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# The effects of zinc supplementation on primary human retinal pigment epithelium

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ABSTRACT

Population-based and interventional studies have shown that elevated zinc levels can reduce the progression to advanced age-related macular degeneration. The objective of this study was to assess whether elevated extracellular zinc has a direct effect on retinal pigment epithelial cells (RPE), by examining the phenotype and molecular characteristics of increased extracellular zinc on human primary RPE cells. Monolayers of human foetal primary RPE cells were grown on culture inserts and maintained in medium supplemented with increasing total concentrations of zinc (0, 75, 100, 125 and 150 μM) for up to 4 weeks. Changes in cell viability and differentiation as well as expression and secretion of proteins were investigated. RPE cells developed a confluent monolayer with cobblestone morphology and transepithelial resistance (TER) > 200 Ω*cm$^2$ within 4 weeks. There was a zinc concentration-dependent increase in TER and pigmentation, with the largest effects being achieved by the addition of 125 μM zinc to the culture medium, corresponding to 3.4 nM available (free) zinc levels. The cells responded to addition of zinc by significantly increasing the expression of Retinoid Isomerohydrolase (RPE65) gene; cell pigmentation; Premelanosome Protein (PMEL17) immunoreactivity; and secretion of proteins including Apolipoprotein E (APOE), Complement Factor H (CFH), and High-Temperature Requirement A Serine Peptidase 1 (HTRA1) without an effect on cell viability. This study shows that elevated extracellular zinc levels have a significant and direct effect on differentiation and function of the RPE cells in culture, which may explain, at least in part, the positive effects seen in clinical settings. The results also highlight that determining and controlling of available, as opposed to total added, zinc will be essential to be able to compare results obtained in different laboratories.

## 1. Introduction

According to the population-based study conducted in Rotterdam, those with the highest quartile of zinc nutrition have a lower risk of progression in age-related macular degeneration (AMD) [1]. Decreased levels of total zinc in the peripheral blood serum and the retinal pigment epithelium (RPE)/choroid [2–6] have also been associated with development of AMD. Accordingly, the Age-Related Eye Disease Study (AREDS) trial showed that the progression to advanced AMD was significantly reduced in patients who received daily supplementation of 80 mg zinc, especially in combination with a cocktail of antioxidants [7]. The mechanism behind this beneficial zinc effect remains elusive, but understanding the processes affected by zinc may lead to improved or novel treatment strategies for AMD.

Zinc is the second most abundant trace element in the human body [8–10]. Zinc toxicity is rarely observed *in vivo* [11,12], although in cell culture experiments zinc overdose can trigger cell death [13–16]. In the eye, zinc is present in high concentrations [17–19] where the majority of ocular zinc is localised to the RPE/choroid complex [20]. Zinc deficiency or zinc overload in the RPE can lead to a variety of problems

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[21–25]. However, the actual concentration of biologically active or available zinc ions (*e.g.*, the small fraction of total zinc ions not tightly bound by protein and other ligands) is not determined; therefore, comparison of results between laboratories is usually difficult. Active uptake and prolonged retention of zinc has been shown in RPE *in vivo* [26] and the presence of exchangeable zinc in RPE has been demonstrated [27,28] and was localised to the Golgi apparatus [29], melanosomes, and lysosomes [30–33].

Based on protocols for culturing and differentiating human RPE cells [34–36] there are abundant high affinity zinc-binding proteins such as serum albumin in the culture medium and it is hypothesized that RPE cells in culture are in a potentially zinc-deficient environment. In this report, we cultured primary human RPE cells in a zinc-enriched environment by supplementing the culture medium with different concentrations of added zinc sulphate and found that under these conditions 3.4 nM free zinc could accelerate RPE differentiation, alter gene expression, and modify secretion of AMD-specific proteins.

## 2. Materials and methods

### 2.1. RPE cell culture

Primary human foetal RPE cells were purchased from ScienCell™ Research Laboratories at passage 1 (P1). Cells were propagated and frozen as P2 cells. For the experiments below, P3 cells were seeded onto laminin-coated 24 well plastic culture plates with glass cover slip or porous cell culture inserts (Millipore Millicell-HA Culture Plate Inserts, PIHA 01250) with the density of 125,000 cells per square centimetre and cultured in Epithelial Cell Medium (EpiCM, ScienCell™ Research Laboratories) for one week to allow optimal propagation, following the manufacturer's instructions. Following this period cells were differentiated in the so-called "Miller medium" [35] with or without 75, 100, 125, 150 or 200 μM added zinc sulphate (Sigma-Aldrich) apically, and when the cell culture insert was used, non-supplemented medium basally for 28 days. Medium was changed twice a week and cells were maintained at 37° C and 5% $CO_2$. Prior to change of culture medium, trans-epithelial resistance (TER) was measured using the EVOM2 Epithelial Voltohmmeter and STX2 electrodes (World Precision Instruments). Both apical and basal media were collected at the time of the change of medium for toxicity measurements using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) to monitor cell viability. Pigmentation was imaged using a Nikon LZM1500 binocular microscope at 1 × magnification and the coverage of pigment at different zinc concentrations was determined by ImageJ software (ImageJ v.1.51p; background subtraction and thresholding then analysing particle size distribution).

### 2.2. Determination of free zinc in the medium

Zinc concentrations in the Miller medium were calculated using MINEQL, a chemical equilibrium programme for personal computers [37], as well as measured using fluorescent sensors essentially as described [13]. Briefly, the known affinities [23,38] and concentrations of the 25 most important zinc ligands were included in the calculation; other potential ligands (*e.g.* vitamins, growth factors) were not included in the calculation due to their low concentrations (< 10 nM) and/or weak affinities ($K_D$ > 1 μM). Bioavailable ("free") zinc was held to include $Zn^{2+}$ together with its labile complexes with weak, rapidly exchangeable ligands such as water, hydroxide, or chloride: *e.g.*, ZnOHCl, $ZnCl_2$, *etc*. The results were corrected for temperature, ionic strength, and pH, and the presence of dissolved $CO_2$ in the medium was included. The accuracy of this calculation method was previously demonstrated for other media [39] and sea water [40]. We used a variation on our previously described fluorescence-based zinc biosensors [41–43] to measure the free zinc in the growth medium. The sensor employed one of two variants of apocarbonic anhydrase II to provide sensitivity to the appropriate free zinc concentrations, and a polymeric form of ABDN (7-amino-(2′-hydroxyethyl)-benz-2-oxa-1,3-diazole-4-sulfonamide) which exhibits a 50 nm blue shift in its peak fluorescence emission when bound to holocarbonic anhydrase compared to its unbound form which is observed in the absence of zinc, when both are entrapped in a porous gel. The ratio of fluorescence emission at 550–600 nm is thus a measure of the proportion of carbonic anhydrase with zinc bound, which in turn is a simple function of the free zinc concentration [41]. The variants of the carbonic anhydrase were wild type bovine CA II and H94N human CA II, which exhibited apparent $K_D$'s under the conditions of the experiment of 0.10 ± 0.033 and 0.71 ± 0.06 nM, respectively (data not shown).

### 2.3. Immunohistochemistry (IHC) and confocal microscopy

After 28 days of zinc supplementation, specimens were rinsed in PBS (3 × 5 min), fixed for 10 min in 4% (v/v) paraformaldehyde in PBS, and stored in 0.4% paraformaldehyde pending sectioning and immunohistochemical analysis. The membrane inserts were carefully excised, then cryopreserved by immersion in 30% (w/v) sucrose (in PBS) overnight at 4° C, followed by 30% sucrose plus Tissue-Tek® O.C.T.™ compound (Sakura) at 50%: 50% (vol:vol) for 2 h, and finally in 100% (v/v) O.C.T. for 1 h. Cryopreserved samples were embedded in OCT and 20 μm sections were generated on a cryostat (Bright Instruments). Sections were rinsed with PBS and blocked with 5% (v/v) donkey serum in PBT (PBT: PBS containing 0.5% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100) for 1 h at room temperature. The sections were co-labelled with polyclonal goat anti-human Apolipoprotein E (1/500 in PBT, Millipore) and monoclonal mouse anti-human PMEL17 (clone HMB45, 1/2000 in PBT, Dako) antibodies for 1 h at room temperature. Sections were rinsed for 3 × 5 min in PBS, after which they were incubated in Alexa Fluor 546-conjugated donkey anti-goat IgG and Alexa Fluor 488-conjugated donkey anti-mouse IgG (both 1/200 in PBT, Life Technologies) for 1hr at room temperature. After removal of the secondary antibodies by rinsing the sections with 3 × 5 min in PBS, nuclei were stained with Hoechst 33342 (1.5 μg/ml in PBS, Life Technologies) and mounted on slides with Vectashield antifade mounting medium (Vector Laboratories). Immunofluorescence was imaged using a Zeiss LSM700 confocal microscope and analysed by ZEN 2 software (Carl Zeiss Microscopy GmbH).

### 2.4. Transmission electron microscopy (TEM)

After 28 days of zinc supplementation, specimens for TEM were fixed in a solution of 1% (v/v) glutaraldehyde and 1.5% (v/v) paraformaldehyde in 0.1 M PBS at pH 7.2. Specimens were post-fixed with 1% (w/v) osmium tetroxide in 0.1 M PBS for 50 min, dehydrated and embedded in Araldite. For EM, ultra-thin sections were cut and stained with 1% (w/v) uranyl acetate and Reynolds' lead citrate. Semi-thin sections for light microscopy were also cut and stained with Toluidine Blue. EM was performed using a JEOL JEM-1010 Transmission Electron Microscope. Images were collected using a Gatan Orius CCD camera and converted from Digital Micrograph DM3 format to 8-bit TIFF images for analysis at 4008 × 2762-pixel resolution.

### 2.5. Mass spectrometry and label-free quantitative analysis

To analyse the secreted proteins with or without 125 μM added zinc at day 28 after zinc supplementation, cells were washed with 1 × 5 min PBS then kept in serum-free "Miller medium" for 24 h, after which the apical secretome and the filter insert for basal secretion were collected and frozen immediately, except when part of the membrane insert was fixed for immunolabeling. Proteins absorbed by the membrane during culturing representing the basally secreted protein pool were directly proteolyzed on the membrane by incubation with ammonium bicarbonate buffer, reduction with dithiothreitol for 30 min at 60 °C,