

# High molecular weight hyaluronan decreases oxidative DNA damage induced by EDTA in human corneal epithelial cells

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## Abstract

**Purpose** To investigate the toxic effects of ethylenediaminetetraacetic acid disodium salt (EDTA), a corneal penetration enhancer in topical ophthalmic formulations, on DNA in human corneal epithelial cells (HCEs), and to investigate whether the effect induced by EDTA can be inhibited by high molecular weight hyaluronan (HA).

**Methods** Cells were exposed to EDTA in concentrations ranging from 0.00001 to 0.01% for 60 min, or 30 min high molecular weight HA pretreatment followed by EDTA treatment. The cell viability was measured by the MTT test. Cell apoptosis was determined with annexin V staining by flow cytometry. The DNA single- and double-strand breaks of HCEs were examined by alkaline comet assay and by immunofluorescence microscope detection of the phosphorylated form of histone variant H2AX ( $\gamma$ H2AX) foci, respectively. Reactive oxygen species (ROS) production was assessed by the fluorescent probe, 2', 7'-dichlorodihydrofluorescein diacetate.

**Results** EDTA exhibited no adverse effect on cell viability and did not induce cell apoptosis in human corneal epithelial cells at concentrations lower than 0.01%. However, a significant increase of DNA single- and double-strand breaks was observed in a dose-dependent manner with all the concentrations of EDTA tested in HCEs. In addition, EDTA treatment led to elevated ROS generation. Moreover, 30 min preincubation with high molecular weight HA significantly decreased EDTA-induced ROS generation and DNA damage.

**Conclusions** EDTA could induce DNA damage in HCEs, probably through oxidative

stress. Furthermore, high molecular weight HA was an effective protective agent that had antioxidant properties and decreased DNA damage induced by EDTA.

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**Keywords:** hyaluronan; EDTA; DNA damage; ROS; corneal epithelial cell

## Introduction

Adjuvants, including surfactants, preservatives, chelating agents, are used in most ophthalmic preparations to enhance transcorneal drug penetration and improve the topical bioavailability of ophthalmic drugs.<sup>1–3</sup> Several studies have shown that these adjuvants exert their effects by inducing a variety of ultrastructural changes in the corneal and conjunctival epithelium.<sup>4–8</sup> A large body of evidence indicates that the long-term use of topical drugs may have adverse effects on the corneal epithelium, causing inflammation, tear film instability, epithelial apoptosis, corneal surface impairment, and the potential risk of failure for further glaucoma surgery.<sup>9</sup>

Ethylenediaminetetraacetic acid disodium salt (EDTA), a calcium chelator that is commonly used as an adjuvant in ophthalmic solutions, acts mainly on the tight junctions by interfering with calcium ions and altering intercellular integrity.<sup>10–13</sup> It has also been reported to disrupt the plasma membrane, thus facilitating intercellular permeability.<sup>10</sup> Although EDTA showed less cytotoxic effect than some other adjuvants, such as

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benzalkonium chloride,<sup>4–8</sup> it has been confirmed that EDTA induced loss of the cellular membrane in isolated rabbit corneas.<sup>14,15</sup> Moreover, EDTA has been shown to influence corneal hydration, evidently as a consequence of alteration of the corneal epithelium.<sup>16</sup>

In addition to causing functional and morphological alterations, EDTA may possibly cause genotoxic effects on corneal epithelial cells. Genotoxicity refers to the potency of biological, chemical, or physical agents to trigger DNA damage, such as DNA single-strand breaks (SSBs), DNA double-strand breaks (DSBs), alkali labile sites (ALSs), and DNA cross-linking. Such damage can lead to malignant transformation and, possibly, to transmission of gene defects to progeny cells. The aim of the present study was to evaluate the genotoxicity of EDTA in corneal epithelial cells and to discover whether there is a molecule that could protect cells from EDTA-induced DNA damage.

Hyaluronan (HA) is a ubiquitous polysaccharide of the extracellular matrix that is abundant in the vitreous body of the eye, the skin, the synovial fluid of articular joints, and the intercellular space of the epidermis. Native HA exists as a high molecular weight nonsulfated glycosaminoglycan polymer in excess of 1000 kDa, composed of repeating disaccharide units of ( $\beta$ ,1–4)-D-glucuronic acid-( $\beta$ ,1-3)-N-acetyl-D-glucosamine.<sup>17</sup> Among extracellular matrix molecules, HA has unique hygroscopic, rheological, and viscoelastic properties.<sup>18</sup> Several studies have shown that high and lower molecular weight HA exhibit different biological effects on cells and tissues.<sup>19,20</sup> High molecular weight HA has been suggested to have an important role in a number of biological processes, including water homeostasis, plasma protein distribution, and matrix structuring,<sup>17</sup> whereas lower molecular mass HA species accumulate at sites of inflammation and tissue injury.<sup>21,22</sup> HA is used as a tear substitute for dry eyes to increase tear film stability and reduce subjective symptoms, such as ocular irritation and burning.<sup>23–25</sup> Thus, HA is a remarkable biopolymer that appears to have an impressive array of biological functions.

In the current study, we investigated the immediate effects of EDTA on DNA in a human corneal epithelial cell line by using two classic and sensitive methods for detecting DNA damage: alkaline comet assay and immunocytochemical assay of a phosphorylated form of H2AX ( $\gamma$ H2AX). The effects of EDTA on cell viability, cell apoptosis, and reactive oxygen species (ROS) formation were also investigated. Further, we examined whether high molecular weight HA (1000 kDa) could influence the effects of EDTA on HCEs. As reported herein, we found that EDTA could induce DNA damage in HCEs and that HA 1000 kDa protected HCEs from the genotoxic effects of EDTA.

## Materials and methods

### Cell culture

Simian virus 40-immortalized human corneal epithelial cells (HCEs) were provided by Dr Zan Pan (New York University, New York, NY, USA) and were cultured in DMEM/F12 (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco), 5  $\mu$ g/ml insulin (Gibco), 0.1  $\mu$ g/ml cholera toxin, 5 ng/ml human epidermal growth factor (Gibco), and 40  $\mu$ g/ml gentamicin and cultured in 25 cm<sup>2</sup> cell culture flasks at 37 °C in an atmosphere of 95% air and 5% CO<sub>2</sub>.<sup>26</sup> Normal culture development was assessed daily by phase-contrast microscopy. Confluent cultures were removed by 0.25% trypsin (Sigma-Aldrich, St Louis, MO, USA) incubation, and cells were counted, plated on sterile glass coverslips for  $\gamma$ H2AX detection, in 96-well plates for cell viability assay, or in six-well plates for alkaline comet assay, ROS, and apoptosis detection.

### Cell incubation

When cells reached approximately 80% confluence, the culture medium was removed. Cells were incubated with EDTA (Sigma-Aldrich) for 60 min at concentrations of 0.00001, 0.0001, 0.001, and 0.01, 0.01% being the most common concentration used in eye drops.<sup>5</sup> An inhibition study was performed using a 30-min 0.2% (w/v) HA 1000 kDa (Freda Biopharm Co., Ltd, Shangdong, China) preincubation followed by treatment with different concentrations of EDTA. EDTA and HA were dissolved in culture medium; thus, culture medium was used as control.

### Cell viability assay

The cell viability of EDTA-treated cells was examined by the MTT test. Cells were seeded into 96-well culture plates at a density of  $1 \times 10^4$  cells/well. At 24 h later, the medium was removed and 200  $\mu$ l of the new medium containing various concentrations of EDTA was added to each well. After 60 min incubation, 20  $\mu$ l of MTT (5 mg/ml in PBS) was added to each well and incubated at 37 °C. After 4 h, the solution was discarded and 150  $\mu$ l DMSO was added to each well. After formazan was dissolved, the absorbance at 490 nm was read on a microtiter plate reader (Bil Tek, Winooski, VT, USA). Relative survival was represented as the absorbance of treated sample/absorbance of control group.

### Alkaline comet assay

Alkaline comet assay, also called alkaline single-cell gel electrophoresis assay, was performed to detect DNA

SSBs and ALSs.<sup>27</sup> The method was performed as previously described with some modifications.<sup>28</sup> Briefly, the cell suspensions were pelleted and resuspended in 0.65% low melting point (LMP) agarose, and 75  $\mu$ l LMP-cell suspension was immediately pipetted onto a fully frosted microscope slide precoated with a 100  $\mu$ l layer of 0.65% normal melting point agarose. A third layer of 75  $\mu$ l of 0.65% LMP agarose was added. Following slide preparation, the embedded cells were lysed by gently immersing the slides in the freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, with 1% Triton X-100 and 10% DMSO added just before use, pH 10). After at least 1 h at 4 °C in the dark, the lysis solution was removed, and the slides were rinsed three times with distilled water. The slides were then placed in a horizontal gel electrophoresis chamber filled with fresh buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min to allow DNA to unwind. Electrophoresis was conducted in the same buffer at 20 V and 300 mA for 20 min. Then the slides were washed twice in a neutralization buffer (0.4 M Tris, pH 7.5) and fixed in methanol for 3 min. The slides were stained with 20  $\mu$ g/ml ethidium bromide and observed using an Olympus AX70 fluorescent microscope (Olympus, Tokyo, Japan). The mean olive tail moment was analysed using ImagePro Plus software (Media Cybernetics Inc., Silver Spring, MD, USA) in 100 cells on one slide in each of three independent experiments.

#### *Immunofluorescent microscopy and quantification of $\gamma$ H2AX foci*

The method was performed as we have previously described.<sup>29</sup> Briefly, after treatment, cells were fixed in 4% paraformaldehyde for 15 min, washed twice with PBS, and permeabilized in 0.2% Triton X-100 (Sigma-Aldrich). After being blocked with 3% blocking serum albumin (Sigma-Aldrich) for 1.5 h to suppress nonspecific antibody binding, samples were incubated with 1 : 1000 mouse monoclonal anti- $\gamma$ H2AX antibody (Upstate Technology, Lake Placid, NY, USA) for 2 h, followed by 1 : 500 fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse secondary antibody (AF488; Invitrogen, Carlsbad, CA, USA) for 1 h. To stain the nuclei, DAPI was added to the cells and incubated for another 15 min. The coverslip was then removed from the plate, mounted on a glass slide, and observed with an Olympus AX70 fluorescent microscope (Olympus). To prevent bias in selection of cells that display foci, all the cells were counted in the field of vision (at least 50 cells of each group in each of three independent experiments). ImagePro Plus was used to count the  $\gamma$ H2AX foci in each cell.

#### *Intracellular ROS production*

The production of ROS was measured with membrane permeable dye 2',7'-dichlorodihydrofluorescein diacetate molecule probes (DCFH-DA, Sigma-Aldrich), using a slight modification of the previously published method.<sup>30</sup> Briefly, cells were collected and centrifuged, and supernatant was discarded. The pellet was washed twice with PBS, resuspended in PBS containing a final concentration of 10  $\mu$ M DCFH-DA, and incubated at 37 °C for 30 min. The cells were then centrifuged and washed three times with PBS. After resuspension with PBS, cells were measured immediately, using flow cytometry (Cytomics FC 500, Beckman Coulter Inc., Miami, FL, USA) to monitor the formation of the fluorescent-oxidized derivative of DCFH-DA at an emission wavelength of 525 nm and an excitation wavelength of 488 nm. ROS were detected immediately after incubation to provide reliable data. Statistical analysis was performed using specialized software (CXP software; Beckman Coulter Inc.). For each sample, at least 10 000 events were analysed in each of three independent experiments. The ROS level was represented as the mean fluorescence intensity (MFI) of DCFH-DA in treated sample/the MFI in control group.

#### *Annexin V-FITC apoptosis detection assay*

The annexin V-FITC/propidium iodide (PI) kit (Biovision, Inc., Mountain View, CA, USA) was used to assess modifications of the cell membrane that are associated with programmed cell death. Experiments were conducted according to the manufacturer's instructions. In short, after treatment with EDTA, cells were collected, counted, centrifuged, and resuspended to  $5 \times 10^5$  cells in 500  $\mu$ l of  $1 \times$  binding buffer. A total of 5  $\mu$ l annexin V-FITC and 10  $\mu$ l PI were added to each sample. The samples were incubated in the dark at room temperature for 5 min. Samples were then examined immediately on the Cytomics FC 500 flow cytometry using the CXP software for data analysis. At least 5000 cells were analysed in each treatment group in each of three independent experiments.

#### *Statistical analysis*

Statistical analysis was performed with the Student's *t*-test and one-way ANOVA followed by the Dunnett multiple comparison test (GraphPad Prism 5 software; GraphPad Software, San Diego, CA, USA). Statistically significant differences between groups were considered to have a *P*-value of <0.05. Results are expressed as the mean  $\pm$  SEM of more than three experiments.

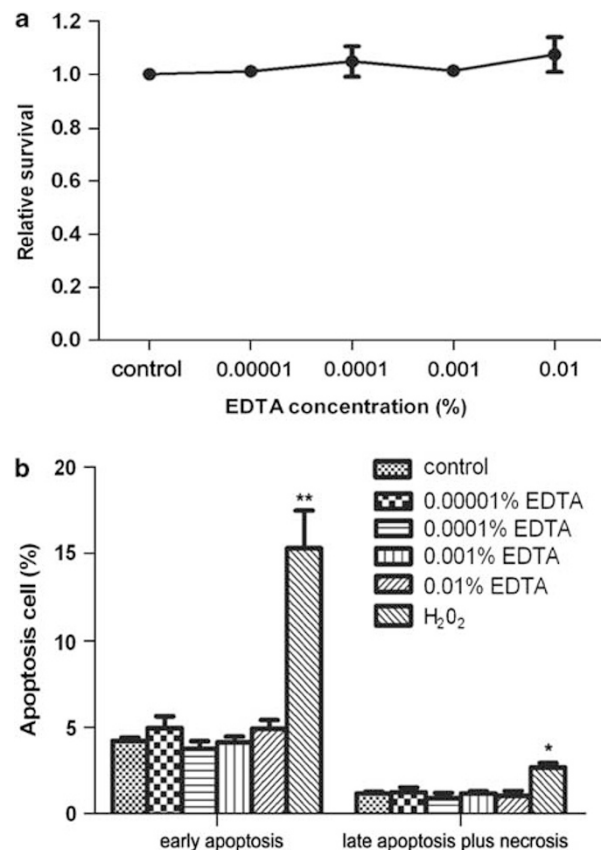
## Results

### Effects of EDTA on cell viability and apoptosis

After 60 min incubation at all the concentrations of EDTA tested (from 0.00001% to 0.01%), no significant loss of HCEs viability was observed ( $P > 0.05$ ; Figure 1a).

To assess the effects of EDTA on cell apoptosis, the percentage of early apoptotic cells was estimated by counting cells that were annexin V-FITC-positive but PI-negative, whereas the percentage of late apoptosis plus necrotic cells was estimated by counting cells that were both annexin V-FITC- and PI-positive. No significant differences in the percentage of apoptotic cells were found for all the concentrations of EDTA tested compared with the control ( $P > 0.05$ , Figure 1b).

Also, with preincubation with HA, there was no significant loss of cell viability or occurrence of cell

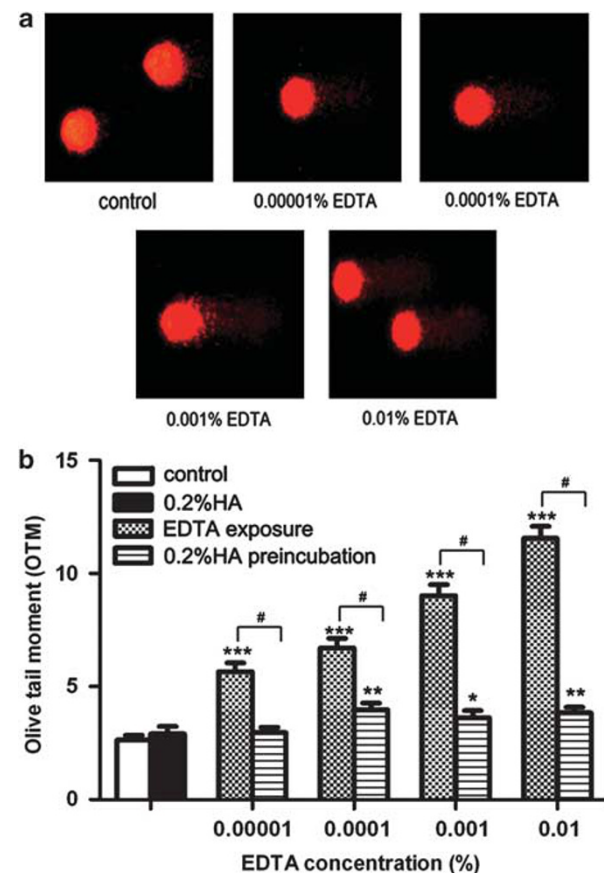


**Figure 1** The effects on cell viability and cell apoptosis induced by EDTA on HCEs. (a) Exposure to different concentrations of EDTA for 60 min did not significantly affect the relative survival of HCEs compared with the control group ( $P > 0.05$ ). The relative survival of control group was set 1. (b) No significant apoptosis nor necrosis was found with treatment of EDTA at all concentrations within 60 min ( $P > 0.05$  compared with control). H<sub>2</sub>O<sub>2</sub> treatment was used as positive control. \* $P < 0.01$ , \*\* $P < 0.001$  compared with control.

apoptosis in EDTA treatment groups compared with the control ( $P > 0.05$ , data not shown).

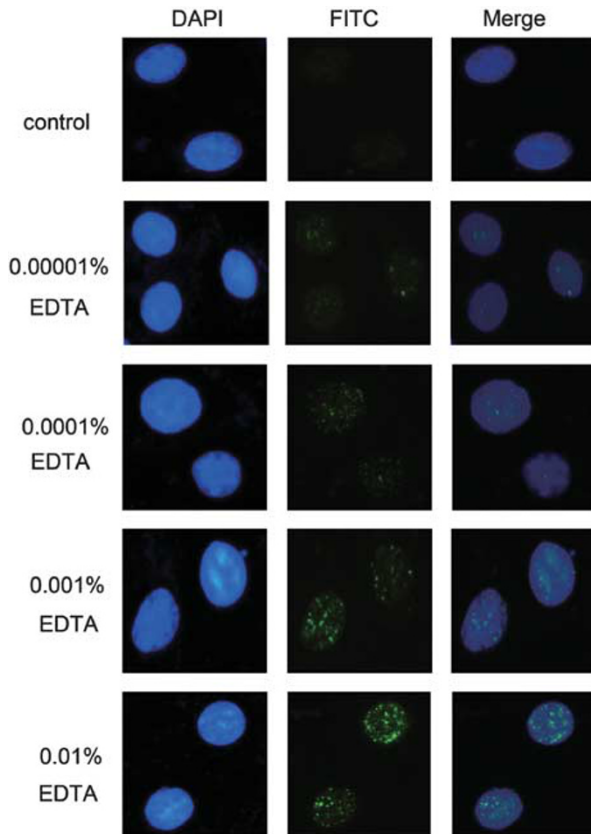
### Alkaline comet assay reveals the presence of SSBs and ALSs induced by EDTA

Under alkaline conditions, comet assay directly correlates SSBs and ALSs with the olive tail moment, which is defined as the product of the distance between the head and the centre of gravity of DNA in the tail and the percentage of DNA in the comet tail.<sup>31</sup> Figure 2a is a



**Figure 2** Alkaline comet assay showed that EDTA induced DNA damage. Under alkaline conditions, the comet assay directly correlates SSBs and ALSs with the olive tail moment (OTM). (a) Representative images of alkaline comet assay after EDTA exposure in HCEs. The cell looks like a comet with a distinct head consisting of intact DNA, and a tail that contains damaged or broken pieces of DNA. (b) Incubation of various concentration of EDTA for 60 min significantly increased OTM. Moreover, a significant increase in OTM in 0.01% EDTA-treated group was observed compared with the control when preincubated with 0.2% HA for 30 min. Furthermore, cells that had been preincubated with HA showed significantly less OTM compared with the EDTA-treated cells. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with control group; # $P < 0.001$  compared with EDTA-treated cells. Magnification,  $\times 200$ .

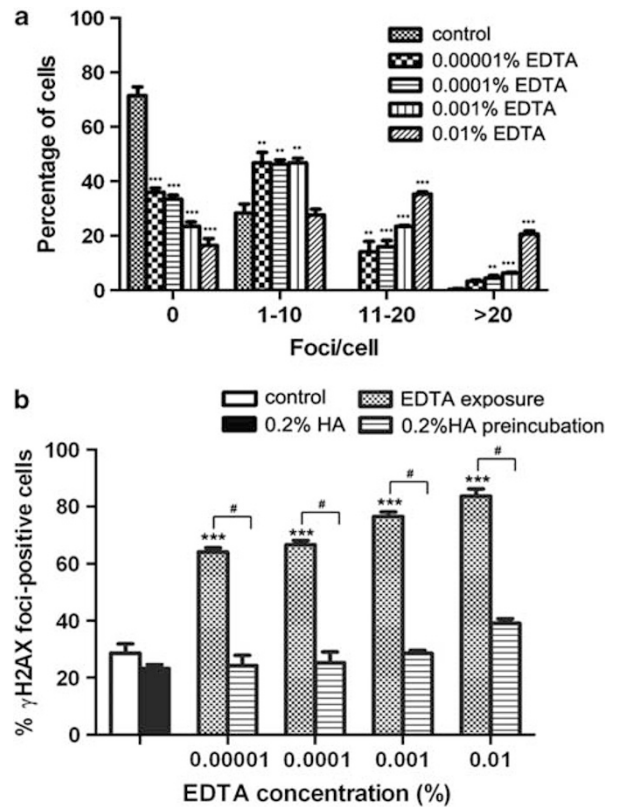
representative image of the results of the comet assay illustrating staining of DNA from individual HCEs after fragmentation. The cell treated with EDTA looks like a comet with a distinct head consisting of intact DNA, and a tail that contains damaged or broken pieces of DNA. After 60 min exposure to EDTA, a dose-dependent increase of olive tail moment was found (Figure 2b). A 60-min incubation of various concentrations of EDTA produced a 2.2-, 2.5-, 3.4-, and 4.4-fold increase in SSBs and ALSs, respectively, compared with the control group ( $P < 0.001$ ; Figure 2b). Cells that underwent 30 min preincubation with HA showed significantly fewer SSBs and ALSs than EDTA-exposed cells that had not undergone preincubation ( $P < 0.001$ ); however, the cells preincubation with HA before exposed to 0.0001, 0.001 and 0.01% EDTA showed a significantly increase in olive tail moment compared with the control cells ( $P < 0.05$ ; Figure 2b).



**Figure 3** Representative images of  $\gamma$ H2AX foci. After 60 min incubation,  $\gamma$ H2AX-foci staining became visible in HCEs after exposure to EDTA at 0.00001% and higher. Higher concentration of EDTA produced more number of  $\gamma$ H2AX foci in each cell. The nuclei stained by DAPI exhibit in blue, whereas the  $\gamma$ H2AX foci stained by FITC exhibit in green. Magnification,  $\times 400$ .

**DSBs induced by EDTA in  $\gamma$ H2AX foci formation detection**

$\gamma$ H2AX (phosphorylation at Ser139 of histone variant H2AX) foci formation has been used as a sensitive biomarker for DNA DSBs.<sup>32</sup> The number of  $\gamma$ H2AX foci is quantitatively the same as that of DSBs.<sup>33</sup> In our study,  $\gamma$ H2AX foci assay exhibited a dose-dependent increase in DSBs, similar to the trend that was seen in the alkaline comet assay. After 60 min incubation,  $\gamma$ H2AX staining became visible in HCEs after exposure to EDTA at concentrations of 0.00001% and higher (Figure 3). As shown in Figure 4a, the percentages of cells with 0  $\gamma$ H2AX foci in four concentrations of EDTA-treated groups were  $35.94 \pm 1.48\%$ ,  $33.41 \pm 1.50\%$ ,  $23.48 \pm 1.58\%$ , and  $16.40 \pm 2.58\%$ , respectively, each of which was



**Figure 4**  $\gamma$ H2AX foci formation in HCEs induced by EDTA. (a) Quantitative analyses of  $\gamma$ H2AX foci formation (Foci/cell). Higher concentration of EDTA yielded more number of  $\gamma$ H2AX foci. (b) Percentage of  $\gamma$ H2AX foci-positive cells. After 60 min incubation, all EDTA tested cells showed significant increase of the percentage of  $\gamma$ H2AX foci-positive cells. Preincubation with HA for 30 min before the EDTA caused a significant decrease in the number of  $\gamma$ H2AX foci-positive cells; however, the cells exposed to 0.01% EDTA showed a significant increase in the number of  $\gamma$ H2AX foci-positive cells compared with the control cells.  $**P < 0.01$ ,  $***P < 0.001$  compared with control group;  $\#P < 0.001$  compared with respectively EDTA-treated cells.

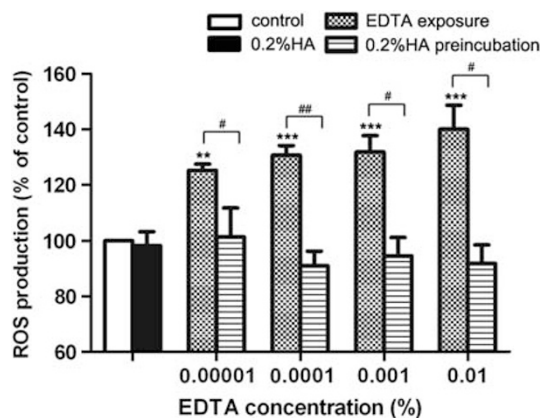
significantly less than in the control cells ( $71.36 \pm 3.22\%$ ,  $P < 0.001$ ). In addition, higher concentrations of EDTA produced more  $\gamma$ H2AX foci ( $P < 0.01$  compared with the control cells). The percentage of  $\gamma$ H2AX foci-positive cells (arbitrarily defined as those cells with  $\gamma$ H2AX foci number  $> 0$ ) in all concentrations was counted, and it ranged from  $64.06 \pm 1.48\%$  to  $83.6 \pm 2.58\%$  ( $P < 0.001$  compared with the control cells; Figure 4b). Furthermore, preincubation with HA for 30 min before the EDTA exposure resulted in a significantly smaller number of  $\gamma$ H2AX foci-positive cells compared with EDTA-exposed cells without HA preincubation ( $P < 0.001$ ; Figure 4b).

### ROS increased after EDTA treatment

After incubation for 60 min, there was a dose-dependent increase of ROS production in each EDTA-treated group. The MFI of the various concentrations of EDTA increased to  $125.2 \pm 2.26\%$ ,  $130.6 \pm 3.56\%$ ,  $131.8 \pm 5.89\%$ , and  $140 \pm 8.71\%$  compared with control groups, respectively, each of which was significantly higher than that found in the control cells ( $P < 0.01$ ; Figure 5). However, HA preincubation inhibited the increase in ROS generation induced by EDTA ( $P < 0.01$  compared with the EDTA-exposed cells; Figure 5).

### Exposure to HA did not have any significant effect on HCEs

Exposure to HA alone for 30 min did not produce any significant changes in cell viability, DNA damage, ROS generation, or cell apoptosis compared with controls.



**Figure 5** EDTA treatment induced ROS production in HCEs. All four concentrations of EDTA induced significant increase of ROS after 60 min incubation. 0.2% HA preincubation significantly decreased the ROS formation induced by EDTA.  $**P < 0.01$ ,  $***P < 0.001$  compared with control cells;  $\#P < 0.01$ ,  $\#\#\#P < 0.001$  compared with respective EDTA-exposed cells. The ROS production was represented as the MFI of DCFH-DA in treated sample/the MFI in control group.

### Discussion

Corneal epithelial cells, which form the outermost layer of the cornea, have important roles in maintaining metabolic homeostasis and corneal barrier integrity. EDTA is commonly used as a drug penetration enhancer in most ocular medications, and the most common concentration of EDTA used in eye drops is 0.01%.<sup>5</sup> It has been reported that EDTA can disrupt the plasma membrane, thus facilitating intercellular permeability.<sup>10</sup> EDTA has been occasionally reported to exhibit adverse effects on corneal epithelium at concentrations higher than that commonly used in eyedrops.<sup>4,6-8</sup> However, it is unknown whether EDTA has genotoxic effects on HCEs at clinical concentrations. Unlike the *in vitro* situation, ophthalmic drugs applied *in vivo* are almost instantly diluted by tears. Therefore, in our study, 0.01% EDTA was used as the highest concentration. After a 60-min incubation at concentrations of EDTA ranging between 0.00001 and 0.01%, no significant changes on cell survival or induction of cell apoptosis was observed. However, we found that EDTA could induce DNA damage in HCEs even at low concentration for a short incubation time. Thus, our study has revealed new evidence of genotoxic effects of EDTA on the HCEs.

The alkaline comet assay, a sensitive method for direct visualization of DNA damage on the level of a single cell, is capable of detecting DNA SSBs and other lesions that could induce SSBs, such as ALSs.<sup>27,34-36</sup> The phosphorylated form of histone variant H2AX ( $\gamma$ H2AX) has an important role in the recruitment of DNA repair and checkpoint proteins to sites of DNA damage, especially at DNA DSBs.<sup>37-42</sup> Within a few minutes of exposure to various stimuli that induce DSBs, H2AX can be phosphorylated by members of the phosphatidylinositol 3-kinase family at the serine residue in the highly conserved SQ motif and form foci at the damaged DNA sites.<sup>43-47</sup> Furthermore, it has been demonstrated that the number of  $\gamma$ H2AX foci detected by immunofluorescence is quantitatively the same as that of DSBs.<sup>48</sup> Therefore,  $\gamma$ H2AX foci formation has been suggested as a specific and sensitive indicator for DSBs.<sup>49-52</sup>

Our results of alkaline comet assay demonstrated that the level of SSBs induced by EDTA at all concentrations was significantly higher than that in control group (Figure 2a). Moreover, EDTA-induced DSBs, detected by the  $\gamma$ H2AX foci formation test, were also observed in HCEs at different doses (Figure 3 and Figure 4a). In concordance with these results, alkaline comet and  $\gamma$ H2AX foci assays showed that EDTA-induced SSBs and DSBs were positively correlated with EDTA dosage. Although EDTA could induce SSBs and DSBs of HCEs,

the results of cell apoptosis indicated that DNA damage induced by EDTA did not result in apoptosis; it is possible that EDTA-induced DNA damage could be repairable.

The mechanism by which EDTA induces DNA damage is still unclear. However, it is well known that ROS, which are generated as a by-product of normal mitochondrial activity in aerobic cells, can cause severe damage to cellular macromolecules, especially the DNA, if not properly controlled.<sup>53</sup> Meanwhile, many extracellular stimuli can influence the redox cycling pathway, causing the formation of ROS and eventually leading to DNA damage.<sup>54,55</sup> These redox cycles could be repeated and could generate numerous ROS capable of inducing amplified DNA damage, including SSBs and DSBs formation.<sup>56</sup> Atilano *et al*<sup>57</sup> found that hydrogen peroxide could cause mitochondrial DNA damage in corneal epithelial cells. In our study, a dose-related increase in ROS levels was observed in HCEs exposed to various concentrations of EDTA (Figure 5). Therefore, we postulated that the surplus ROS produced by EDTA disturbs the balance between the oxidation and reduction systems, eventually leading to DNA damage.

In accordance with previous *in vitro* studies,<sup>4,6-8</sup> we have demonstrated that EDTA has certain immediate toxic effects on HCEs. Because EDTA is necessary to enhance ocular penetration in topical ophthalmic preparations, we further investigated whether HA, a well-known biopolymer, is able to reduce the genotoxicity of EDTA. In the present study, we confirmed that single exposure to 0.2% HA 1000 kDa for 30 min did not show any toxic effects on HCEs. In addition, although SSBs and DSBs existed in cells treated with 0.01% EDTA, HA preincubation for 30 min effectively reduced the SSBs and DSBs induced by EDTA in HCEs (Figure 2b, Figure 4b). Moreover, a significant decrease was shown in superoxide anion production (Figure 5). A possible explanation is that HA may serve as a scavenger of free radicals and as an antioxidant.<sup>18</sup> HA is rich in hydroxyl functions, which can potentially absorb ROS.<sup>58</sup> Moreover, HA can bind to specific cell-surface receptors, for example, CD44, which has been demonstrated to be expressed in HCEs,<sup>58</sup> to initiate certain intracellular signal transduction pathways. Some of the pathways activated, might be involved in regulating cellular redox status, and thus could inhibit the intracellular ROS generated by EDTA exposure. Thus, the decreased formation of ROS led to decreased DNA damage in HCEs. Although HA did not inhibit the DNA damage completely, our study demonstrated that 1000 kDa HA is an effective protective agent that has antioxidant properties and partially inhibited DNA damage induced by EDTA.

## Conclusion

In conclusion, our study showed that the corneal penetration enhancer EDTA could increase ROS formation and cause DNA strand breaks in HCEs at concentrations lower than 0.01%, but that it did not have an effect on cell viability or induce cell apoptosis. In addition, high molecular weight HA, a tear substitute, which has no toxic effect on HCEs, can significantly reduce all the EDTA-induced toxic effects observed. Although it is possible that most of the DNA damage, including SSBs and DSBs could be repaired, the remaining breaks might lead to further mutations in progeny cells. Even for those repaired damages, mis-repair may also occur, which eventually could also lead to disastrous effects on cells. Therefore, long-time use of topical drugs containing EDTA might raise health concerns. However, the experiments conducted in this study, in particular, by using the monolayer cell culture system, may not reflect the real situation *in vivo*. Unlike the *in vitro* situation, it is difficult to predict the effects of ophthalmic drug concentrations *in vivo*; in addition, the availability of drug changes dynamically when blinking. Therefore, further investigation is needed to confirm the significance of these findings *in vivo*.

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## Summary

### What was known before

- EDTA is a common adjuvant in topical ophthalmic formulation.

### What this study adds

- EDTA can cause DNA damage in corneal epithelial cells in clinical concentration.
  - High molecular weight HA can reduce all the EDTA-induced toxic effects in corneal epithelial cells.
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## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

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