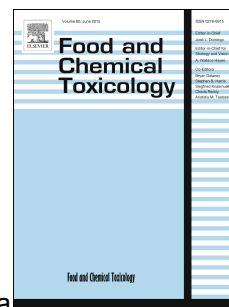


Accepted Manuscript

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Allergens of permanent hair dyes induces epidermal damage, skin barrier loss and IL-1 α increase in epidermal in vitro model

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1. Introduction:

Permanent hair dyes are formed by a mixture of compounds that after successive reactions with a dye precursor (i.e., PPD) and a coupler agent (Ex: resorcinol) under oxidative conditions results in hair coloring (Morel and Christie, 2011). However, these mixtures contain by-products of which the sensitizing and/or irritant toxic properties have not yet been elucidated (SCCP/1198/08). It is known that the oxidation of PPD produces a mutagenic chemical which is also an extreme skin sensitizer named Bandrowski's base (Brown and Corbett, 1979, Corbett, 1998 and Ghosh and Sinha, 2008). Although many authors discuss that some of the by-product formed could be the key to skin sensitization, the exact mechanism remains unclear. Therefore, still some research results remain inconclusive, while other studies indicate that the direct use of aromatic amines or the indirect formation of them during a hair dye coloring procedure could be related to skin toxicity and may even play an important role in the etiology of some human cancers (Baan et al., 2000).

The main concern is based on the fact that permanent hair dye formulations are formed by a complex mixture of skin sensitizers and aromatic amines, among other products, that may result on the formation of even more toxic by-products (Aeby et al., 2009, SCCS/1311/10). According to (Aeby et al., 2009, SCCS/1311/10) there is a lack of information dealing with toxicity of these mixtures as most studies investigate hazard potential of individual ingredients and for this they need to be better investigated. In addition, considering the high statistic of usage, evaluating the risk of exposure to these dyes is important as it may affect a wide population of users.

According to Aeby et al., 2004; 2009, the oxidation of PPD releases intermediates that may form protein adducts such as PPD-benzoquinone and PPD-benzoquinonediimine. Seo et al., 2012, showed that the mixture of some of these hair dye ingredients are responsible for increasing the risk of developing contact dermatitis and has also been associated to increase of hair loss. Another source that can contribute to skin allergy after exposure of these dyes is the generation of reactive oxygen species (ROS) (Kidd, 2003, Bickers, 2006). Topical exposure to irritants and sensitizers are known to increase levels of ROS that can disrupt the epidermal barrier and activate several cellular pathways including the increase of cytokines involved in allergic reactions (Byamba et al., 2010., Kim et al., 2012., Corsini et al., 2013., Larbi et al., 2007., Martin, 2005., Rinnerthaler et al., 2015).

Following the trend to replace animal testing for *in vitro* alternatives (Jung et al., 2014; Lemper et al., 2014) there are currently four commercial kits; Epiderm @ (MaTek corporation), EPSKINTM (SkinEthic laboratories), (modified EpiDermTM. SIT test method, SkinEthicTM RhE test method, recommended by the European center for validation of alternative methods (ECVAM) that allow *in vitro* evaluation of skin irritants (ECVAM) (ESAC Peer Review Panel for the skin irritation Validation Study-Consensus report, www.ecvamjri.it). To contribute to this issue, the Organization for Economic Co-operation and Development (OECD) has described a complete protocol guideline that allows validation of new RhE models for skin irritation as described in TG 439 (OECD, 2015b). Skin irritation is induced when a compound penetrates the stratum corneum reaching and damaging living layers of the epidermis, consequently, there is an increase of pro-inflammatory cytokines, such as IL-1 α that trigger a skin immune reaction contributing to skin irritation (Fartasch et al., 1995, Wood et al., 1996, Wood et al., 1997, Spiekstra et al., 2005, Spiekstra et al., 2007). Following the TG439 OECD guideline, our group

has recently established a RhE that allows identification of in vitro skin irritation (Pedrosa et al., 2017). Furthermore, we have also shown that reconstructed skin models are efficient tools to screen toxicity of hair dyes, allowing us to observe early symptoms of skin irritation as damaging of living epidermal compartment (Zanoni et al., 2015). Therefore, the knowledge that these RhE are appropriated methods to determine irritant potency of chemical compounds (Teunis et al., 2014; Spiekstra et al., 2009; Dos Santos, 2011) and a unique platform to evaluate hair dye toxicity using similar methodology to human exposure without animal experimentation. The aim of this study was to: (I) assess free radical formation of a mixture of PPD, Resorcinol (RES) and H_2O_2 (II) evaluate skin irritation using RhE (III) evaluate filaggrin expression and morphological epidermal changes.

2. Results

2.1 Reaction between permanent hair dye ingredients: PPD, Resorcinol and H_2O_2 leads to hydroxyl radical formation ($OH^\cdot$)

As reference sensitizers used in permanent hair dyes, PPD (p-phenylenediamine) and Resorcinol (RES) were used. H_2O_2 was used as oxidizer. The concentration used was 10x lower than that recommended by SCCPN, and selected according to our previous data (Zanoni et al., 2015).

The ability of the mixture of the three ingredients commonly used in permanent hair dye formulations (PPD, RES and H_2O_2) to induce Reactive Oxygen Species (ROS) was investigated Fig. 1. To determine the specific free radical formed, we used 280 mM 5,5- dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trapping agent to obtain a stable Electro Spin Resonance (ESR) signal. As seen on in Fig.1: (A) indicates that PPD and Resorcinol alone are not able to form free radicals, although when H_2O_2 was added as shown on (B) an unstable signal is observed that reassembles a free radical. Aiming to stabilize this signal of the specific free radical formed (C) was tested on same conditions as (B) although with the addition of 280mM of DMPO. The ESR analysis confirmed the release of hydroxyl radicals ($OH^\cdot$) with the characteristic 1:2:2:1 peak intensity and $a^N=14.9$ G, $a^H=14.9$ G in line with our previous observations after oxidation of PPD with H_2O_2 (Zanoni et al., 2015). These results suggest that oxidation of PPD in the presence of RES is determinant to contribute to the release of hydroxyl radical ($OH^\cdot$).

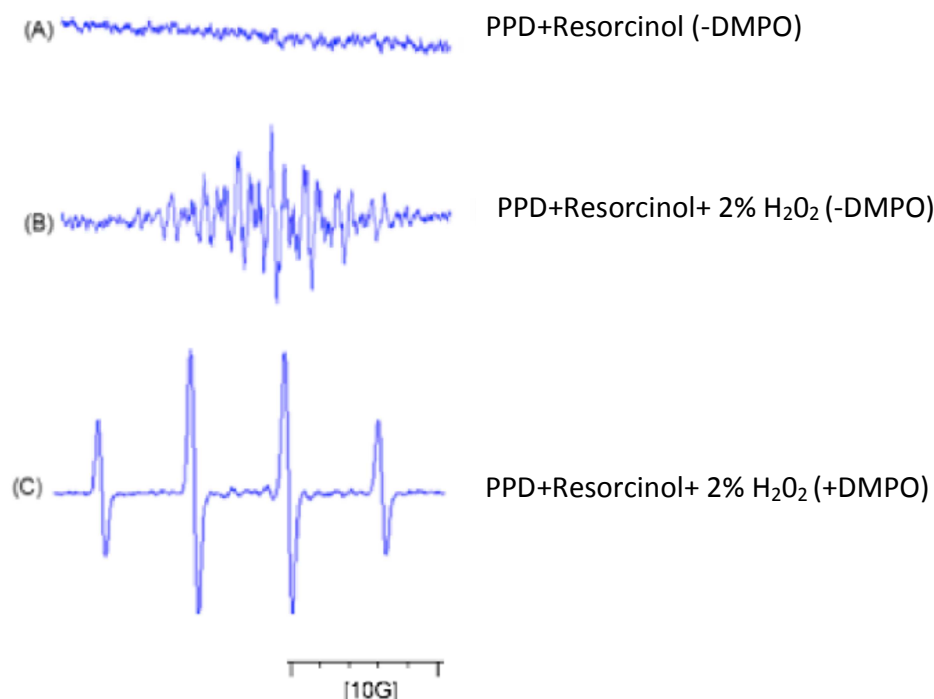


Figure 1: Mixture of 2000 µg/mL PPD and 2000 µg/mL Resorcinol in 2% of H₂O₂ results on the formation of (OH[•]) radical. (A) PPD and RES in absence of DMPO, (B) PPD and RES in 2 % H₂O₂ in absence of DMPO indicates a signal without definition of the type of free radical formed, (C) PPD and RES in 2 % H₂O₂ in presence of DMPO indicates a defined signal of a hydroxyl radical (OH[•]) formation.

2.2 *in vitro* evaluation of skin irritation potential of a mixture of contact allergens used in permanent hair dyes

To investigate the irritant potential of the mixture used in permanent hair dyes and to compare the mixture with the ingredients alone, we applied the OECD TG 439 MTT method to the RhE as previously adapted by (Pedrosa et al., 2017). Sodium dodecyl sulfate (SDS) (5%)

used as positive control was applied topically to RhE for 1 hour followed by further incubation of ± 18 hours. The RhE exposed to SDS showed 78% decrease in viability (Fig. 2). In contrast, hair dye ingredients applied individually or as a mixture failed to be classed as irritants because viability was higher than 50%, cut-off recommended by the OECD TG 439 to consider a chemical as skin irritant. Despite these findings, and as PPD and Resorcinol are labeled as sensitizers we furthermore investigated their ability to enhance other parameters related to skin irritation as described by ECVAM (Pedrosa et al., 2017).

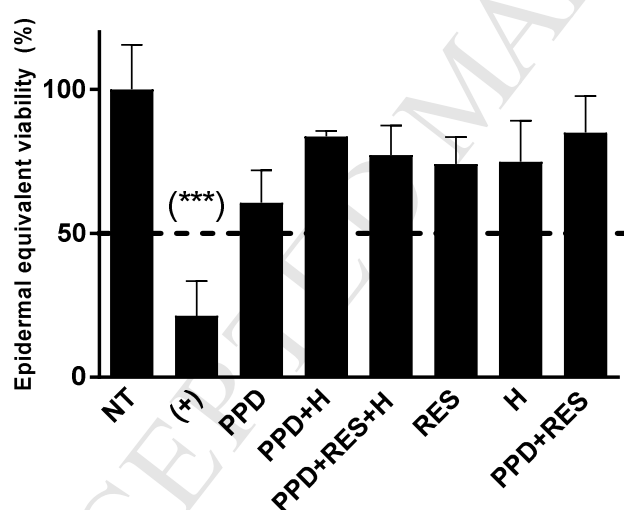


Figure 2: Epidermal Equivalent (RhE) viability after exposure to PPD, H₂O₂, RES and the following mixtures PPD/RES, PPD/H₂O₂ and PPD/H₂O₂/RES. A substance was classified as an irritant if viability after 1 h exposure was below 50% compared to the non-treated control. Sodium dodecyl sulfate (SDS) (5%) positive control was correctly classified as irritant. PPD and Resorcinol were incubated at 2000 μ g/mL alone and in combination, while H₂O₂ was tested at 2%, Mean of three independent experiments n = 3, where (***) p<0.0001.

2.3 Mixtures of permanent hair dye compounds and H₂O₂ decrease RhE viability and increase IL-1 α release

To further investigate the irritant potential of the hair dye mixture, the chemicals were next topically applied individually or as a mixture for 24 hours (instead of 1 h) using RhE following the principle of *in vitro* irritation assays as recommended by ECVAM. As shown in Fig. 3, topical exposure for 24h to the mixtures PPD/RES/H₂O₂ and PPD/H₂O₂ greatly decreased RhE viability to less than 20% which coincided with an increase of IL-1 α release. The results show that, the individual ingredients did not result in a decrease in cell viability and only a significant increase in IL-1 α release for H₂O₂. These results indicate that the mixture of ingredients under the tested conditions increases parameters related to skin irritation, whereas isolated ingredients did not.

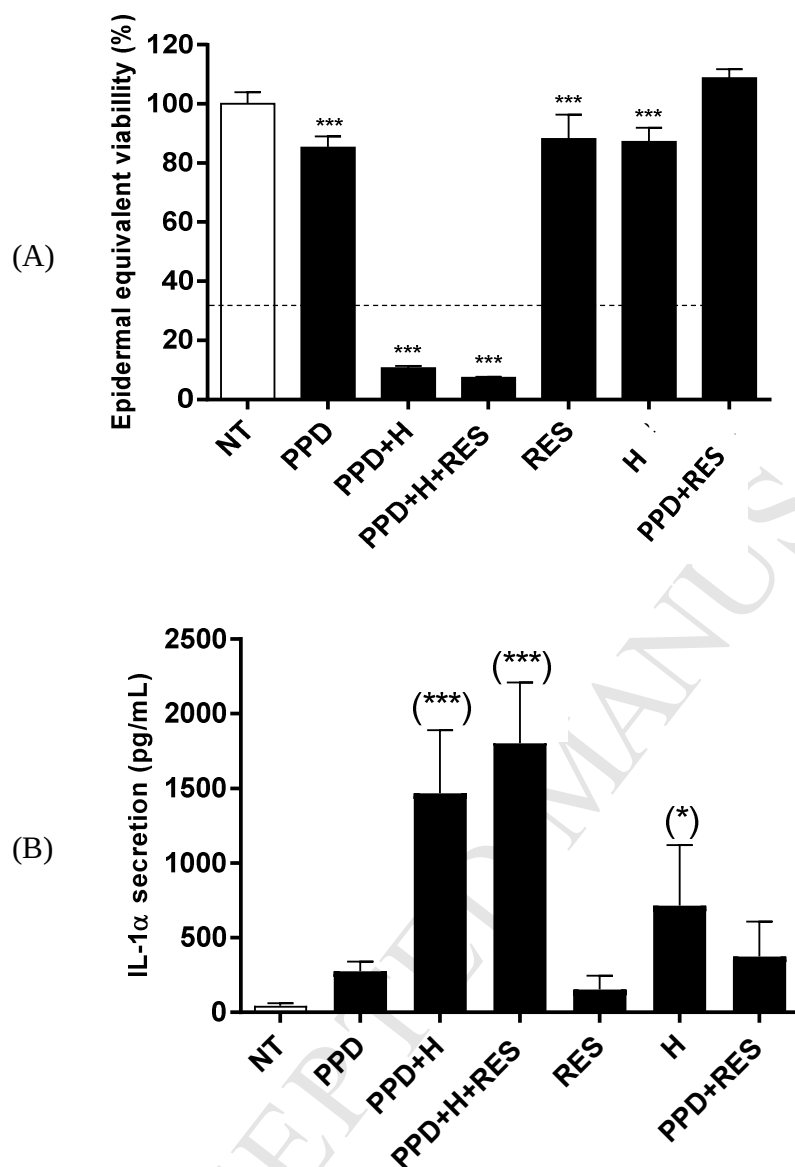


Figure 3: (A) RhE viability (MTT assay) after exposure to PPD, H₂O₂, Resorcinol and the following mixtures PPD/RES, PPD/H and PPD/H/RES after 24 hours topical exposure. (B) Evaluation of IL-1 α (ELISA) using the same test conditions. RhE were treated on the following conditions, with NT (Non-treated), PPD (2000 μ g/mL), RES (2000 μ g/mL) and 2% H₂O₂ alone and combined for 24 hours. Results are expressed as mean $\pm$ SD, n=3.

2.4 Permanent hair dye mixtures but not individual ingredients induce morphological changes in the epidermis

Next the extent of morphological changes induced in RhE after topic exposure to the hair dye ingredients and their mixtures was determined. After 12 days of culturing RhE at an air-liquid interface a fully differentiated epidermis was obtained. (Figure 4). Histological analysis of hematoxylin-eosin stained RhE sections exposed to PPH/H₂O₂ (and PPD/RES/H₂O₂ revealed the presence of significant epidermal damage when compared to the non-treated RhE. Exposure to the mixtures resulted on stratum corneum loosening and upper epidermal layer detachment, both typical signs of cytotoxicity. Furthermore, topic exposure to PPD/H₂O₂ mixture altered granular layer cell formation with an increased number of apoptotic cells becoming visible. Exposure to PPD/H₂O₂/RES resulted on an apparent dilation of the extracellular spaces at the lower basal living keratinocytes layer. Moreover, basal layer keratinocytes presented hyperchromatic cell nuclei. These results suggest that the extent of epidermal damage caused by the three ingredients mixture extends to the basal layer, while exposure to PPD/H₂O₂ reaches only the upper living cells of epidermis as shown by TUNEL assay, which evaluates apoptosis caused by DNA strand breaks. Furthermore, the percentage of epidermal TUNEL-positive cells after PPD- H₂O₂ exposure rate was higher on the upper living epidermal layer compared with PPD-H-RES in the basal layer, suggesting deeper cellular damage after incubation of three ingredients (Fig. 4). No morphologic difference or apoptosis induction was observed on the topical exposure of ingredients tested alone at concentration of 2000 µg/mL (data not shown).

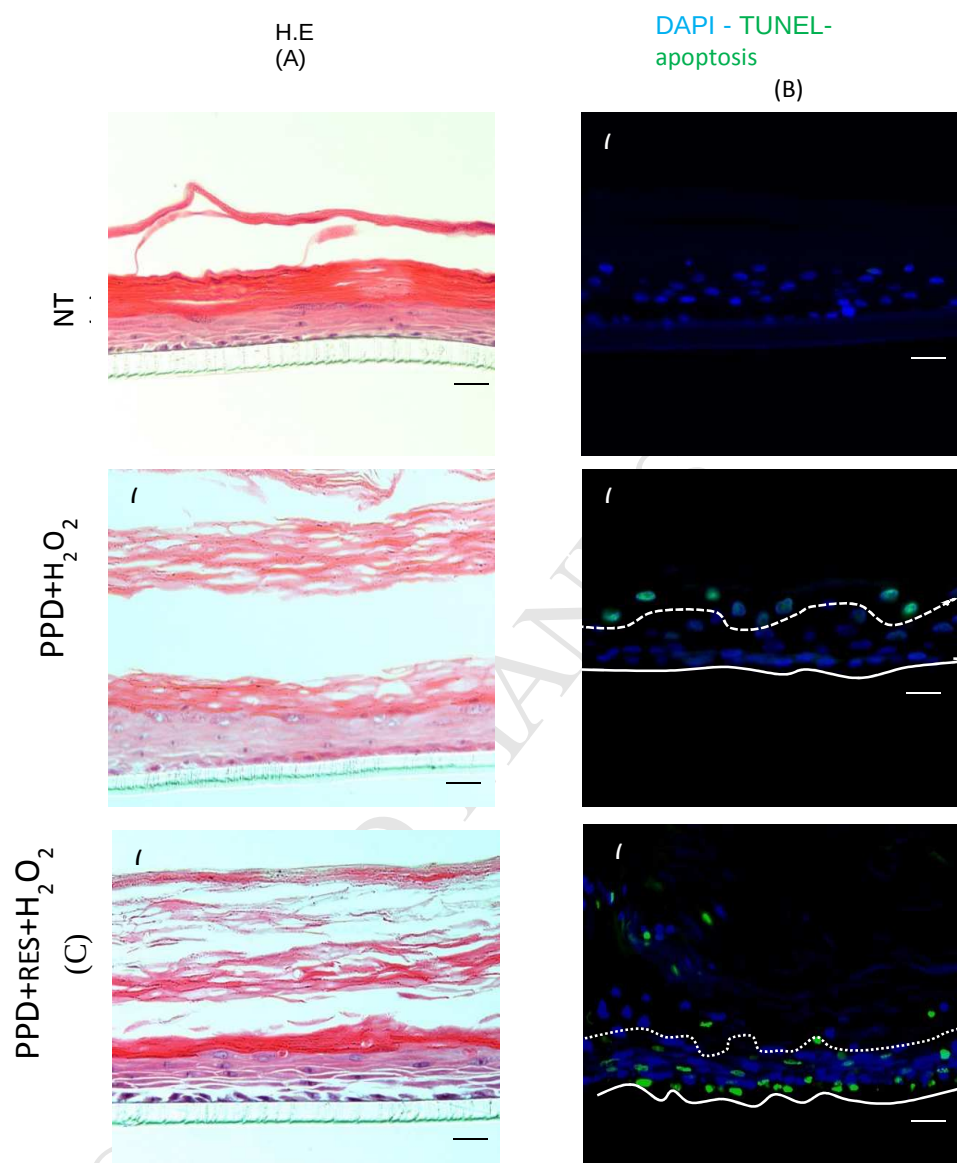


Figure 4. (A) H.E staining and (B) Apoptosis (TUNEL assay) induction in RhE after topical exposure to hair dye mixtures. Non-treated (NT) (A), PPD +H₂O₂ (B) and PPD+Resorcinol +H₂O₂ (C). Bar 100 μ M (II) Immunofluorescence in reconstructed epidermis after topical exposure to hair dye mixtures. DAPI detection normal healthy cells marked in blue and Fragmented DNA apoptosis keratinocytes are marked in green. Bar 100 μ M.

2.5 Mixtures of permanent hair dye ingredients result in filaggrin depletion in RhE

To determine whether the tested mixtures resulted in filaggrin depletion, we next evaluated filaggrin expression using immunofluorescence analysis of RhE tissue sections (Fig. 5). The results show that non-treated RhE showed similar expression to normal human skin with filaggrin (green fluorescence) being expressed in the granular layer and stratum corneum. When RhE is exposed to of the hair dye mixtures, filaggrin expression decreased.

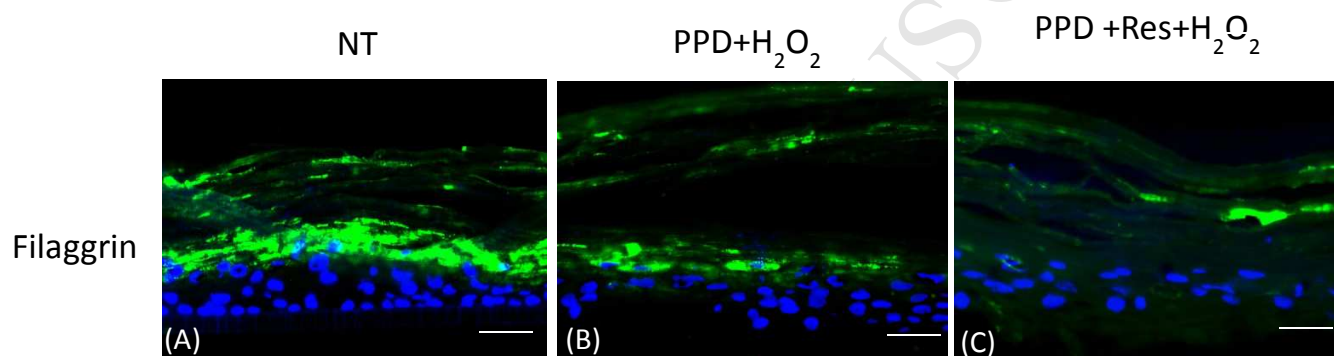


Figure 5. Filaggrin expression in RhE after topical exposure to hair dye mixtures. Non-treated (NT) (A), PPD +H₂O₂ (B) and PPD+Resorcinol+H₂O₂ (C). DAPI detection cell nuclei (blue) and filaggrin expression (green). Bar 100 μ M.

3. Discussion

It is apparent that ingredients used for permanent hair dyes may be considerable allergens to some users as their etiology vary from weak to strong skin sensitizers (Schuttelaar and Vogel, 2016; Pongpairoj et al., 2016). Hazard toxic potential drawbacks from mixture of these ingredients have recently been identified (Aeby et al., 2004). Emerging evidence indicates that by-products can, in some circumstances, be related to the development of skin allergy as they may form stronger skin sensitizers (Kawakubo et al., 2000, Krasteva et al., 1993, Meyer and Fischer, 2015) and p-benzoquinone diamine (BQDI) a highly protein reactive oxidation product of PPD (Meyer and Fischer, 2015). Other by-products formed during mixture of hair dye ingredients do not have their toxic/ allergic potential evaluated. Therefore, the real danger etiology from these mixtures are unknown and is a matter of investigation in this study.

Previous studies have shown that reactive oxygen species (ROS) are generated in human skin by contact allergens (Corsini, Galbiati and Nikitovic et al., 2013). Regarding permanent hair dyes, exposure to PPD, a strong skin sensitizer, has been frequently involved in skin toxicity (Schnuch et al., 1997; Lee et al., 2008). Recently, our group has found that the oxidation of PPD by H_2O_2 results in the release of hydroxyl radicals ($OH\cdot$) that may be associated with oxidative stress in human keratinocytes (Zanoni et al., 2015). Although this is an important finding, in a real hair dye formulation the presence of a coupler is fundamental to form the hair dye that will permanently change the color of the hair fiber. In this article, we show that the reaction among PPD and RES in the presence of 2% H_2O_2 also induces ($OH\cdot$) formation. The release of these free radicals could be a possible reason for enhancing skin toxicity resulting in epidermal disruption that could eventually increase skin allergy or irritation induction after topical

exposure. Although it is frequently observed that some consumers may develop allergic contact dermatitis and other allergic reaction due to the mixture of skin sensitizing ingredients in permanent hair dye formulations, there is yet no clear answer on the toxic potential of these mixtures to human skin (SCCS/1311/10). Hence, we hypothesize that ($\text{OH}\cdot$) formation could contribute to enhance skin toxicity upon exposure to these dyes.

In a recent publication, Hudari et al., 2014 described that the oxidation of PPD and RES by H_2O_2 resulted on the formation of the following by-products: PPD, RES, Bandrowski's base (BB) and p-quinonediimine (PQD). The following compounds PPD, RES and BB are considered respectively strong, moderate, and extreme skin sensitizers. PQD is a nucleophilic molecule able to directly bind to Cysteine, Lysine and other amino acids resulting on hapten complex formations (Eilstein et al., 2006,2007,2008). Moreover, other formed products such as indoaniline red and indoaniline green (Hudari et al., 2014) do not have their toxic, allergic, or irritant potential elucidated yet. According to the literature there is a lack of data regarding this subject (SCCS/1311/10) and therefore, here, we investigated and compared the irritant potential of some key ingredients and their mixtures used for permanent hair dying purposes after topic exposure to RhE as an attempt to generate *in vitro* data that could be related to real skin exposure.

For a long time, PPD has been associated to the development of skin allergy reactions in sensitized individuals (Sosted et al., 2006, Liden et al., 2016). Despite this, it remains unclear whether it is PPD or the autoxidation by-products that activates the skin immune system (Sieben et al., 2002, Aebly et al., 2009, Coulter et al., 2008, Krasteva et al., 1993). In addition, the mechanism induced in human skin after exposure to mixture of ingredients of hair dyes containing PPD remains unclear and is a matter of subject to this work. Hence, in sensitized

individuals some skin sensitizers also have some irritant properties the differentiation among them may be difficult as a mixture of both responses may also occur (Turton and Hooson, 1994). By measuring Interleukin 1 alpha (IL-1 α) (an early marker used for screening of skin irritation of both *in vivo* and *in vitro*) skin irritancy induction could be predicted (Gibbs et al., 2002, Coquette et al., 2003, Spiekstra et al., 2005, 2009, dos Santos et al., 2011, Teunis et al., 2014). Regarding this subject, here, we evaluated the irritant potential of each ingredients tested alone and after reaction among them using an our RhE model designated for skin irritation evaluation (Pedrosa et al., 2017). Our findings show that under the test conditions which included a 1 h topical exposure of RhE, the ingredients and their mixtures were not classified as irritants and no significant epidermal damage was observed (according to OECD TG49 guideline). Considering recurrent complaints from exposure to permanent hair dyes due to allergic reactions and the unknown toxic effect of this mixture to human skin, we decided to use another approach recommended by ECVAM to evaluate *in vitro* skin irritants that is based on longer period of exposure to irritant compounds. The test principal consists on a 50 % decrease of RhE viability followed by an increase of the inflammatory cytokine IL-1 α upon topical skin exposure to irritants (ECVAM). These events stimulate further production of pro-inflammatory cytokines and chemokines that directly contribute to skin immune system activation as an attempt to regulate skin disturbance (Spiekstra et al., 2002,). Our findings indicate that under these tested conditions a mixture of two or three ingredients decreased RhE viability to under 20% and consequently resulted in a significant increase on levels of IL-1 α release. Moreover, morphological changes in keratinocytes upon topical exposure of mixtures to RhE were now observed. This is a particularly interesting finding and therefore indicate that in the tested conditions the irritant potential of these mixtures occurs after longer periods of topical exposure

to RhE while this is not observed when ingredients are tested alone. Thus, considering that these combinations involve a mixture of skin sensitizers and other compounds that have not yet had their toxic properties elucidated, we hypothesize that the mixtures could form more toxic by-products resulting in a possible additive or even synergistic effect, explaining the reason that irritation on RhE was observed after 24h of topic exposure to the mixtures. Besides these events, skin allergy induction involves other complex factors and mechanisms that may contribute for its development. In normal skin, the epidermis (outermost layer) forms a protective barrier against external environment toxicants. Once this barrier is damaged several multiple skin health impairments including increased trans epidermal water loss, redness, and susceptibility to infection or irritation by external factors may occur. Therefore, skin barrier disruption could be another preponderant key factor that contributes to allergy skin induction and even predispose to Atopic Dermatitis or similar conditions (Bonnart et al., 2010). The magnitude of these injuries is often influenced by stratum corneum damage, chronic or acute exposure, and the anatomical location of the damage. Underlying deficiencies in the process responsible for development of a normal, healthy stratum corneum barrier have been linked to the etiology of skin disorders including psoriasis, eczema, and atopic dermatitis (Motta et al., 2001; Cookson, et al., 2001; Sator et al., 2003, Proksch et al., 2002). Also, when the stratum corneum is damaged, the living epidermis is more directly exposed to external factors and the innate immune system can be more easily triggered (Bendetto, Kubo and Beck, 2012). Therefore, to understand the level of this damage, we evaluated hair dye exposure resulted in filaggrin depletion. Filaggrin is an essential skin protein produced by keratinocytes that regulates epidermal permeability and barrier function. The depletion of filaggrin leads to multiple epidermal morphological changes and is frequently observed in skin allergy diseases such as ichthyosis vulgaris and atopic

dermatitis (Brown et al., 2012, Thyssen et al., 2014, Nomura et al., 2008). Our results showed that the mixtures PPD/H/R and PPD/H modulated epidermal morphology and expression of filaggrin in stratum corneum, although results were more pronounced after mixture of three ingredients. Therefore, it is possible that frequent exposure to this mixture could damage the epidermal barrier increasing skin contact to by products that have yet not their toxicity elucidated. Here, using H.E staining we have shown that the mixture of the three components increases epidermal damage when compared to mixture of the two ingredients. Furthermore, TUNEL analysis indicates that exposure to oxidized PPD increased keratinocyte apoptosis on the upper layer of epidermis, while exposure to the three components increased apoptosis in the deeper basal layer of RhE. The specific molecular mechanisms that results in allergy induction and the possible mode of action of the skin exposure to permanent hair dyes mixture has not been elucidated yet; however, recent findings have shown that epidermal barrier dysfunction is involved in many skin diseases process (Nakatsuji et al., 2011). Considering the data shown here, we suggest that the key event to skin allergy induction related to permanent hair dyes is possibly related to a number of toxic by-products formed after PPD oxidation and reaction with RES.

4. Conclusion:

Topic exposure to mixture of ingredients PPD/H₂O₂ and PPD/H₂O₂/RES to RhE for 24 hours increased epidermal damage, decreased viability, increased release of cytokine of IL-1 α , increased apoptosis and reduced filaggrin expression. The hazard effects were more pronounced when RhE was exposed to PPD/H₂O₂/RES when compared to PPD/H₂O₂. Some of these parameters are associated to epidermal irritation reactions and appear after longer periods (24 h) of topic exposure to RhE. At the tested conditions, no sign of irritation appeared after 1 hour of

exposure, these effects were not observed when using same concentrations of the individual ingredients, indicating the more hazard effect of the hair dye mixture. To conclude, we suggest that by-products formed during the reaction of the ingredients together with (OH[•]) formed could contribute to skin barrier loss which is another important key event in the development of allergic contact dermatitis. Also, we demonstrated that the use of RhE to evaluate skin irritation after exposure to hair dyes is a useful model for further in-depth studies on the molecular processes of permanent hair dye mixtures in inducing allergic responses.

5. Materials and Methods

5.1. Electron spin resonance (ESR)

Using a Bruker EMX100 ESR spectrometer ESR measurements were performed. Instruments settings were: modulation amplitude 1 G; receiver gain, 5.0 10⁴; time constant, 5ms; sweep time, 84 s; microwave power 20mW with frequency, 9.76 GHz, and power. A mixture between PPD, H₂O₂ and Resorcinol was held at lower concentration than the allowance for commercial hair dye formulations of hair dyes (SCCS/1443/11, 2012) to fulfill and be suitable for *in vitro* conditions. To obtain a stable ESR signal 5,5- dimethyl-1-pyrroline-N-oxide (DMPO) was used as a spin trapping agent at the concentration of 280 mM. Fenton reaction was prevented by the addition of Chelex (1 mg/mL) to all incubations.

5.2 Cell dissociation from foreskin: keratinocyte isolation,

Skin cells were obtained from foreskin donated tissue obtained from surgery held at the University of Sao Paulo Hospital (HU-USP). Next, isolation of cells was described as previously shown by our group (Sandri et al, 2016 and Brohem et al., 2011 with minor changes. Briefly, immediately after surgery the foreskin tissue was kept in phosphate-buffered saline (PBS) containing antibiotics penicillin–streptomycin (Pen-Strep; Gibco, Life Technologies, Carlsbad, CA). Next, excess fat and blood tissue were carefully removed from samples and fragmented followed by incubation with 4 mg/mL dispase II (Roche, Life Sciences, Branford, CT) overnight at 4C. The next day, using two tweezers epidermis was carefully removed from dermis. Then, to provide cell dissociation epidermis was placed in 0.05% trypsin-EDTA for 15 min at 37C and dermis was incubated in 1 mg/mL collagenase (Gibco, Life Technologies) for 3 h in water bath for 3 hours at 37C. Keratinocytes were cultured in KGM-Gold Bullet Kit medium (Lonza KGM gold keratinocyte basal medium, without Ca^{2+} , Walkersville, MD), and fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies) supplemented with 10% fetal serum.

5.3 Epidermal equivalents reconstruct (RhE)

Human keratinocytes isolated from foreskin were maintained in culture using a specific keratinocyte medium (KGMTM gold and KGM single kit Lonza) supplemented with 1 μM of isoproterenol and kept in incubator at 37 °C at 7.5% CO_2). The cells were plated at a concentration of 2.5×10^5 cells in a 12 well plate trans well membrane with pore 0.4 μm . Cells

were maintained for 24 hours submerged in 1:1 KGMTM/RAFT. Then, each trans well was transferred to a deep well plate for air-liquid interface kept with 12mL of RAFT medium at the bottom. This was cultured for 12 days so that epidermis would differentiate.

5.4 Skin irritation

In vitro skin irritation assays correlate decrease on epidermis viability that is measured by mitochondrial activity using tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to a direct increase of cytokine IL-1 α release by keratinocytes in presence of an irritant as a consequence of keratinocyte damage (Bronnenberg et al., 2007, Coquete et al., 2003, De Jongh et al., 2006, Faller et al., 2002, Gibbs et al., 2002, Spiekstra et al., 2005). *In vitro* skin irritation test was performed based on OECD TG 439 guideline using the RhE model previously published by our group (Pedrosa et al., 2017). For this, the following ingredients and mixtures were topically exposed to RhE, PPD, RES, H₂O₂ and the following mixtures: P/H₂O₂/R, P/H₂O₂, and P/R for 15 minutes at the final concentration of 2000 μ g/mL for 15 minutes exposure, then, tissue was rinsed with PBS and maintained in the incubator with 5% CO₂ at 37°C. Then, RhE was transferred to 24-well plates and incubated with 300 μ L of MTT (1 mg/mL) for 3 h at 37 °C and 5% CO₂. Next, tissue was washed with PBS and added to a plate containing 2 mL of isopropanol at room temperature for 2 h. Extracted isopropanol (200 μ L) containing formazan was then transferred to a 96-well plate, and the OD of the resulting purple dye was measured at 570 nm using an ELISA plate reader. Isopropanol was used as a blank. The concentrations of the ingredients tested was 10 times lower than the

maximum commercial allowance in hair dyes formulations according to (SCCNP). Three skin replicates were used for each of the test chemical and control groups. Sodium dodecyl sulfate (SDS) (2,5%) was used as positive chemical and PBS pH 7.4 was used as negative chemical. After exposure, test materials were removed by repeated rinsing with phosphate buffered saline (PBS) and skin models were transferred to the new plates for the viability test.

5.5 RhE viability test and cytokine IL-1 α determination

The same compounds at same conditions tested before were topically exposed for 24 hours in RhE. The topic exposure of 24 hours followed by epidermal viability and IL-1 α is a standard procedure used in our laboratory to predict *in vitro* skin irritation in RhE (Gibbs et al., 2002, Spiekstra et al., 2005, 2009). Furthermore, this time of exposure was also chosen based on a publication of Lademann et al., 2008, that found remaining hair dye in human skin 24 hours after exposure, even after washing. The RhE were added to 24 well plate for viability determination, where, 300 μ L of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.3 mg/mL) was added to each skin model followed by 24 hours of incubation at 5% CO₂ incubator. Next, epidermal equivalents were washed with PBS pH 7.4 and incubated in 24 well plate containing 2mL isopropanol per well that was placed into a shaker for 2 hours, so the insoluble formazan products were extracted. Then, 200 μ L of the isopropanol extract was transferred onto a 96-well plate to measure the optical density (OD) at 570 nm. To achieve more information regarding the allergenic potential of each compound and their respective mixture, the released IL-1 α level evaluated. For this, an immunoassay kit 24h for human IL-1 α was

purchased from R&D Systems (Minneapolis, MN, USA). After coating the 96-well plate with capture antibody, 100 μ L of media obtained from treated RhE models were added to the wells and incubated for 2 hours at room temperature. After washing the wells, detection antibody was added to the wells and the plates were incubated at room temperature for 2 hours. Following the washing steps, secondary antibody with streptavidin-horse radish peroxidase was added to the well and the plate was incubated for 20 minutes. After incubation and washing the plate, substrate solution was added and incubated for 20 minutes. After incubation, stop solution containing 2N sulfuric acid was added to the wells and OD was measured at 450 nm using a microplate reader. The concentration was calculated using a standard curve.

5.6 Histopathological analysis: Hematoxylin/Eosin (H.E) staining and immunofluorescence

For the histopathological examination, epidermal equivalents were fixed in 10% (v/v) neutral buffered formaldehyde at 4°C for 12 h. Then, 5 μ M paraffin sections of the tissues were mounted on glass slides and stained with hematoxylin and eosin for morphological analysis. For immunofluorescence analysis, paraffin sections were maintained at 55°C for deparaffinization, followed by washing in xylene and hydration in decreasing series of alcohol and PBS. Antigen recovery was performed twice in citrate buffer pH 6.0 for 5 min at 95 °C. The immunolabeling assay was carried out using the mouse monoclonal antibodies filaggrin rabbit (Abcam-24584) dilution 1:1000. A commercial kit with anti-rabbit immunoglobulin secondary antibody (LSAB+ SystemHRP; Dako, Carpinteria, CA) was used in combination with diaminobenzidine (Sigma-Aldrich) according to the manufacturer's instructions. All images were obtained by optical

microscopy and analyzed by NIS-Elements software (Nikon Instruments, Melville, NY). For each image, we performed 3 epidermal.

5.7 TUNEL analyses of epidermal equivalents

TUNEL assay was performed using a Fluorescein-FragELTM DNA fragmentation detection kit that allows the recognition of apoptotic nuclei in paraffin tissue sections. Epidermal equivalent treated with the PPD, RES, H₂O₂, PPD/H/RES, P/H and P/R were embedded in paraffin and placed onto glass slides at 5- μ m-thick sections. The glass slides were submitted to deparaffinization and rehydration where they were immersed in xylene and different percentages of ethanol. Then permeabilization of the specimen was done by the addition of 100 μ l of 20 μ g/mL proteinase K followed by incubation at room temperature for 20 min. Next, glass slides were washed with PBS and followed by the equilibration and labelling reaction were 60 μ l of a mixture of 57 μ l of Fluorescein-FragELTM TdT Labelling Reaction Mix with 3 μ l TdT Enzyme. The slides were then placed onto a humid chamber followed by incubation of 1.5 h at 37 °C. Then, glass slides were mounted using Fluorescein-FragELTM Mounting Media what allowed cell population to be seen using a DAPI filter for 330-380nm while labelled nuclei analysis could be visualized using a filter, 465-495nm. Fluorescence microscopy image acquisition was performed using a 12-bit Hamamatsu Digital Camera C10600 ORCA-R (Hamamatsu Corporation, Bridgewater, NJ) connected to a Nikon ECLIPSE 90i Microscope (Nikon Instruments, Inc.,

Melville, NY) on a Dell Optiplex 960 desktop computer running Windows 7 Enterprise (Microsoft Corporation, 2009).

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- Permanent hair dye formulations are formed by a complex mixture of skin sensitizers
- The specific mechanism that leads to skin allergy and irritation remains unknown
- Among other hair dyes, permanent are the most produced and consumed worldwide
- Reconstructed skin models are efficient tools to screen toxicity of these dyes