

Effects of hair dye ingredients on the oxidative stress response: Modulation of the mRNA expressions of *NRF2*, *HO-1*, and *FOS* in HaCaT keratinocytes

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Our group has demonstrated that adulteration of henna products with the potent contact allergen *p*-phenylenediamine (PPD) changes the toxicological profile of henna by modulation of the keratinocyte and dendritic cells network involved in skin sensitization.¹ To better understand the keratinocyte response, this study evaluated the effects induced by synthetic hair dye ingredients on the oxidative stress response in HaCaT keratinocytes. The messenger RNA (mRNA) expressions of the following genes were then investigated: nuclear factor erythroid 2-related factor 2 (*NRF2*); heme oxygenase 1 (*HO-1*), a phase II xenobiotic-metabolizing enzyme gene partially regulated by *Nrf2* through the antioxidant response element (ARE); and *Fos* proto-oncogene (*FOS*).

MATERIAL AND METHODS

HaCaT human immortalized keratinocytes, obtained from the Rio de Janeiro Cell Bank (Brazil), were first used to perform the cytotoxicity analysis to obtain noncytotoxic concentrations of each test material that maintain cell viability $\geq 80\%$ (CV_{80}).¹ Cells, previously seeded overnight in cell culture flasks (25 cm^2) at 1.5×10^5 cells/mL, were then exposed to each test material at noncytotoxic concentrations (CV_{80}) for 4 hours for gene expression analysis, based on previously described treatment protocol.² After that, total RNA was isolated using TRIzol reagent (Invitrogen, Grand Island, New York), following manufacturer's instructions. RNA integrity was measured using the Nanodrop 2000 UV-Vis spectrophotometer (260 nm; Thermo Fisher Scientific, Waltham, Massachusetts). RNA samples were then used for complementary DNA synthesis using a recombinant reverse transcription kit (iScript gDNA Clear Synthesis kit,

Bio-Rad, Hercules, California). Quantitative real-time polymerase chain reaction analysis was performed using a cyanine dye-based hot start polymerase chain reaction kit (SsoAdvanced Universal SYBR Green Supermix; Bio-Rad, Thermo Fisher Scientific). Normalization was made with *HPTR1* as the reference gene and fold change was calculated by the DDCT (delta-delta-CT) method. Data were calculated as average log₂-fold change in independent biological replicates $\pm$ standard deviation. Assessed genes and primers are listed in Table S1. Statistical analysis was performed using Graph Pad Prism software version 5.0 for Windows 98 (San Diego, California). The intergroup variation was measured by one-way analysis of variance followed by Bonferroni's test. Statistical significance was established as $P < .05$.

RESULTS

According to the literature, animal data for the chemicals tested in this study showed a concordance of 75% in relation to human data (Table S2). Gene expression results for *NRF2*, *HO-1*, and *FOS* are shown in Figure 1A-C, respectively. When compared with control (nonexposed cells), nonsensitizer reference chemicals (dimethyl sulfide and sodium dodecyl sulfate) did not trigger changes in the mRNA expression of the investigated genes, whereas skin sensitizer reference chemicals (eugenol and 2,4-dinitrochlorobenzene) promoted a reduced *NRF2* and *FOS* mRNA expression and a significant increase of *HO-1* mRNA levels ($P < .001$). *NRF2* mRNA levels were also showed significantly reduced in cells exposed to the hair dye ingredients ($P < .05$), with the exception of 1,4-diaminoanthraquinone (14D), where reduction was not significant. Moreover, six of ten hair dye ingredients, including lawsone (LAW), 2-amino-3-hydroxypyridine (2A3), pyrogallol, hydroquinone, resorcinol, and the highly allergenic

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PPD ($P < .0001$), triggered an increase of *HO-1* mRNA levels. In the same way, all hair dye ingredients, except three (LAW, Basic Red 51, and 5-amino-o-cresol), showed increased *FOS* mRNA expression, in particular 2A3 and 14D ($P < .05$). Therefore, these data highlight that hair dye ingredients investigated have skin sensitization potential

by disrupting the oxidative stress response in HaCaT keratinocytes via transcriptional changes of *NRF2*, *HO-1*, and *FOS* expression.

DISCUSSION

Skin sensitizers usually promote oxidation of Kelch-like ECH-associated protein 1 (Keap1) cysteine residues which results in the dissociation of the Nrf2-Keap1 complex in the cell cytoplasm, translocation of the transcription factor Nrf2 to nucleus, and then regulation of antioxidants and xenobiotic-metabolizing enzyme genes through the ARE (eg, *HO-1*).^{3,4}

Regarding this, our data showed that, after 4 hours of exposure, *NRF2* mRNA expression in HaCaT keratinocytes is decreased by synthetic hair dye ingredients. This suggests that, after 4 hours, *NRF2* transcript levels seem to be downregulated when the Nrf2 protein pathway is activated to promote increase in its downstream target genes such as *HO-1*. Thus, an earlier increased expression of *NRF2* may have been missed because RNA was extracted 4 hours after the exposure of test materials. Haslam et al have also observed that the *NRF2* transcript levels were reduced in a human scalp hair follicle model exposed to the Nrf2 activator sulforaphane for 24 hours.⁵ Furthermore, the authors showed regulation of the downstream Nrf2 target genes, including *HO-1*. Here, the upregulation of *HO-1* was not observed for all chemicals, suggesting that other cell signaling pathways may be involved. In fact, it has been shown that skin sensitizers can induce *HO-1* expression in KeratinoSens cells through dual regulation triggered by Nrf2 and other DNA-binding factors such as BTB and CNC homology 1 (Bach1).

A gene signature has been established in HaCaT keratinocytes, which includes 10 genes modulated by skin sensitizers, among which *HO-1* and *FOS* were the most affected.² Corroborating this, our findings also showed that the hair dye ingredients promoted, in a general way, an increase of *FOS*. This biomarker has been related to inflammatory and general stress response and Toll-like receptor signaling and cytokine network triggered by contact allergens.²

In conclusion, these markers seem suitable for use in a keratinocyte-based *in vitro* model for skin sensitization prediction of hair dyes. Moreover, our results support the need to introduce ingredients with lower allergenic potential in hair coloring products to ensure safety of human health.

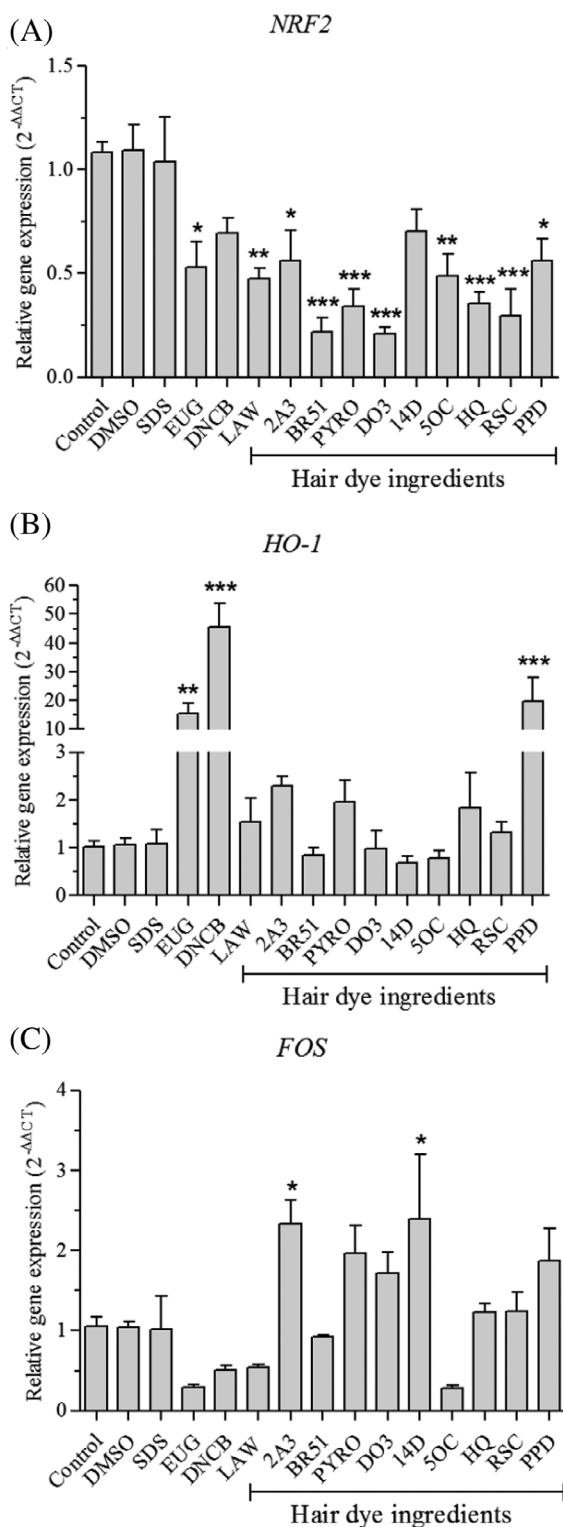


FIGURE 1 Legend on next column.

FIGURE 1 Effects of hair dye ingredients on the modulation of mRNA expression of (A) *NRF2*, (B) *HO-1*, and (C) *FOS* in HaCaT keratinocytes. Dimethyl sulfoxide (DMSO; 0.1%), sodium dodecyl sulfate (SDS; 64 μg/mL), 2,4-dinitrochlorobenzene (DNCB; 2 μg/mL), and eugenol (EUG; 26 μg/mL) were used as reference controls. The following numbers in parentheses represent the noncytotoxic concentrations (μg/mL) tested for each hair dye ingredient: 1,4-diaminoanthraquinone (14D; 1), 2-amino-3-hydroxypyridine (2A3; 324), lawsone (LAW; 110), 5-amino-o-cresol (5OC; 15), hydroquinone (HQ; 17), *p*-phenylenediamine (PPD; 40), resorcinol (RSC; 288), Disperse Orange 3 (DO3; 10), Basic Red 51 (BR51; 3), and pyrogallol (PYRO; 60). * $P < .05$, ** $P < .001$, and *** $P < .0001$ vs control. *FOS*, Fos proto-oncogene; *HO-1*, heme oxygenase 1; *NRF2*, nuclear factor erythroid 2-related factor 2

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Table S1. qPCR primer sequences and associated GenBank accession numbers.

Gene	Name	Primer sequence (5' – 3')	GenBank number
<i>NRF2</i>	Nuclear factor, erythroid 2 like 2	AGT GGA TCT GCC AAC TAC TC CAT CTA CAA ACG GGA ATG TCT G	S74017.1
<i>HO1</i>	Heme oxygenase 1	CAT GAC ACC AAG GAC CAG AG AGT GTA AGG ACC CAT CGG AG	GQ221778.1
<i>FOS</i>	Fos proto-oncogene	TGC CTC TCC TCA ATG ACC CTG A ATA GGT CCA TGT CTG GCA CGG A	CR542267.1
<i>HPRT1</i>	Hypoxanthine phosphoribosyltransferase 1	TGA CAC TGG CAA AAC AAT GAC GGT CCT TTT CAC CAG CAA GCT	CR407645.1

Table S2. Reference controls and hair dye ingredients with skin sensitization potential. HaCaT keratinocytes were exposed to each chemical for 24 h and cytotoxicity analysis was conducted using MTT assay to obtain the CV₈₀ values.

Test materials ¹	CAS No	Human classification ²	Animal classification ²	Solvent	CV ₈₀ (µg/mL)
Reference controls					
Dimethyl sulfoxide (0.1%, DMSO)	67-68-5	Negative	Negative	Medium	-
Sodium dodecyl sulfate (SDS)	151-21-3	Negative	Weak	Medium	64
Eugenol (EUG)	97-53-0	Positive	Weak	DMSO	26
2,4-Dinitrochlorobenzene (DNCB)	97-00-7	Positive	Extreme	DMSO	2
Hair dye ingredients					
Lawson (LAW)	83-72-7	NA	Equivocal	DMSO	110
2-Amino-3-hydroxypyridine (2A3)	16867-03-1	Positive	Negative	DMSO	324
Basic red 51 (BR51)	77061-58-6	NA	Negative	Medium	3
Pyrogallol (PYRO)	87-66-1	Positive	Negative	Medium	60
Disperse orange 3 (DO3)	730-40-5	Positive	Very weak	DMSO	10
1,4-Diaminoanthraquinone (14D)	128-95-0	Positive	Moderate	DMSO	1
5-Amino- <i>o</i> -cresol (5OC)	2835-95-2	Positive	Moderate	DMSO	15
Hydroquinone (HQ)	123-31-9	Positive	Strong	Medium	17
Resorcinol (RSC)	108-46-3	Positive	Strong	Medium	288
<i>p</i> -Phenylenediamine (PPD)	106-50-3	Positive	Extreme	DMSO	40
Concordance vs. Human data	-	-	75%	-	-

Abbreviations: NA – data not available by literature; CAS No – Chemical Abstracts Service Number.

¹Basic red 51 was acquired from Santa Cruz Biotechnology (Dallas, Texas). All other reagents were obtained from Sigma-Aldrich (St. Louis, Missouri). Before the cell exposure, solutions of all chemicals tested were freshly prepared by dilution in DMSO or medium. The final DMSO concentration in the cell-based assay did not exceed 0.1% (v/v).

²Classification based on the human repeated insult patch test (HRIPT), murine local lymph node assay (LLNA) and/or guinea pig maximization test (GPMT)/Buehler test (BT). Information extracted from de Ávila et al. (2019).