Micronuclei assessment in the urothelial cells of women using hair dyes and its modulation by genetic polymorphisms

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Abstract

Increases in the frequency of micronuclei (MN) in exposed cells can be used as a measure of genotoxicity. Hair dyes contain chemicals that are eliminated by urine and could be genotoxic to urothelial cells. To address this question, we evaluated whether hair dye use is associated with an increase in the frequency of MN in urothelial cells, and whether this association is modified by NAT1 (N-acetyltransferase 1), NAT2 (N-acetyltransferase 2) and GSTM1 (glutathione-S-transferase M1) genotypes. We included 92 women participating as controls in a bladder cancer case-control study in Spain. Of those, 72 had adequate number of cells to be included in the MN analysis. There were no significant differences in the mean MN frequency in women using hair dyes in the last month (9.88 MN/1000 cells), in comparison with the MN in unexposed women (9.50 MN/1000 cells). No statistically significant differences in MN frequency were observed by type of hair dye or color of the hair dye. Comparison of subjects in the highest quartile of MN frequency (≥12 MN/1000 cells) and those in the lowest quartile (<4 MN/1000 cells) suggested an association between hair dye use and elevated MN frequency (OR 14.2 (95% CI 0.81–247.8; P = 0.069)). None of the polymorphisms examined significantly modified association between hair dye use and frequency of MN. Findings of an increased frequency of MN in urothelial cells of hair dye users suggest a possible genotoxic effect of hair dye compounds and need confirmation in larger studies.

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1. Introduction

The use of hair dyes is a very popular habit. In developed countries, over one-third of women older than 18 years, and 10% of men older than 40 years, uses coloring hair products. There are three major types of hair dyes: permanent dyes, semi-permanent dyes and temporary rinses. Permanent dyes are the most common, accounting for roughly three quarters of all hair dye used [1].

Although different mutagenic and carcinogenic compounds have been historically found in many brands of hair dye products [2–4], few ingredients of currently commercial hair dyes are known to cause cancer in animals [5]. Nevertheless, when chromosome aberrations were evaluated in people exposed (by use or occupationally) to hair dyes, significant increases in the peripheral lymphocytes were observed [6]. On the other hand, the levels of sister-chromatid exchanges did not increase with the use [7], or in occupational exposure [8]. In addition, although the levels of DNA damage measured with the comet assay did not increase in a study with professional hair colorists [8], a significant increase has been recently reported, using the same assay, in human volunteers after hair dying [9].

Epidemiological studies have examined risk of bladder cancer and of other neoplasms in hairdressers, barbers and users of hair dyes. The International Agency for Research on Cancer (IARC) [1], evaluated hairdressers and barbers as occupations entailing exposures that are probably carcinogenic to humans. An excess bladder cancer risk has been detected in two recent case-control studies that included information on personal use of permanent hair dyes [5,10], or in subgroups of women using dyes [11], but the overall results are not consistent [12,13]. Large studies, including the American Cancer Society cohort study [14], and the National Bladder Cancer Study in the USA [15], indicated no overall increased risk of bladder cancer among hair dye users. No increased risks were found among users of hair dyes in a recent study in Spain [16]. Two recent meta-analyses both yielded a pooled relative risk of 1.01 [17,18]. Two studies evaluated the role of genetic polymorphisms in relation to hair dye use and bladder cancer risk. In one study risk was higher among subjects with a NAT2 and CYP1A2 (cytochrome P450, family 1, subfamily A, polypeptide 2) ‘slow’ phenotype, who were exclusive permanent hair dye users [5] while no statistically significant associations were observed in the other study [16].

The inconclusive evidence linking hair dye use and genotoxic risk provides impetus to examine new information on potential mechanisms of action of hair dyes. The use of the MN test with exfoliated epithelial cells has been suggested as a good biomarker for monitoring individuals with elevated risk of genetic damage resulting from exposure to mutagenic/carcinogenic agents [19]. Increases in the MN frequency of bladder cells have been observed in smokers [20], and in people drinking water with high levels of arsenic [21], as well as in schistosomiasis patients who has severe inflammation of the epithelial tissue in the bladder as well as cancer [22]. We have recently published the results of an epidemiological study in Spain [16] that did not identify an increased risk of bladder cancer among users of hair dyes. In the present analysis we examine the same exposure but using a sensitive and validated biomarker. Specifically, we evaluate in the same population the MN frequency in the urothelial cells of women using hair dyes recently (in the last month) in comparison to the frequency obtained in women that did not use hair dyes. In addition, we evaluate MN frequency in relation to the GSTM1, NAT1 and NAT2 genotypes that have been previously associated with bladder cancer risk and could be important for the metabolism of some components of hair dyes [5,16,23].

2. Materials and methods

2.1. Subjects

The study was conducted as an adjunct to the Spanish Bladder Cancer (SBC) Study, a hospital-based case-control study of 2490 subjects in five areas of Spain (2172 men; 318 women). Controls were mainly hospitalized for trauma or minor surgery. Detailed information on the design of the study and the selection of studies has been published [24]. Information on hair dye use among women participating in the study who agreed to provide information on the hair dye component of the study, was obtained through a computer-aided telephone interview (n = 128 cases; 131 controls) [16]. Information was
requested on type of hair dye, color, mode of application and time period/frequency of application. Of the 131 female controls with information of hair dye use, 92 provided urine samples for MN analyses, and samples from 72 of these women had enough number of cells to be included in the MN analyses. The frequency of hair dye used in the final sample (72) does not significantly differ from the initial group (131). The Ethic Committees of all the participant hospitals approved the study.

2.2. Urine sample collection and slide preparations

First void urine samples were collected at home in plastic vials (about 50 mL) and sent to the laboratory where they were processed as soon as possible. Cell samples were concentrated by centrifugation (10 min at 1500g), the supernatant was discarded and cells were washed in a NaCl solution (0.9%). Cells were centrifuged again and resuspended in new NaCl solution (0.09%). After a new centrifugation, cells were fixed in 5 mL of a fresh fixative solution (methanol/acetic acid, 3:1) added drop by drop. After 1–2 washings with the fixative solution, the pellet was resuspended in 1 mL of fixative solution and dropped onto pre-cleaned microscope slides. Cell density was confirmed by using a phase contrast microscope (400×) and adjusted by adding fixative solution. Slides were air dried overnight and stored at 4 °C in a black box until the staining.

2.3. Scoring procedure

Cells were stained with a DNA-specific stain, namely 1 µg/mL 4',6-diamino-2-phenylindole dihydrochloride (DAPI), what avoids possible scoring artefacts. One scorer under an Olympus BX50 fluorescence microscope (1000×) scored a total of 2000 cells/donor wherever possible on coded slides. The criteria for MN evaluation were those suggested by Stich et al. [25]. The frequency of cells with MN and the total number of MN were determined for each analyzed subject. Only those cells with a typical morphology corresponding to the urothelial cells were scored. This criterion avoids any kind of bias, since in woman many squamous cells (with no urothelial origin) are observed. Although bacteria are present in some few urine samples, they do not interfere with the scoring.

2.4. Genotype assays

DNA for genotype assays was extracted from leukocytes using the Puregene® DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Genotype assays were performed at the core genotyping facility (CGF) of the Division of Cancer Epidemiology and Genetics, National Cancer Institute. Genotype assays for NAT1, NAT2 and GSTM1 have been previously described [23]. All genotypes under study were in Hardy–Weinberg equilibrium among the studied population.

2.5. Statistical methods

Linear regression was used to examine differences in the frequency of MN in relation to hair dye exposure. Odds ratios (OR) and 95% confidence intervals (95% CI) for the association between high (>75th percentile) and low (≤75th percentile) levels of MN frequency and hair dye use were estimated using unconditional logistic regression models, adjusting for age (continuous) region and smoking status (never, former, current smokers). In some analyses regions are clustered to avoid over dispersion of the data. Additional adjustment for coffee consumption did not modify results and coffee was therefore not included in the final models. Multiplicative interactions between genotypes and hair dye use were evaluated using stratified analyses and conventional logistic models in STATA v.8.0.

3. Results

The description of the population under study is shown in Table 1. The average age of the selected women was 66.5 ± 6.7 years with an age range from 39 to 79 years. Women were mainly non-smokers, with only

<table>
<thead>
<tr>
<th>Description of the study population</th>
<th>Recent hair dye usea</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (n = 27)</td>
<td>Yes (n = 45)</td>
<td>Total (n = 72)</td>
</tr>
<tr>
<td>Age mean (SD)</td>
<td>66.6 (9.2)</td>
<td>66.3 (10.4)</td>
<td>66.5 (9.6)</td>
</tr>
<tr>
<td>Smoking n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>26 (96.3)</td>
<td>40 (88.9)</td>
<td>66 (91.7)</td>
</tr>
<tr>
<td>Ever</td>
<td>1 (3.7)</td>
<td>5 (11.1)</td>
<td>6 (8.3)</td>
</tr>
<tr>
<td>Coffee n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular</td>
<td>6 (22.2)</td>
<td>11 (24.4)</td>
<td>17 (23.6)</td>
</tr>
<tr>
<td>Never</td>
<td>21 (77.8)</td>
<td>34 (75.6)</td>
<td>55 (76.4)</td>
</tr>
<tr>
<td>Region n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barcelona</td>
<td>9 (33.3)</td>
<td>9 (20.0)</td>
<td>18 (25.0)</td>
</tr>
<tr>
<td>Vallés + Alicante + Tenerife + Asturias</td>
<td>9 (33.3)</td>
<td>18 (40.0)</td>
<td>27 (37.4)</td>
</tr>
<tr>
<td>Education n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less primary</td>
<td>12 (44.4)</td>
<td>30 (66.7)</td>
<td>42 (58.3)</td>
</tr>
<tr>
<td>Primary + higher primary</td>
<td>15 (55.5)</td>
<td>15 (33.3)</td>
<td>30 (41.7)</td>
</tr>
</tbody>
</table>

a Recent use was defined as use within one month of urine collection.
three current smokers and three ex-smokers. Sampled people came from five different areas of Spain: Barcelona, Vallès, Asturias, Alicante and Tenerife.

The distribution of the MN frequency in the study population is shown in Fig. 1. The mean (SD) MN frequency of the overall population was 9.72 ± 0.82 MN/1000 cells, and did not vary by hair dye exposure: 9.90 MN/1000 cells (±0.78) observed in women using hair dyes during the last month vs 9.50 MN/1000 cells (±2.45) observed in women who did not use hair dyes in last month. No substantial changes were observed when adjusting for smoking and coffee consumption neither when adjusting for age and region.

Table 2
Odds ratios (OR), 95% confidence intervals (95% CI) for micronuclei frequency (≤75% vs >75%) and different characteristics of hair dye use

<table>
<thead>
<tr>
<th>Characteristics of hair dye use</th>
<th>No. MN frequency ≤75%/&gt;75%</th>
<th>OR± (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Used hair dyes last month</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not using hair dyes last month</td>
<td>21/6</td>
<td>1.00 –</td>
</tr>
<tr>
<td>Used hair dyes last month</td>
<td>30/12</td>
<td>1.46 0.44–4.81</td>
</tr>
<tr>
<td>Type of hair dyes normally used</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never used</td>
<td>14/3</td>
<td>1.00</td>
</tr>
<tr>
<td>Only permanent</td>
<td>27/10</td>
<td>1.61 0.35–7.42</td>
</tr>
<tr>
<td>Other</td>
<td>11/4</td>
<td>1.25 0.15–10.37</td>
</tr>
<tr>
<td>Tone of color of hair dyes normally used</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never used</td>
<td>14/3</td>
<td>1.00</td>
</tr>
<tr>
<td>Light (light brown, blond)</td>
<td>28/11</td>
<td>2.02 0.43–9.51</td>
</tr>
<tr>
<td>Dark (dark brown, black, red)</td>
<td>9/4</td>
<td>1.45 0.23–9.33</td>
</tr>
<tr>
<td>Color of hair dyes normally used</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never used</td>
<td>14/3</td>
<td>1.00</td>
</tr>
<tr>
<td>Brown (dark or light)</td>
<td>10/4</td>
<td>1.32 0.20–8.49</td>
</tr>
<tr>
<td>Blond, black, red</td>
<td>25/10</td>
<td>1.99 0.42–9.50</td>
</tr>
</tbody>
</table>

± Odds ratios are adjusted for age, region of residence and smoking status.

Table 3
Mean (SD) MN frequency by NAT1, NAT2 and GSTM1 genotypes in the study population

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Mean (SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*4/*4</td>
<td>32</td>
<td>9.64 (7.31)</td>
<td>0.67</td>
</tr>
<tr>
<td>*4/*10 or *4/*10</td>
<td>12</td>
<td>8.64 (5.43)</td>
<td></td>
</tr>
<tr>
<td>NAT2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate and rapid</td>
<td>24</td>
<td>8.71 (4.66)</td>
<td>0.21</td>
</tr>
<tr>
<td>Slow</td>
<td>39</td>
<td>10.94 (7.89)</td>
<td></td>
</tr>
<tr>
<td>GSTM1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+ and +/−</td>
<td>38</td>
<td>9.73 (7.11)</td>
<td>0.79</td>
</tr>
<tr>
<td>−/−</td>
<td>25</td>
<td>10.21 (6.91)</td>
<td></td>
</tr>
</tbody>
</table>

Subjects were categorized as having high (≥12 MN/1000 cells, 75th percentile) or low (<12 MN/1000 cells) MN frequency. Use of hair dyes in the last month was associated with high frequency of MN but the association was not statistically significant (Table 2, P = 0.536). Similarly, MN frequency was associated with use of permanent hair dyes compared to non-permanent, to light colors compared to dark and to non-brown colors compared to brown, but none of these results were statistically significant (Table 2). Comparison of subjects in the highest quartile of MN frequency (≥12 MN/1000 cells) and those in the lowest quartile (<4 MN/1000 cells) suggested an association between hair dye use and elevated MN frequency (OR 14.2 (95% CI 0.81–247.8; P = 0.069)).

GSTM1 (null vs present), NAT1 (wt/wt vs *10 carriers) and NAT2 (slow vs rapid/intermediate acetylators) genotypes were not significantly associated with MN frequency (Table 3). None of the polymorphisms examined significantly modified association between hair dye use and frequency of MN.

4. Discussion

The easy detection of genotoxic damage in exfoliated epithelial cells has led to renewed interest in analyzing early carcinogenic changes in epithelial tissues [25]. In this context, the use of MN as a biomarker of genotoxic effects has been of special relevance. MN are acentric fragments or complete chromosomes that fail to attach to the mitotic spindle during cytokinesis and are excluded from the nuclei. Thus, the MN assay is a system that allows the detection of both clastogenic and aneugenic effects. In the present study, micronuclei in urothelial cells have been evaluated to determine the possible association between genetic damage and use of hair dyes in Spanish women. Exfoliated urothelial...
cells have several advantages with respect to peripheral blood lymphocytes, as a non-invasive sample collection, but less studies exist on inter individual differences due to endogenous and environmental influences on MN in exfoliated cells as well as assay validation as compared with MN in PBL. In addition, MN in urothelial cells is theoretically associated with bladder genotoxic exposure contrarily to MN in lymphocytes which is associated with cancer risk.

In our overall group, the frequency of MN was 9.72 ± 0.81 MN/1000 cells, which agrees with results reported by other authors. The reported MN frequencies have a wide range, which would depend of several factors including sex, age, habits as well as undoubtedly the staining applied and the scoring criteria. Thus, low values as 1.2 or 1.4 MN/1000 cells have been reported [26,27], although higher frequencies as 5.18 and 6.9 MN/1000 cells have also been found [28,29]. Although the frequency obtained in our study is high, similar values have also been reported in literature. A recent study [30], gives a frequency of 9.24, practically the same that our MN frequency. It must be remembered that our group is constituted by old women and micronuclei formation increases directly with both sex and age parameters at least in peripheral blood lymphocytes [31]. In this context it is interesting to indicate the existence of the human micronucleus (HUMN) project, an international database comparison study on the data obtained in human lymphocytes [32]. This project shows that the majority of studies found an increased level of MN in PBL with increased age and a higher level in elderly women as compared with similar age group of men. However as a limited number of studies has been conducted evaluating MN in exfoliated urothelial cells it can only be speculated that age might also influence on the level of MN in these cells. Although there is no data relating MN frequency and age, the low repair capability associated to elder people would also affect the incidence of MN in our study group. In addition, staining criteria may be also an important factor modulation MN frequency. In this case, it must be emphasized that we have used DAPI staining and the scoring has been carried out under fluorescence microscope, which permits a better and clear detection of MN.

Results of our study indicated that the use of hair dyes in the last month was associated with a higher frequency of MN, but this increase was modest and not statistically significant. In our study, we did not have information on specific brands of hair dyes but evaluated normal use of hair dyes in a population sample. Hair dyes is a complex group where permanent dyes differ in composition compared to semi-permanent dyes, although carcinogenic agents have been found in both types of products. Permanent dyes typically contain para-phenylenediamine (PPD) or derivatives of PPD [33], that have been reported to increase the tumors in the liver of mice. Thus, the type of hair dye is a factor to be taken into account when evaluating the genotoxicity of hair dyes. The color of the hair dye was also related to risk; the incidence of non-Hodgkin’s lymphoma was higher in women using dark colors compared to those using light colors [34]. A study evaluating the presence 4-aminobiphenol (4-ABP, a known bladder carcinogen) in hair dyes, detected it in eight of the 11 hair dyes examined and particularly in black, red and blonde hair dyes but not in brown hair dyes [35]. Nevertheless, our results indicate that neither type nor the color used by the study participants seems to have any significance with respect to the MN frequency in bladder cells.

It is well documented that micronuclei formation is augmented in urothelial cells of smokers [20,36,37] and this increase in the genetic damage has been correlated with the increased bladder cancer observed in smokers [38]. Such increases have also been found in people who quit smoking more than 2 years before the study, indicating that toxic effects of smoking in basal tissue persists [20]. This finding is supported by data from the National Bladder Cancer Study (USA) that showed that ex-smokers had an increased risk of bladder cancer, although risk for ex smokers was lower than in current smokers [39]. The observed lack of effects of tobacco smoking in our study is probably due to the small number of current smokers (3) and ex-smokers (3) included in our study. With respect to coffee consumption, no data have been previously obtained that relate coffee drinking with genotoxic damage in urothelial cells.

Although no studies have been published on the genetic damage induced in epithelial cells as consequence of hair dye use, three biomonitoring studies using lymphocytes as target cells have been reported. Although an early study found that levels of sister chromatid exchanges did not increase with the use [7], a second study [6], observed increases in the frequency of chromosome aberrations in the peripheral lymphocytes after repeated hair dying. More recently, a significant increase in the DNA damage of human lymphocytes from human volun-
teers after hair dying was reported based on the comet test [9]. In addition, two further studies of occupational exposure to hair dyes have also been reported, both showing no increases in the frequency of sister chromatid exchanges [8], or the levels of DNA damage measured with the comet assay in professional hair colorists [8], which may indicate a higher risk associated to personal hair dye than from professional exposure. These in vivo results emphasize the need of complementary in vitro genotoxicity studies to have more clear information on the risk of hair dye exposure.

Although further studies on large scale are needed, our results comparing the two extreme quartiles of MN distribution suggest an association between hair dye use and genetic damage in the urothelial cells, which may correlate with the bladder cancer risk. In addition, our results support the advantages of the MN assay in urothelial cells as a biomarker of early genotoxic effects on bladder epithelia, including bladder cancer, caused by exposure to chemicals. Detecting urothelial genotoxic damage in exposed subjects can be interesting for cancer prevention, as it has been demonstrated in different groups exposed to chromium [40], arsenic [41] and mineral jelly [27]. In addition, micronuclei can also arise as consequence of genomic instability as has been observed in individuals suffering of ataxia telangiectasia, Bloom and Fanconi anemia syndromes [42,43], reinforcing the predictive value of micronuclei in the prediction of human cancer hazards.

The level of genetic damage in cells is considered a good biomarker of effect, which may indicate a future cancer risk [44]. Increases in the frequency of these biomarkers can be a consequence of both exposures to genotoxic agents and/or genetically determined individual factors. Thus, it is crucial to determine which genetic and environmental factors are responsible for a particular increase in the frequency of these biomarkers. Because we found a small effect in the MN frequency related to the use of hair dyes, the question is whether some genetic polymorphism modulates the genotoxic effect measured in the bladder epithelial cells. The NAT2 slow acetylator phenotype and the GSTM1 null genotype have been associated with bladder cancer risk [23,45,46], and it has been postulated that this results in decreased capacity to detoxify aromatic amines and polycyclic aromatic hydrocarbons [47,48]. Thus, these genetic variants are good candidates to examine in relation to MN modulation.

In addition, permanent hair dye use has been associated to bladder cancer risk and permanent hair dyes content arylamines, which are the putative carcinogens of bladder cancer development [49]. Gago-Dominguez and colleagues [49], observed that the association between hair dye and bladder cancer is principally confined to the NAT2 slow acetylaters. In contrast, a recent preliminary study examining hair dye containing [14C]-Para-phenylenediamine (PPD) did not show variations in the urinary profile between slow and intermediate NAT2 acetylators [50]; only 0.43% of the applied hair dye is found in urine, suggesting that the human acetylation rate of PPD after topical application is independent of the NAT2 genotype status. Acetylated metabolites of PPD, such as MAPPD (N-monoacetylated) and DAPPD (N,N-diacyethylated), do not induce micronuclei in in vitro studies, contrarily to its parental form PPD. Thus, these acetylated conversion products are considered to be detoxified metabolites that are biologically less reactive than the parent molecule PPD [51]. This work indicates the complexity and importance of processes, such as metabolism and penetrance of the different hair dyes, in their risk evaluation. Although we had small statistical power to evaluate gene–environment interactions, our results indicate that the genotypes (NAT2, NAT1, GSTM1) analyzed do not modify the frequency of MN in urothelial cells, irrespectively, of the hair dye use, providing no support for a role for hair dye use in bladder tumor carcinogenesis, nor its modulation by the genetic variants analyzed.

Acknowledgments

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