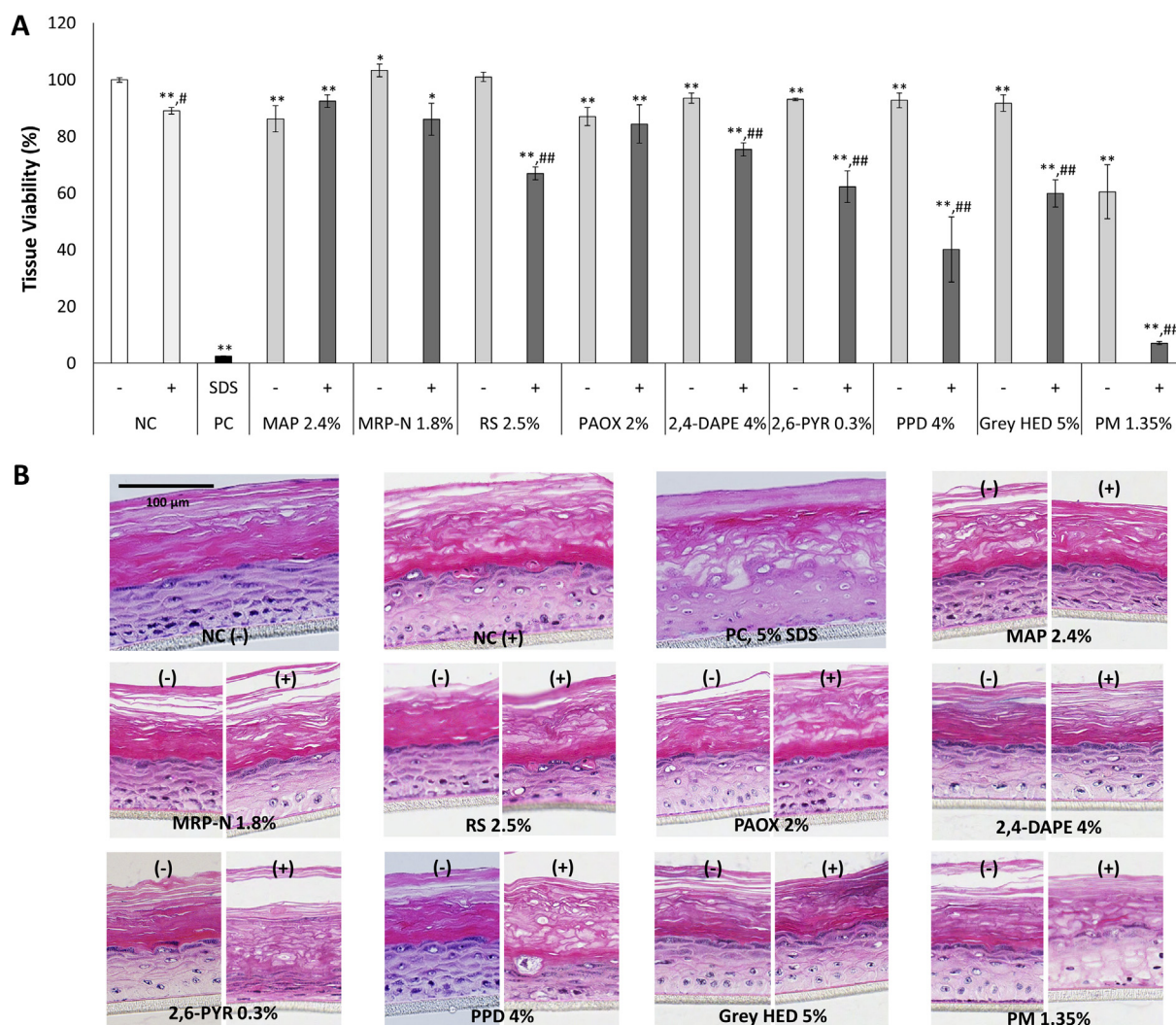


Fig. 2. Skin irritation potential of hair dyes with or without 6% of H_2O_2 solution on Epiderm™ model.

(A) Relative viability treated with 9 hair dyes at the maximum permitted concentration with and without 6% of H_2O_2 solution. (B) Epiderm™ tissues used for the MTT assay were stained with hematoxylin and eosin. NC; negative control, PC; positive control, SDS; sodium dodecylsulfate 5%. Scale bar is 100 μm.

evaluation of skin sensitization and irritation would provide more reliable information on the local toxicity of hair dyes. In the present study, we investigated the sensitizing and irritant potency of 9 hair dye substances by using 4 types of alternative animal test methods.

Here we utilized DEREK, Direct Peptide Reactivity Assay (DPRA) and KeratinoSens™ assay, and found that 6 of the 9 tested chemicals were sensitizers in common (Table 3). There existed inconsistent sensitization predictions for MAP, RS and PAOX across three assays. But although positive in decision, MAP, RS and PAOX were commonly determined as a relatively low-degree skin sensitizer by DPRA, KeratinoSens™ and DEREK, respectively. Interestingly, MAP and RS were classified as skin sensitizers by ECHA (ECHA, 2018), which was based on positive results (EC3 0.24% and 6%) from LLNA (Basketter et al., 2007; SCCP, 2007). Incidentally, PAOX was negative in LLNA (EC3 > 50%) (SCCP, 2006), which resulted in non-classification (ECHA, 2018). In this regard, DEREK yielded most concordant results but it is not surprising since DEREK produces predictions based on existing data. KeratinoSens™ failed to predict RS correctly and it was attributable to its prohaptenic nature, which requires metabolic activation (Emter et al., 2010) but it was correctly predicted by DPRA and hCLAT (Hennen and Blomeke, 2017). In case of MAP, DPRA failed to predict it positively while KeratinoSens™ produced the positive prediction. However, in a previous study, MAP was predicted as negative

by both KeratinoSens™ and DPRA (Urbisch et al., 2016), suggesting that these substances may be borderline sensitizers. Actually, MAP, RS and PAOX have hydroxyl at meta-positions, which is less easier than the para-position to be oxidized into quinone or quinone-imine to manifest skin sensitization (Aptula et al., 2009), explaining the borderline results across these assays. In addition, these results alert possible false prediction by these *in vitro* assays for these meta-sensitizers.

Skin irritation testing is also an essential requirement for the development of cosmetics and hair dye products (Su et al., 2017). Irritant contact dermatitis (ICD), caused by skin irritation reaction, is as frequent as allergic contact dermatitis (ACD). ICD accompanies skin itchiness and redness, and even inflammation in severe cases (Corsini and Galli, 1998). Especially hair dyes are the most probable cause of the skin irritation, since the highest ICD annual incident rates were found in hairdressers (46.9 per 10 000 workers per year) (Dickel et al., 2002). Nevertheless, reliable data about the skin irritation potency of hair dyes are still very limited (Frosch, 1989) compared to those about the skin sensitization. One of the main objectives of our investigation was to use the reconstructed human epidermis models (Epiderm™) to predict the irritant potential of 9 hair dyes in the presence of H_2O_2 . This study demonstrated that H_2O_2 synergistically increases the skin irritation potential of hair dyes (Table 3). Especially, PPD and PM, strong-sensitizing agents, alone did not affect the skin irritant potential. On the

Table 3

Overall evaluation of skin sensitization potency and irritation for 9 hair dye chemicals.

Chemical	Sensitization				Irritation		
	DEREK	DPRA	Kerato-Sens™	ECHA (2018) ^a	Epiderm™ classification		ECHA (2018) ^a
					(–) H ₂ O ₂	(+) H ₂ O ₂	
MAP	Sensitizer	Non-sensitizer	Sensitizer	Cat 1 (H317)	Non-irritant	Non-irritant	–
MRP-N	Sensitizer	Sensitizer	Sensitizer	Cat 1 (H317)	Non-irritant	Non-irritant	2 (H315)
RS	Sensitizer	Sensitizer	Non-sensitizer	Cat 1B (H317)	Non-irritant	Non-irritant	2 (H315)
PAOX	Non-sensitizer	Sensitizer	Sensitizer	–	Non-irritant	Non-irritant	2 (H315)
2,4-DAPE	Sensitizer	Sensitizer	Sensitizer	Cat 1B (H317)	Non-irritant	Non-irritant	2 (H315)
2,6-PYR	Sensitizer	Sensitizer	Sensitizer	Cat 1 (H317)	Non-irritant	Non-irritant	2 (H315)
PPD	Sensitizer	Sensitizer	Sensitizer	Cat 1A (H317)	Non-irritant	Irritant	–
Gray HED	Sensitizer	Sensitizer	Sensitizer	Cat 1 (H317)	Non-irritant	Non-irritant	2 (H315)
PM	Sensitizer	Sensitizer	Sensitizer	Cat 1 (H317)	Non-irritant	Irritant	–

^a ECHA (European Chemical Agency Database, <http://echa.europa.eu/search-for-chemicals>, accessed June 26th, 2018). H317 in ECHA was considered to correspond to UN GHS Category 1, indicating skin sensitization. Sub-category 1A, substances showing a high frequency; Sub-category 1B, a low to moderate frequency of occurrence in humans. H315 in ECHA was considered to correspond to UN GHS Category 2, indicating skin irritation.

other hand, combined with H₂O₂ they induced a higher degree of the skin irritation in Epiderm™. Some weak- and moderate-sensitizers, RS, MAP and MRP-N, also reduced the tissue viability in the presence of H₂O₂ although they did not fall below the cutoff. Of note, with the histological examination of tissue remaining after MTT assay, we could further confirm the damage which would be of a great value for colorful substances without wasting another costly tissues.

Hair dye products can cause irritating skin symptoms such as skin itchiness and redness (Lisby and Baadsgaard, 2006). The present study revealed that the skin sensitization as well as skin irritation can be induced by hair dyes and their combination with H₂O₂. The degree of skin sensitization and irritation was different across hair dyes reflecting that safer hair dyes can be developed for which our study may be helpful.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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