

Assessment of *in vitro* genotoxicity effect of homosalate in cosmetics

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ABSTRACT: Many cosmetics contain UV filters as active ingredients for skin protection. Homosalate (HMS) is one of the most widely used organic UV filter. Despite the widespread use of these products, data about their genotoxic effects are lacking. Genotoxicity assessment is an important part of biosafety evaluation; therefore, we aimed to investigate genotoxic effects of homosalate as organic UV filter. The cytotoxicity effects of homosalate were assessed by trypan blue exclusion. Homosalate was incubated with four different concentrations for 30 min and 120 min. Our results revealed that HMS increased the DNA migration both the time and concentration manner. DNA damage was higher in cells that had been incubated with the greatest concentration of 200 µg mL⁻¹ (p<0.05). Results obtained by the present study indicate that homosalate would be genotoxic at higher concentration and incubation time. Further studies should be carried out with different test systems (*in vivo* and *in vitro*) related to its genotoxic effects and incubation time should be extended. This may be helpful for genotoxicity evaluation of compounds with similar characteristics and their impact of human population.

KEYWORDS: Comet assay; homosalate; DNA damage; genotoxicity.

1. INTRODUCTION

Organic UV filters protect human skin from UV radiation and prevent light-induced product degradation in many cosmetic products such as lipsticks and lip balms, make-up, perfumes, facial creams, aftershaves, hand creams, face powders and hairsprays by absorbing UV light [1]. UV filters are divided in three types: organic (chemical), inorganic (mineral, physical) and insoluble organic pigments [2]. Inorganic UV filters comprise of particles that act by scattering, reflecting or absorbing the passage of UV radiation [3]. Organic UV filters are able to absorb UV rays through chemical reactions, whereas inorganic UV filters incident UV radiation and their importance increases day by day due to negative effects of UV radiation and skin cancer. Generally, organic and inorganic UV filter combinations are used to achieve a sufficient Sun Protection Factor [4].

Increasing application of these substances in all age groups brings with the responsibility against healthcare, economy and environment [5]. Nowadays, there is a growing concern regarding the safety of organic UV filters, mainly when applied on the skin in variable conditions. It is also reported that some skin-absorbed UV filters could result unknown systemic toxic effects [6]. However, recent studies have shown that organic UV filters in certain formulation -after absorption through the skin- have the potential of being metabolized and excreted [7,8]. Now that many organic UV filters are lipophilic, they can easily bioaccumulate and biomagnify in aquatic ecosystems, where their impact is a big concern. These persistent chemicals have been determined in various water samples [9], fish [10] and several biological samples of human such as breast milk [11] and placenta [12]. Therefore they should be considered as environmental contaminants.

Although the acute and subchronic toxicities of these compounds are considered to be rather low; their long-term exposure may result in various adverse and serious health effects like mutagenicity, genotoxicity, ontogeny, endocrine disruptors, and photoallergic reactions [2,13].

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Organic UV filters are often classified as 7 group; 1-Anthranilates, 2-benzophenones, 3-camphors, 4-cinnamates, 5-dibenzoylmethanes, 6-p-aminobenzoates 7- salicylates [13]. Among these salicylates, 3,3,5-trimethylcyclohexyl 2-hydroxybenzoate (homosalate- HMS) can be found in many products (Figure 1) [7]. The content of UV filters in products are regulated by EU Cosmetic Directive and according to Directive 76/768/EEC, the maximum allowed concentration of HMS in cosmetic products can be 15% in the United States, Australia and 10% in the European Union (EU), Korea and Japan [14, 15].

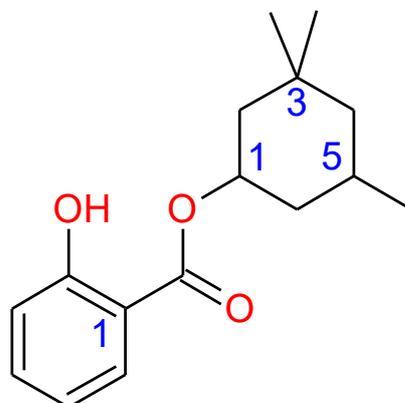


Figure 1. The Chemical structure of homosalate.

As a member of salicylate-type UV filters, HMS contains an ester bridge between phenol and alkyl-substituted cyclohexane rings. Its unique ortho-disubstituted chemical structure provides an important advantage for this molecule of being chosen as a stable ingredient in UV filter formulations for more than fifty years [16]. The genotoxic activities of phenolics [17, 18], esters [19, 20] and cyclohexane [21] were previously reported. The presence of alkyl-substituted cyclohexane ring can drastically change the lipophilicity of the whole molecule, might possibly have big impact on ADME (absorption, distribution, metabolism, excretion) [22] and as well as toxicity properties. In addition, toxicological profile of HMS has been reviewed recently [23].

Although the daily consumption of UV filters is low, the long-term exposure to organic UV filters can cause to serious toxic effects including mutations and DNA damage [24]. DNA damage induced by mutagens can play a key role in the process of carcinogenesis and inherited genetic diseases [25]. DNA damage can occur through a variety of processes including electron transfer, hydrogen abstraction, triplet-triplet energy transfer, or generation of reactive oxygen species. Formation of so-called DNA adducts may result in teratogenesis, mutagenesis and carcinogenesis [26]. Genotoxicity is the capability of a chemical to alter the genetic material of the cell, and is one of the earliest effects of most carcinogens [2].

Despite the widespread use of HMS, data about their genotoxic effects are lacking. Therefore, in this study, the potential genotoxic effect of HMS has been evaluated using the single cell gel electrophoresis (SCGE-comet) assay in isolated human peripheral lymphocytes. This study provides directly toxicity data beneficial for the evaluation of potential risk of HMS for both ecology and human health.

2. RESULTS

In the experiments, human peripheral blood lymphocytes were exposed to different concentrations (10, 50, 100 and 200 $\mu\text{g mL}^{-1}$) of HMS for 30 and 120 min after treatment, cell viability was determined by using the trypan blue exclusion method and it was found to be above 90% for all samples. The data indicate that all concentrations of HMS did not produce statistically significant differences in cell viability when compared to control ($p > 0.05$). According to the data obtained from three separate experiments, DNA damage was significantly increased at 200 $\mu\text{g mL}^{-1}$, compared with those of untreated cells. The increase was dose dependent.

Exposure to all concentrations of HMS for 120 min caused significant increases in the experiment. The toxicity of HMS at 10, 50 and 100 $\mu\text{g mL}^{-1}$ concentration was not statistically significant ($p > 0.05$). The cell viability percentage of lymphocytes after exposure to increasing concentrations of homosalate for 30 and 120 min is shown in Figure 2.

The results of in this study revealed that HMS increased the DNA migration in both concentration and time dependent manner. DNA damage was higher in cells which had been incubated with 200 $\mu\text{g mL}^{-1}$ and 120 min ($p < 0.05$). The cell viability percentage and total comet scores (TCS) in lymphocytes after exposures of homosalate are shown Table1.

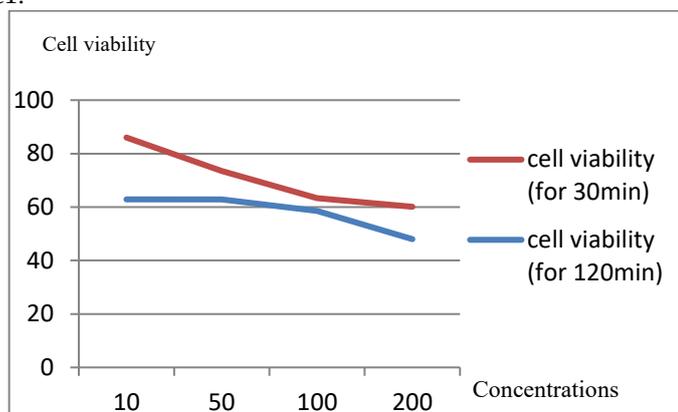


Figure 2: Cytotoxicity in lymphocytes after exposures to increasing concentrations of HMS for 30 and 120 min

Table 1: Cell viability percentage and total comet scores in lymphocytes after exposures of HMS at different concentrations for 30 and 120 min

Samples	Time (min)	Concentration ($\mu\text{g mL}^{-1}$)	Cell viability % (Mean \pm SD)	TCS ^a (Mean \pm SD)
Negative control (DMSO)	30	10	91.80 \pm 1.20	15.30 \pm 2.34
Positive control (H ₂ O ₂)	30	25	90.79 \pm 5.12	156.1 \pm 2.23
HMS	30	10	86.01 \pm 7.3	50.20 \pm 5.2
	30	50	73.51 \pm 1.20	51.8 \pm 3.8
	30	100	63.30 \pm 3.12	52.1 \pm 4.3
	30	200	60.11 \pm 5.28	54.6 \pm 2.5
HMS	120	10	62.88 \pm 1.23	50.12 \pm 4.5
	120	50	62.82 \pm 5.1	55.89 \pm 9.1
	120	100	58.6 \pm 8.3	57.31 \pm 3.4*
	120	200	47.99 \pm 7.3	68.1 \pm 1.2*

^aTCS (Total comet score) =0 \times No Migration+1 \times LowMigration+2 \times MediumMigration+3 \times High Migration+4 \times Extensive Migration, * $p < 0.05$

3. DISCUSSION

Sunscreens are more likely prone to macroscopic tests (prevention of skin erythema and sunburns) rather than microscopic ones like genotoxicity, understanding the cellular level of protection against skin cancer [27]. UV filters are necessary to a through risk/benefit evaluation, which includes acute skin damage like sunburn, chronic skin damage such as DNA damage, photo induced immune suppression, reproductive toxicity, genotoxicity, carcinogenicity and mutagenesis. More, UV filters should be determined a strict toxicological safety evaluation prior to approval [23, 28].

Although the daily use of UV filters is low, the long-term exposure to UV filters can cause to genotoxic effects. There are very few studies about the genotoxic effects of organic UV filters [24, 29]. Despite the widespread use of HMS, a few studies are present about the *in vivo* and *in vitro* effects of HMS, data about their genotoxic effects in human are lacking [23]. To the best of our knowledge, we report here the first study involving human lymphocytes to assess the genotoxic effect of homosalate using comet assay. The study performed by Imamoviä *et al.* [30] about HMS's genotoxicity on plants seems to be the only work reported so far, in which the genotoxic effects of chlorinated homosalate by-products occurred in the presence of free chlorine using Allium test on onion root and they found genotoxic effects of HMS [30].

HMS is strongly lipophilic and the presence of alkyl-substituted cyclohexane ring on HMS can drastically change the lipophilicity of the whole molecule [22] and as well as toxicity properties. HMS may be attributed to high affinity binding to the lipophilic components of the skin layers. HMS absorb into the systemic circulation from skin after topical application of the gel formulation in rats [8]. Schlumpf *et al.* [10]

also found residues of HMS in breast milk samples. Particularly, 15.09 % of the study participants used the sunscreens containing HMS, and complied the use of products containing these UV with their presence in milk. Although the daily use of HMS-containing filters is low, the environmental problem caused from their long-term input should not be neglected [24].

The genotoxicity of benzophenones (BPs), widely used in UV filters, were tested using the SOS/UMU assay. These results of the study revealed that the major contributor to the genotoxicity of the PBs was the ortho, para-di-substitution and the increasing hydroxy substitution on the benzene ring. In addition, the higher the dispersion of the substituent species on the two benzene rings, the lower the genotoxicity exhibited by the compound [24]. HMS contains an ester bridge between phenol and alkyl-substituted cyclohexane rings [16]. The DNA damage observed in this work might possibly be due to free radical formation: a sunscreen (as RC-CR' where Rs are organic functional groups) will absorb solar photons having sufficient energy to be able to break numerous covalent bonds. Carbon-centered free radicals are quite reactive and can assault and change other molecules in the closest perimeter. In addition, since sunscreens will eventually meet molecular oxygen from air, these free radicals could possibly form organic peroxy radicals. Hydrogen atom elimination, addition and electron transfer mechanisms are known to be the main pathways hydroxyl radical goes into reaction with organic compounds. As in homosalate, .OH may react with thymine (addition to a double bond) ending up with thymine radical and -with oxygen- thymine peroxy radical. These reactions can possibly cause DNA damage over strand breakage, cell mutation, and finally, cell death [13]. Therefore; the chemical stabilization of HMS in any topical dosage form seems to be critical.

The latest study about organic UV filters' genotoxic effects was reported by Sharma *et al.* [2]. The genotoxic effects of ethylhexyl methoxycinnamate (EHMC), a member of organic UV filters, have been reported using both bioassays (SOS chromotest and Umu C test). The author was observed significant genotoxicity of EHMC at the highest concentrations. Afterwards, the genotoxic potential of EHMC was evaluated using the comet assay by measuring DNA damage on TK-6 cell line (the human-derived lymphoblastoid) and HL1-hT1 (human adult stem cell). Both cell lines showed higher DNA damage by EHMC. The results of our study are in agreement with Sharma *et al.* [2]. Sevinc [31] reported the genotoxic effects of octocrylene and avobenzene alone and in combination using comet assay. The author reported that significant DNA damage was observed in higher concentrations of avobenzene and octocrylene. The level of DNA damage was higher with combination forms of these chemicals. Our results are in parallel with Sharma *et al.* [2] and Sevinc [31] UV filters may attack DNA either directly or indirectly by free radicals to produce damage in the form of adducts or cell death. According to these results, it has been suggested that UV filters may contribute to long term skin damage [13] and might represent a human hazard.

4. CONCLUSION

In conclusion; our study showed for the first time that, the potential genotoxic effect of HMS has been evaluated using the single cell gel electrophoresis (SCGE-comet) assay in isolated human peripheral lymphocytes. We conclude that based on the results of the comet assay, HMS has high potential to interact with DNA and to cause primary DNA damage in human lymphocytes as an *in vitro* model. However, further studies should be carried out with different test systems related to its genotoxic effects and incubation time should be extended. This may be beneficial for genotoxicity evaluation of compounds with similar characteristics and their impact of human population.

5. MATERIALS AND METHODS

5.1. Reagents and chemicals

HMS (CAS No: PHR1085) was supplied by Sigma. Other chemicals used in the comet assay were purchased from the following suppliers: low melting agarose (LMA), high melting agarose (HMA), Triton-X-100, EDTA, ethidium bromide, histopaque-1077, phosphate-buffered saline (PBS) tablets were purchased from Sigma; sodium chloride (NaCl), sodium hydroxide (NaOH), dimethylsulfoxide (DMSO) from Merck.

5.2. Blood samples and cell preparation

For each experiment, 5 mL heparinized whole blood samples were collected by venipuncture from female author not exposed to radiation or drugs. The alkaline version of the comet assay was performed according to Singh *et al.* [32] with a minor modification. The triplicate experiments were provided by blood samples from the same donor at different time intervals. Peripheral blood lymphocytes were isolated by

Histopaque 1077 density gradient centrifugation, washed with phosphate-buffered saline (PBS) and suspended in 1 mL PBS. Cell viability for isolated human lymphocytes was determined by trypan blue exclusion method and it was found to be above 90% for all samples. Lymphocytes were incubated with 10, 50, 100 and 200 µg/mL HMS and blank control at 37 °C for 30 min and for 120 min separately. Selected concentrations were similar to concentrations reported in previous studies [2, 24, 31]. Parallel with HMS, negative controls were set up at the same temperature and exposure time by incubating lymphocytes with DMSO (final concentration 1%). Positive controls consisted of cells treated with H₂O₂ (final concentration 10µM) were incubated at 37 °C for 5 minutes. After incubation, the lymphocytes were mixed with 100 µL of 0.7% (w/v) low melting agarose, rapidly mounted on pre-coated microscopic slides covered with a coverslip and let 0.7% high melting agarose (w/v) solidify at +4 °C. Once coverslips were gently removed, the slides were immersed in an ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 with 1% Triton X-100 and 10% DMSO) for at least 2 h at +4 °C. At the end of lysis process, the slides were placed in fresh electrophoresis buffer (5 M NaOH, 0.2 M EDTA) and left for 20 min to allow DNA to unwind as well as labile alkali damage to be expressed. Electrophoresis was performed for 30 min at 300 mA and 15V +4 °C (ThermoEC MidiCell Primo, ABD; BioRad Power Pac, England). After electrophoresis, the slides were removed from the tank, gently washed 3 times for 5 min in fresh neutralization buffer (0.4 M tris, pH 7.5) in order to neutralize excess alkali. Slides were stained with 50 µL of ethidium bromide (20 µg/mL) per each slide. 15 min after staining, the slides were evaluated at 200X objective on a fluorescence microscope (Olympus BX50, Optical Co. GmbH, Hamburg, Germany). The experiment was done in triplicate. For each sample, 100 cells (50 for each replicate slide) were analyzed. The damage is represented by an increase of DNA fragments that have migrated out of the cell nucleus during electrophoresis. Cells were graded by eye into five categories corresponding to the extent of DNA migration as described by Collins *et al.* [33]. Undamaged cells were defined as cells with an intact nucleus without a tail and damaged cells had the appearance of a comet as shown in Figure 3.

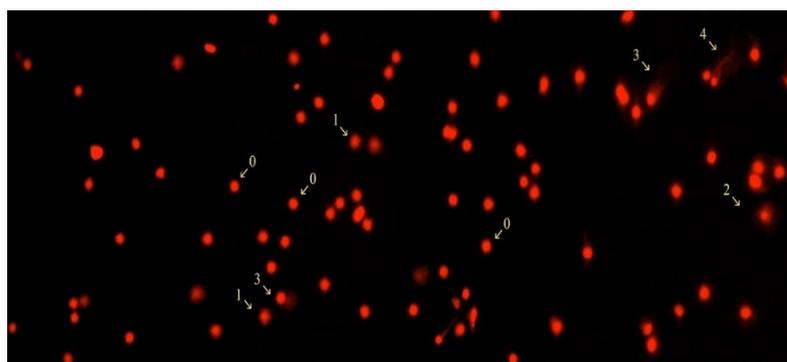


Figure 3. Lymphocytes were incubated with 200 µg/mL HMS for 120 min. (0: No migration, 1:Low migration, 2:Medium Migration, 3:High Migration, 4: Extensive Migration)

DNA damage can be scored by using parameter total comet score (TCS). TCS was evaluated according to the following equation formula,

$$\text{TCS} = 0 \times \text{nomigration} + 1 \times \text{low migration} + 2 \times \text{medium migration} + 3 \times \text{high migration} + 4 \times \text{extensive migration}$$

5.3. Statistical analysis

The SPSS 20 was used for statistical analysis. Statistical comparison of the results from negative and positive controls and test groups treated with HMS were carried out by one-way analysis of variance (ANOVA). A non-parametric Kruskal Wallis test was used to determine whether DNA damages were statistically different from each other.

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Conflict of interest statement: The authors declare that there are no conflicts of interest.

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