Journal of Research in Pharmacy

Research Article

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Eugenol aggravates UVA-induced cytotoxic and genotoxic response in HaCaT human keratinocytes

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Received: 8 September 2021 / Revised: 22 November 2021 / Accepted: 30 December 2021

ABSTRACT: Eugenol is a phenolic compound isolated from clove essential oil. It is used in dentistry, fragrance, cosmetic, and food industries. According to the fragrance ingredient safety assessment report, eugenol does not cause phototoxic reactions and genotoxicity. However, its effect on UV-induced cytotoxicity and genotoxicity has not been well examined. Here in this study, cytotoxic and genotoxic effects of eugenol are investigated on UVA-induced damage using human keratinocyte cells (HaCaT). HaCaT cells were treated with increasing concentrations of eugenol (10-500 µM) for 1 hour and irradiated with 5-10-15 J/cm² UVA. 24 hours later the neutral red uptake (NRU) assay was used to evaluate cytotoxicity. For genotoxicity assay cells were exposed to 1-10 µM eugenol for one hour and non-cytotoxic UVA irradiation doses (1, 2.5 J/cm²) were used. The alkaline comet assay was carried out immediately after the UVA irradiation to measure the genotoxic potential of eugenol. The cytotoxicity assay results indicate that eugenol caused a cytotoxic effect in a dose-dependent manner in HaCaT cells and increasing doses of UVA-irradiation enhanced the cytotoxic effect of eugenol. The alkaline comet assay results showed that eugenol causes DNA single-strand breaks and increasing doses of UVA-irradiation aggravates the genotoxic potential of eugenol. These data demonstrate that eugenol has cytotoxic and genotoxic potential and eugenol aggravates UVA-induced cytotoxic and genotoxic response in HaCaT human keratinocytes.

KEYWORDS: Eugenol; UVA; DNA damage; human keratinocyte; cytotoxicity; genotoxicity.

1. INTRODUCTION

The human skin is exposed to solar ultraviolet radiation (UV) every day. UV is divided into three wavelength ranges: UVC (200-280 nm), UVB (280-320 nm), and UVA (320-400 nm). Only 10% of UVB and almost 90% of the UVA reach the Earth's surface [1].

UVB radiation has a higher energy level than UVA radiation and directly can damage the DNA of epidermal cells. It is thought to be responsible for skin cancers whereas UVA radiation caused DNA damage is minimal but it can penetrate further into the dermal layers and indirectly affect the DNA by generating reactive oxygen species (ROS), [2]. Excessive accumulation of ROS causes oxidative damage to the cellular components such as cellular macromolecules, DNA and mitochondria [3]. The evidence about the harmful effects of UVA promotes skin aging, carcinogenesis, and immunosuppression is increasing [4]. UVA-induced ROS generation can promote single-strand and double-strand DNA breaks [5,6]. UV-induced both direct and indirect DNA damages can result in chromosomal aberrations and disturb DNA replication, transcription and translation. These changes can subsequently result in cell cycle arrest and apoptosis [7]. In addition to the harmful effects directly caused by UV, chemical compounds in drugs or compounds that occur during the metabolism of drugs or chemicals in cosmetic products applied on the skin may interact with UV radiation. Chemical modifications resulting from these interactions may worsen the cytotoxic and genotoxic effects caused by UV irradiation [8].

Eugenol is one of the major constituents of clove essential (*Syzygium aromaticum*) oil (70-90%) and it is a volatile phenolic compound [9]. It is bioactive and known to possess several pharmacological properties like antimicrobial, antioxidant, anticancer, anti-inflammatory, etc. [10]. It is used as a topical anesthetic and analgesic for oral application in dentistry. Also, it is traditionally used as a topical anesthetic by direct application on the gums [11]. Also, eugenol is used in perfumeries, flavoring and cosmetic industries for strong, spicy and clove-like fragrances [12]. Essential oils are often applied directly to the skin as fragrance

How to cite this article: Jannuzzi AT. Eugenol aggravates UVA-induced cytotoxic and genotoxic response in HaCaT human keratinocytes. J Res Pharm. 2022; 26(2): 354-361.

http://dx.doi.org/10.29228/jrp.133

ISSN: 2630-6344

because of that it is important to know about adverse skin reactions [13]. Additionally, eugenol is determined to be cytotoxic to human dermal and endothelial cells and gingival fibroblasts [14,15].

According to the risk assessment result of the Research Institute of Fragrance Materials, eugenol does not raise a concern about genotoxicity [16]. On the other hand, there are several studies showed that eugenol has a genotoxic potential with different genotoxicity assays in different models [17,18] and eugenol is determined to increase sensitivity to H_2O_2, a common reactive oxygen intermediate which is widely used as a genotoxic agent [19].

The effect of eugenol on UVA-induced damage to the skin has not been well examined and remains unclear. The alkaline comet assay is a powerful, fast and easy method to assess primary DNA damage as single-strand DNA breaks. It allows the analysis of the potential photoprotective or phototoxic effects of chemicals in terms of genotoxicity when irradiated with UV radiation [8]. Therefore, in this study, the cytotoxic and genotoxic effects of eugenol in UVA irradiated human keratinocyte cells have been investigated.

2. RESULTS

2.1. HaCaT cells show UVA sensitivity

The cells were exposed to 1, 2.5, 5, 10 and 15 J/cm² UVA. According to the neutral red uptake (NRU) cytotoxicity assay results, increasing doses of UVA showed an increased cytotoxic effect on HaCaT cells. The cell viability was reduced significantly at 10 J/cm² ($p<0.05$) and 15 J/cm² ($p<0.0001$), Figure 1.

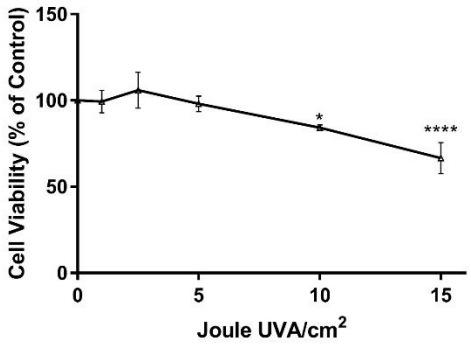

Figure 1. NRU cytotoxicity assay results after different doses of UVA irradiation in HaCaT cells. The data were presented as the mean ±SD. *$p<0.05$, ****$p<0.0001$ vs control.

2.2. Eugenol increases UVA-induced cytotoxicity in HaCaT cells

According to the NRU cytotoxicity assay results, eugenol showed dose-dependent cytotoxic effects in HaCaT cells compared to the control group ($p<0.001$, $p<0.0001$). The cell viability did not significantly change for the cells that were kept in the dark ($p>0.05$), (Figure 2a). 5 J/cm² UVA irradiation did not cause a significant cytotoxic effect ($p>0.05$), (Figure 2b) but increasing doses of UVA caused significantly increased cytotoxicity compared to control groups ($p<0.01$, $p<0.0001$), (Figure 2c, 2d). Eugenol treatment intensified the cytotoxic effect of UVA irradiation significantly compared to UVA irradiated group ($p<0.001$, $p<0.0001$), (Figure 2b-d). Increasing doses of UVA caused increased cytotoxicity for the same eugenol concentrations up to 50 µM (Figure 2). 100-250-500 µM eugenol concentrations cytotoxic effects did not change with 5-10-15 J/cm² UVA irradiation compared to the control group which was kept in dark ($p>0.05$).

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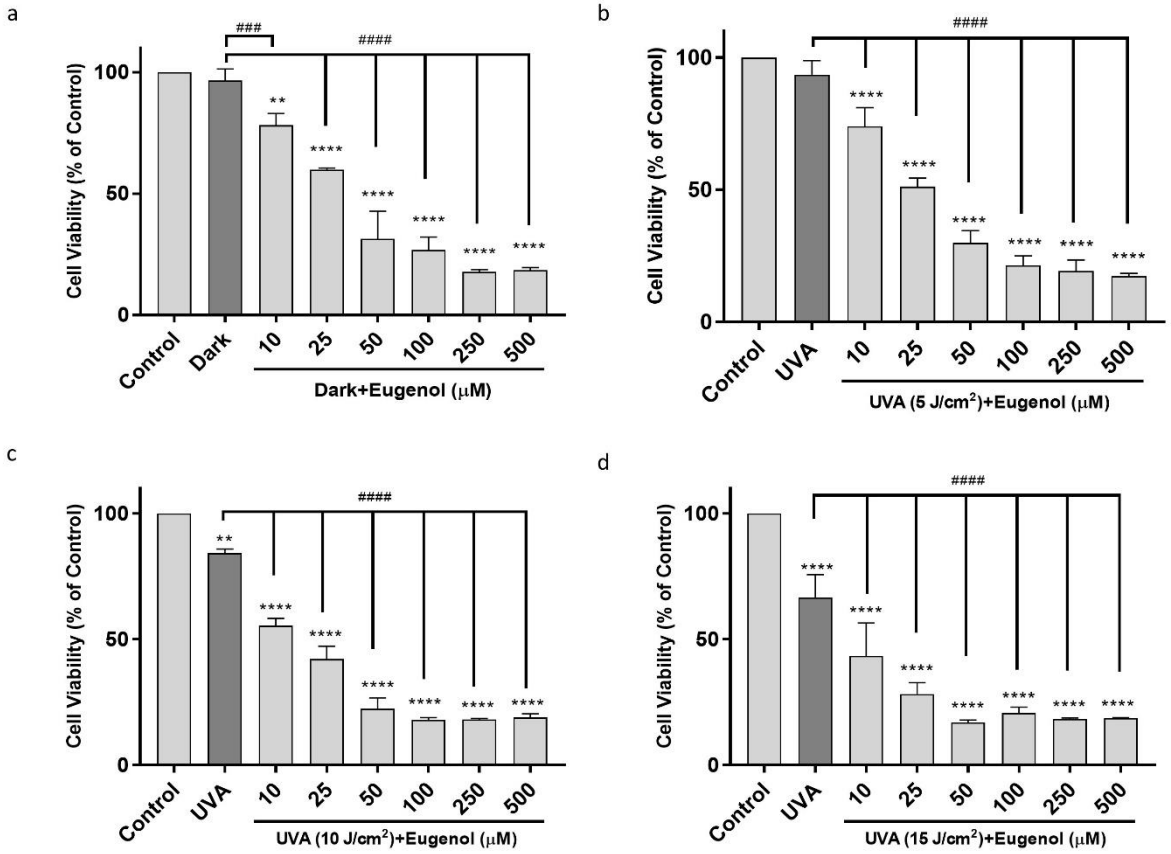

Figure 2. NRU cytotoxicity assay results of eugenol concentrations and different doses of UVA irradiation in HaCaT cells. The data were presented as the mean ±SD. ** p<0.01, ****p<0.0001 vs control, ###p<0.001, ####p<0.0001 vs UVA.

2.3. UVA-induced DNA damage was increased with eugenol treatments

The alkaline comet assay was used to determine DNA damage. UVA irradiation caused increased DNA damage and DNA tail intensity %raised with 1 J/cm² (p>0.05) and with 2.5 J/cm² irradiation (p<0.05), (Figure 3).

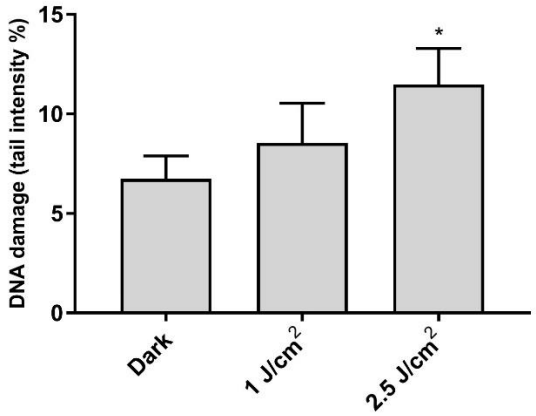

Figure 3. DNA damage % after 1, 2.5 J/cm2 UVA irradiation in HaCaT cells. The data were presented as the mean ±SD. *p<0.05 vs dark.

http://dx.doi.org/10.29228/jrp.133
J Res Pharm 2022; 26(2): 354-361

According to the study results, up to 10 µM eugenol concentrations did not induce DNA damage in HaCaT cells. However, eugenol with 1 J/cm² UVA irradiation resulted in increased DNA damage. The increasing concentrations of eugenol caused more genotoxic effect and these increases were significant at 5 and 10 µM eugenol concentration compared with 1 J/cm² control group ($p<0.0001$). 2.5 J/cm² UVA irradiation and eugenol treatment caused dose-dependent DNA damage and the DNA damage was significant at the concentrations of 2.5 µM ($p<0.05$), 5, and 10 µM ($p<0.0001$) eugenol compared with 2.5 J/cm² control group. From 2.5 µM to 10 µM eugenol concentration increased UVA irradiation caused significantly higher DNA damage at the same concentrations of eugenol treatments when compared to groups that did not receive UVA irradiation (dark) (Figure 4), ($p<0.05$, $p<0.001$, $p<0.0001$).

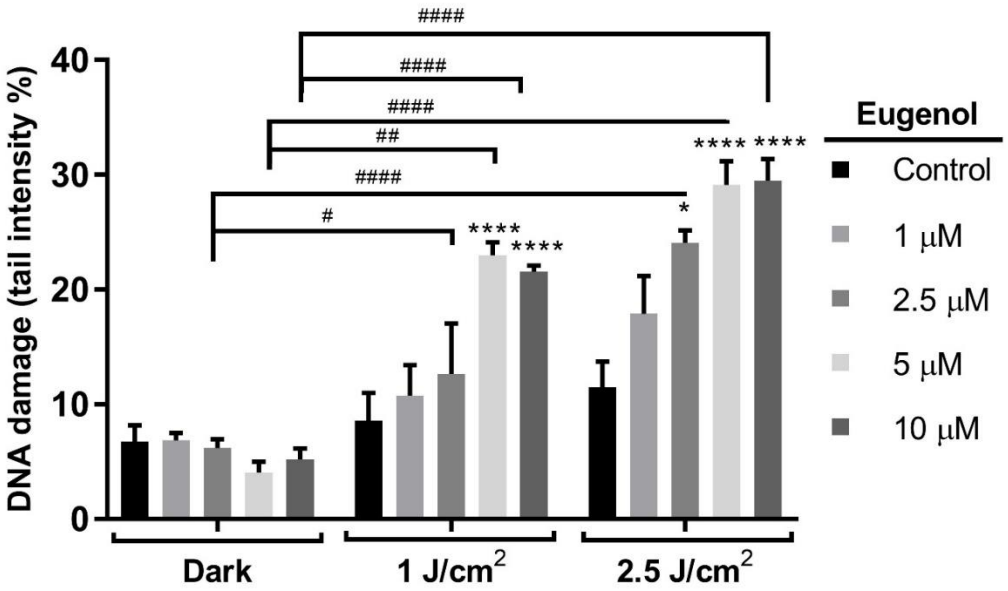

Figure 4. DNA damage % in 1, 2.5, 5, 10 µM eugenol treated HaCaT cells after 1, 2.5 J/cm2 UVA irradiation. The data were presented as the mean ±SD. *$p<0.05$, ****$p<0.0001$ vs control. #$p<0.05$, ####$p<0.0001$ vs dark.

3. DISCUSSION

Solar UV radiation is known to cause adverse effects to the skin. UVA and UVB wavelengths can penetrate the skin and both can induce reactive oxygen species generation [20]. UVA and UVB can cause a deleterious effect in the human skin by triggering damage in cellular membranes, proteins, lipids, and DNA [21]. UVA can reach deeper than UVB and the harmful effects of UVA promote skin aging, carcinogenesis and immunosuppression [4]. UVA-induced toxic mechanisms mainly depend on indirect mechanisms which are activated by the generation of ROS and increased oxidative stress has a central role in UVA-induced DNA damage [22]. Also, UVA exposure is reported to cause micronucleus formation and chromosome instability [23,24] DNA damage [25] and photoaging [26].

Eugenol is widely used in fragrances, cosmetics, dental applications and as a flavoring agent in various foods [27]. Using different methodologies many authors evaluated the cytotoxic, genotoxic, phototoxic, developmental, local, respiratory and skin sensitization potential of eugenol to assess the safety of eugenol [16]. A study has suggested that eugenol is highly cytotoxic even at low concentrations to human fibroblast and endothelial cells [15]. Kalmes and Blömeke investigated the eugenol impact in human HaCaT keratinocytes up to 600 µM concentrations. They found eugenol showed inhibitory activity with WST-1 assay and BrdU cell proliferation assay but cell propidium iodide staining did not show cell death [28]. Similarly, the current study indicates that eugenol shows cytotoxic activity in human keratinocytes. According to the eugenol safety assessment of the Research Institute of Fragrance Materials topical application of eugenol did not cause phototoxic reactions in vivo. Also, eugenol is found to be a weak skin sensitizer [16]. Former reports indicated the genotoxic potential of eugenol. However, no study has so far examined the genotoxic effects of eugenol after UV irradiation. Da Silvia Gündel et al. reported that eugenol showed moderate toxicity at the highest concentration studied and did not cause a genotoxic effect on mononuclear cells of peripheral blood

following 4-hour exposure [29]. In line with these findings, in our study alone eugenol treatments showed a dose-dependent cytotoxic effect on human keratinocytes and non-cytotoxic concentrations of eugenol did not cause DNA damage.

Eugenol was determined to have genotoxic activity on V79 cells at 2500 µM with chromosomal aberration assay [17]. A previous study showed that eugenol induces single and DNA double-strand breaks in Chinese hamster ovary AA8 cells at 250 and 750 µM concentrations [30]. Rompelberg et al. evaluated the antigenotoxic potential of eugenol and found that in vivo treatment of rats with eugenol resulted in a reduction of the genotoxicity of benzo(a)pyrene, but *in vitro* mutagenicity test showed increased genotoxicity with eugenol treatment [18]. Slamenova et al. studied the effects of eugenol on cytotoxicity, DNA and oxidative system in HepG2, Caco-2 and VH10 cell lines [19]. The results of this study showed that eugenol shows cytotoxicity in all three cell lines. Under 600 µM concentrations eugenol significantly increased DNA damage in VH10 fibroblast and to a lesser extent in Caco-2 cells. Also, eugenol increased the H_2O_2-induced DNA damage. Comet assay results of the current study illustrate that noncytotoxic UVA irradiation doses can cause DNA single-strand breaks in HaCaT human keratinocyte cells. Also, nongenotoxic concentrations of eugenol increase UVA-induced DNA damage significantly by causing DNA single-strand breaks. Eugenol can act as an antioxidant compound with low concentrations; however, high concentrations seem to be responsible for prooxidant effects [31]. This characteristic of eugenol may be responsible for worsening the UVA-induced cytotoxicity and genotoxicity in the present study.

4. CONCLUSION

For the first time the current study suggests that UVA-induced cytotoxic and genotoxic response is enhanced with eugenol treatments. The study results may provide preliminary *in vitro* evaluation and contribute to a further understanding of the potential risk of dermal eugenol use under UV radiation.

5. MATERIALS AND METHODS

5.1. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and 10000 U/mL penicillin and 10000 µg/mL streptomycin, trypsin solution, Phosphate saline buffer (PBS) were obtained from Gibco (Carlsbad, CA, USA). Eugenol ≥ 98% was purchased from Sigma Aldrich (Germany). DMSO, neutral red bought from Santa Cruz (CA, USA). Low melting point agarose and normal melting point agarose were from ThermoFisher (Camarillo, CA, USA). All other chemicals were from Sigma Aldrich (Germany) unless otherwise stated.

5.2. Cell culture

Ethical approval is not required for this study since the study does not involve human or animal subjects. The Human Keratinocyte (HaCaT) cell line is kindly provided by Prof. Dr. Betul Yilmaz (Marmara University, Istanbul, Turkey). Cells were cultured in DMEM supplemented with 10% FBS 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified incubator at 37 °C and 5% CO_2.

5.3. Determination of UVA sensitivity of HaCaT cells

Briefly, 1 x10^5 cells were seeded in 96-well plates. After overnight incubation, the media was replaced with a thin layer of phenol red-free DMEM. Then, the cells were exposed to a UVA radiation dose with a UVA lamp (1, 2.5, 5, 10, 15 J/cm^2) from the top of the plate at room temperature. The dose of the radiation was measured with a digital radiometer (Lutron UVA-365SD, Taiwan). Meanwhile, the control plate was kept in dark at room temperature. After the irradiation, the medium was removed and replaced with fresh DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were incubated for 24 hours (h) and the UVA sensitivity of the cells was evaluated with NRU cell viability assay.

5.4. Eugenol treatment with UVA irradiation

Briefly, 1x10^5 cells/well were seeded in 96 well plates. After 24 hours cells were treated with concentrations of eugenol (10-500 µM) along with control (0.5% DMSO) for one hour. Then media was discarded and cells were covered with a thin layer of phenol red-free DMEM. The cells were UVA irradiated with UVA doses 5-10-15 J/cm^2. UVA dose was determined with a digital radiometer (Lutron UVA-365SD, Taiwan). In parallel, another experiment setup was kept in the dark at room temperature. After the irradiation

medium was removed from all of the plates and replaced with a fresh medium and incubated for 24 h. Then, the NRU assay was carried out to determine the cytotoxic effect of eugenol with or without UVA irradiation.

5.5. NRU cell viability assay

After the treatment cells were incubated in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin for 24 h. Then, the medium was discarded and the cells were incubated with fresh DMEM containing 50 µg/mL neutral red dye for 3 h at 37 °C and 5% CO_2. The culture supernatant is removed and the wells were washed with 1x PBS. A mixture of acetic acid, water and ethanol (1:49:50) was used to dissolve neutral red in the cells. After a brief shake, the absorbance was measured at 540 nm with a microplate reader (EPOCH BioTek, USA). The effect of UVA and/or eugenol on cell viability was calculated as the percentage of 0.5% DMSO treated control cells. The assays were performed in triplicate.

5.6. Determination of genotoxicity with the comet assay

Non-cytotoxic UVA doses and eugenol concentrations for HaCaT cells were chosen for genotoxicity experiments. The cells were seeded in 6-well plates and treated with 1-2.5-5-10 µM concentrations of eugenol and 0.5% DMSO as the control for 1 h. UVA irradiation with the 1 and 2.5 J/cm² UVA doses was carried out as aforementioned. Meanwhile, the control plate was kept in dark at room temperature. The comet assay was carried out immediately after the irradiation procedure with a slight modification of the method described by Collins [32]. Briefly, the cells were collected with trypsinization and mixed 1:1 with prewarmed (37 °C) 0.65% low melting point agarose in PBS. The cell mixture was added on the 1% normal melting point agarose precoated microscope slides. The cell mixture was covered with a coverglass and let to solidify for 20 minutes (min). Then, the coverglasses were removed and the cells were lysed in lysis buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 10% DMSO and 1% Triton X-100 (pH=10) at 4 °C for 1 h. Then the slides were placed in the electrophoresis tank horizontally and let for 20 min in electrophoresis buffer containing 0.3 M NaOH and 1 mM EDTA (pH=13) to allow the unwinding of the DNA. The electrophoresis was performed at 300 mA for 20 minutes. The slides were immersed in neutralizing buffer containing 0.4 M Tris-HCl (pH 7) for 10 minutes. After that, the slides were fixed with ice-cold absolute ethanol and air-dried. Ethidium bromide was used to stain the DNA just before slide examination. Randomly selected 100 cells were scored per experiment group under a fluorescent microscope (Olympus BX53, Japan) using a Comet assay IV image analysis system (Perceptive Instruments, UK). The DNA damage level was evaluated from the comet tail intensity%.

5.7. Statistical Analysis

All experiments were repeated at least triplicate and the data were presented as the mean ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism Software 7.0 (GraphPad, USA). Significant differences between groups were analyzed via one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. $P<0.05$ was considered statistically significant.

Acknowledgements: The author would like to thank Prof. Dr. Buket Alpertunga for her insightful comments in this work.

Author contributions: A.T.J.; Conceptualization, Methodology, Investigation, Data collection and analysis, Writing, Supervision

Conflict of interest statement: The authors declared no conflict of interest.

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