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Letter to the Editor

Ozone-induced damage in 3D-Skin Model is prevented by topical vitamin C and vitamin E compound mixtures application

Because its critical location, the skin is the main target of environmental stressors such as ozone (O_3). Although O_3 does not penetrate the deeper layers of skin it is able to react readily with stratum corneum lipids [1]. The toxic effects of O_3 on the uppermost layers, induced either directly by the oxidation of biomolecules or by driving the radical-dependent production of cytotoxic, non-radical species (aldehydes), have repercussions on deeper cellular layers, triggering a cascade of cellular stress and inflammatory responses that can lead to skin pathologies [2,3]. Furthermore, O_3 is able to induce the depletion of cutaneous antioxidants [4]. Therefore, topical application of antioxidants could prevent pollution induces cutaneous damages.

In our recent work, the use of topical antioxidant mixtures (MIXs) has proven an effective defensive approach against O_3-induced oxidative damage in human keratinocytes [5]. In the present study, which is a methodological extension of the one recently published [5], we evaluated the ability of the three MIXs to prevent the noxious effects of O_3 on a customized reconstructed human epidermis (RHE, Episkin), where sebum was applied to reproduce a model resembling “in vivo” expectations [6].

Abbreviations: O_3, ozone; MIXs, antioxidant mixtures; RHE, reconstructed human epidermis; OS, oxidative stress; ROS, reactive oxygen species; HNE, 4-hydroxy2-nonenal; HNE-PA, 4-hydroxy2-nonenal protein adducts; ELISA, enzyme-linked immunosorbent assay; NF-κB, nuclear factor of kappa light polypeptide gene enhancer in B-cells; COX-2, cyclooxygenase-2; Nrf2, nuclear factor, erythroid 2-like 2.

At first, the protective effect of 3 MIXs [MIX1: 15%vitaminC + 1% vitaminE + 0.5%ferulic acid; MIX2: 10%vitaminC + 2%phloretin + 0.5%ferulic acid; MIX3: 1%resveratrol + 1%vitamin E + 0.5%baicalin] against the O_3 cytotoxicity was examined in RHE in presence and absence of sebum. Morphological changes, such as vacuolization of upper cell layers, were evident in RHE w/sebum after exposure to O_3 0.4 or 0.8 ppm for 4 h, whereas the pre-treatment with the MIXs, especially MIX1 and MIX2, appeared to prevent these alterations (see supplementary materials). As evident in Fig. 1a, RHE w/sebum, exposed for 4 h to O_3 (0.4 and 0.8 ppm) showed a dose-dependent LDH release respect to control. No cytotoxic effect was observed in RHE w/o sebum exposed to O_3. Pre-treatment with the three MIXs prevented O_3-induced damage (especially for MIX1 and MIX2).

It is known that O_3 is able to induce oxidative stress (OS) coupled with increased production of ROS. Indeed, RHE exposed to O_3 (0.8 ppm) presented a 3 fold in ROS levels compared to control (Fig. 1b). Interestingly, in the presence of sebum this effect was less pronounced. Pre-treatment with the MIXs demonstrated the ability to diminish ROS levels. Whereas the sebum seemed to have no significant role in O_3-induced generation of ROS in RHE pre-treated with the MIXs (Fig. 1b).

OS can lead to lipid peroxidation and formation of reactive aldehydes, like 4-hydroxy2-nonenal (HNE) [7]. The ability of HNE to form adducts (HNE-PA) with target proteins can altered their functions. Therefore, we evaluated the protective role of the MIXs against O_3-induced HNE-PA formation. As shown in Fig. 1c, the levels of HNE-PA significantly increased in RHE exposed to O_3 for 4 h, and this effect was augmented by the presence of sebum. Indeed, RHE w/o sebum showed an increased level of HNE-PA only at higher O_3 dose (0.8 ppm). Whereas, in RHE w/sebum the levels of HNE-PA were already significantly increased after exposure to 0.4 ppm of O_3 and further escalated with exposure to 0.8 ppm O_3,

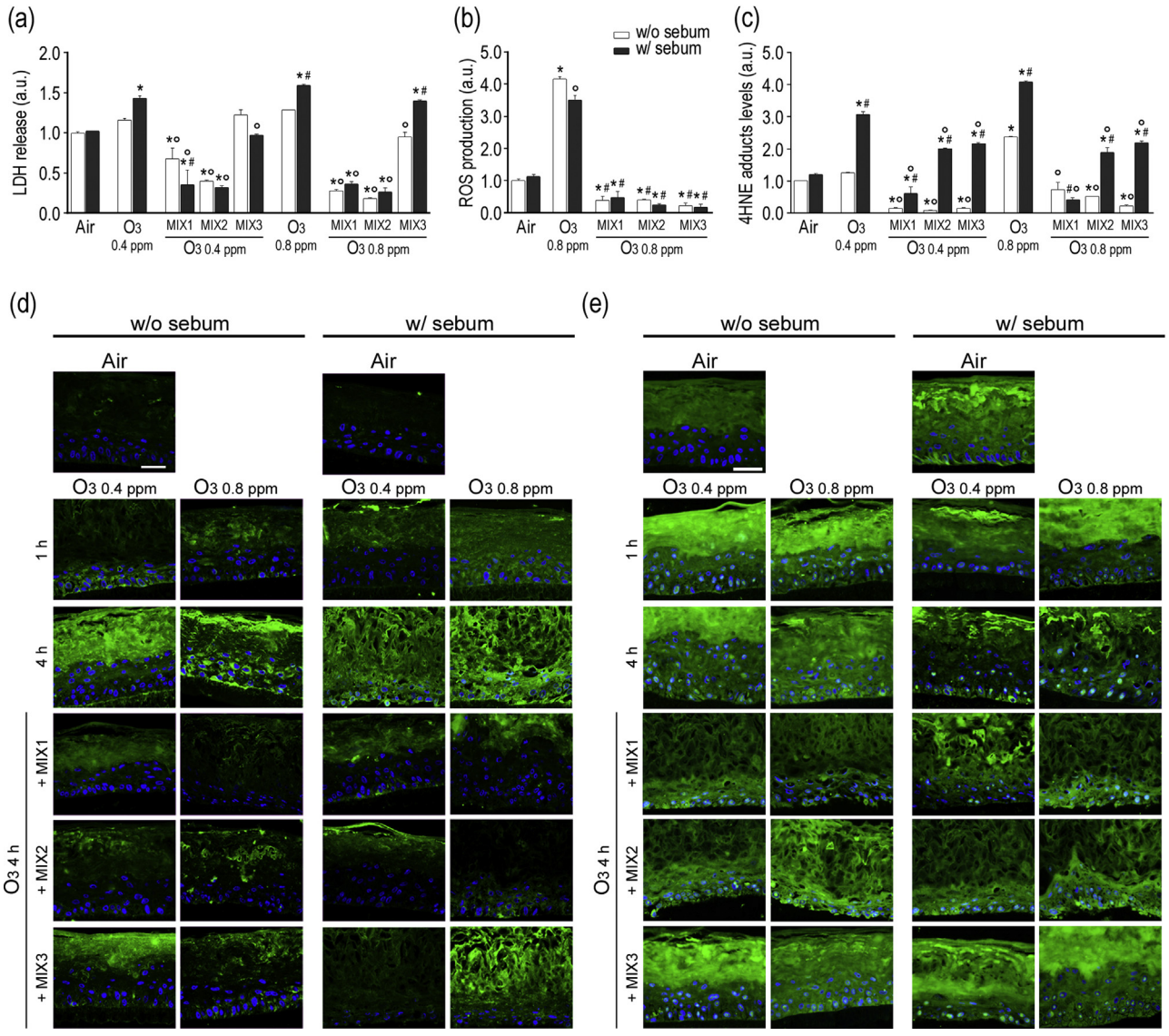

Fig. 1. (a) Cytotoxicity measured by LDH release. Data are averages of 5 experiments ± SEM, $^{*}p < 0.05$ vs air; #p < 0.05 vs w/o sebum; $^{\circ}p < 0.05$ vs O_3. (b) ROS production measured by DCFH-DA staining. Tissues were pretreated with the MIXs for 24 h. Data are expressed in RFU (averages of 5 experiments ± SEM, $^{*}p < 0.05$ vs air; $^{\#}p < 0.05$ vs O_3; $^{\circ}p < 0.05$ vs sebum). (c) HNE levels determined by ELISA. Data are averages of 5 experiments ± SEM; $^{*}p < 0.05$ vs air; $\#p < 0.05$ vs w/o sebum; $^{\circ}p < 0.05$ vs O_3. (d) Immunofluorescence for HNE. (e) Immunofluorescence for Nrf2. Nuclei (blue) stained with DAPI. 5 microscopic fields per slides per experiment were analyzed. Scale bar = 100 μm. Original Magnification × 630.

indicating a dose-dependent effect (2.9 fold and circa 4 fold, respectively). In RHE pre-treated with the MIXs, HNE-PA formation was significantly prevented. These findings were also confirmed by immunofluorescence assay (Fig. 1d). In particular, RHE w/o sebum showed an increased HNE-PA level (green fluorescence) only at later time points (4 h) and at higher O_3 dose (0.8 ppm of O_3). Whereas, in RHE w/sebum, the formation of HNE-PA started at 1 h after the exposure to 0.4 ppm of O_3 with further increase seen at 4 h. This effect was even more evident when RHE w/sebum was exposed to O_3 0.8 ppm, indicating a dose- and time-dependent effect (quantification in supplementary data) and pre-treatment with the MIXs prevented HNE-PA formation as confirmed by ELISA.

To better elucidate the mechanism involved in the protective effects of the MIXs, we evaluated their ability to modulate the transcription factor Nrf2 [8]. The activation of this transcription factor involves its translocation into the nucleus and the transcription of genes involved in cell defense [9]. As showed in Fig. 1e, in absence of stimulus, we observed a baseline activation of Nrf2 (green signal). After O_3 exposure, there was a clear and significant transient activation of Nrf2 (Fig. 1e and quantification in supplementary data). When the RHE was pre-treated with MIXs, Nrf2 induction was even more pronounced (Fig. 1e and quantification in supplementary data). Sebum seems to not affect Nrf2 activation.

To further verify the MIXs ability to protect RHE from O_3-induced proinflammatory effect, we evaluated the activation of the redox sensitive transcription factor NF-κB, [10]. As showed in Fig. 2a, in absence of stimulus, the signal for the p65 subunit of NF-κB (green) was mainly present in cytoplasm. When the tissues were exposed to O_3, there was an evident dose- and time-dependent nuclear increase in p65 (Fig. 2a and quantification in supplementary data). The tissues pre-treated with the MIXs clearly showed a decrease in NF-κB nuclear translocation (Fig. 2a and quantification in supplementary data), demonstrating their ability to inhibit the pro-inflammation responses induced by O_3. The sebum seemed to have no effect in this O_3-stimulated