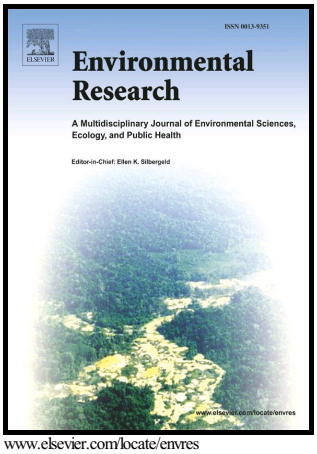

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Volcanic ash from Puyehue-Cordón Caulle Volcanic Complex and Calbuco promote a differential response of pro-inflammatory and oxidative stress mediators on human conjunctival epithelial cells

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Abstract

Volcanic ash could pose a hazard to the ocular surface as it is constantly exposed to environmental particles. We exposed conjunctival cells to Puyehue-Cordón Caulle volcanic complex (PCCVC) or Calbuco ash particles and evaluated proliferation, viability, apoptosis, MUC1 expression, pro-inflammatory cytokines, and oxidative stress markers. Ash particles from these volcanoes vary in

size, composition, and morphology. Our results demonstrate that PCCVC but not Calbuco ash particles induce cytotoxicity on human conjunctival epithelial cells viewed as a decrease in cell proliferation and the transmembrane mucin MUC1 expression; a pro-inflammatory response mediated by IL-6 and IL-8; and an imbalance of the redox environment leading to protein oxidative damage. This is the first *in vitro* study that assesses the biological effect of volcanic ash particles on human conjunctival epithelial cells and the involvement of inflammatory mediators and oxidative stress as the mechanisms of damage. Our results could provide a better understanding of the ocular symptoms manifested by people living near volcanic areas.

Keywords: volcanic ash, conjunctiva, pro-inflammatory cytokines, oxidative stress, MUC1.

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1. Introduction

At least 600 million people live in areas potentially affected by volcanic hazards (Auker et al., 2013). Ash produced during volcanic eruptions and particles remobilized from ashfall deposits can lead not

only to economic losses but also to adverse effects on human health (Gudmundsson, 2011; Hansell, 2006; Horwell and Baxter, 2006).

On 4 June 2011 the latest eruption of the Puyehue-Cordón Caulle volcanic complex (PCCVC) started in the Republic of Chile (40º59' S, 72º11' W), close to the Argentinean border (SERNAGEOMIN, 2011). A new vent from the Cordón Caulle fissure (Collini et al., 2013) ejected about 1000 million metric tons of ash on 7.5 million hectares on the northern Argentinean Patagonia (Gaitán et al., 2011). Ash fallout had a negative impact on the environment affecting electricity and water services, disrupting transport networks, inducing livestock losses, and also affecting public health mostly of nearby residents (Wilson et al. 2013). Villa La Angostura, about 50 km from the volcanic event was one of the population centers that received the greatest ash falls (Canafoglia et al., 2012; Wilson et al., 2013). To a lesser extent, this village was again affected by volcanic ash dispersal on April-May 2015 when the Calbuco volcano, also located on the Southern Andes of Chile (41º20' S – 72º37' W), erupted after decades of quiescence (Reckziegel et al., 2016; SERNAGEOMIN, 2015.).

Several studies have particularly focused on the volcanic ash effects on the respiratory system and on the physicochemical characterization of the ash particles (Damby et al., 2016; Horwell et al., 2003b; Horwell et al., 2010; Tomasek et al., 2016). Some factors that could predict the severity of ash exposure are their concentration and size, the mineralogic composition (in particular the content of free silica), and the surface properties. Additionally, particle surface radicals and transition metals could cause oxidative stress threatening cell integrity (Gudmundsson, 2011; Hansell, 2006; Horwell et al., 2003a; Horwell and Baxter, 2006).

Volcanic ash not only poses a hazard to the respiratory system but it could also harm the ocular surface as it is constantly exposed to environmental particles. The ocular surface epithelia consists of the cornea and conjunctiva and is separated from the environment only by the tear film. Mucins are high molecular weight glycoproteins, essential components of the tear film that protect the ocular surface trapping and removing debris, harmful pathogens from the external environment, and also facilitate lubrication. Mucins can be divided into two subfamilies, 1) the secreted mucins and 2) the membrane-tethered mucins (Gipson, 2004; Govindarajan and Gipson, 2010; Mantelli and Argüeso, 2008). The impact of air pollution from anthropogenic sources on the ocular surface has previously

been studied. Epidemiological results showed that subjects exposed to air pollutants presented ocular discomfort symptoms and instability of the tear film (Jung et al., 2018; Novaes et al., 2010; Torricelli et al., 2013). In addition, it has been demonstrated that corneal and conjunctival epithelial cells in presence of urban air pollution particles elicit a pro-inflammatory response mediated by Interleukin (IL) 6, an alteration of the intracellular redox balance, and a differential expression of mucins (Fujishima et al., 2013; Lasagni Vitar et al., 2018, 2015; Tau et al., 2013).

Moreover, ocular symptoms due to ash exposure have been reported (Carlsen et al., 2012; Fraunfelder et al., 1983; Kimura et al., 2005; Lombardo et al., 2013). However, up to date there are no *in vitro* studies assessing the biological effect of volcanic ash particles on ocular surface and the involvement of inflammatory mediators and oxidative stress on the mechanisms of damage.

Thus, the purpose of this work was to assess the effect of volcanic ash from PCCVC and Calbuco on human conjunctival epithelial cells evaluating proliferation, viability, apoptosis, MUC1 expression, pro-inflammatory cytokines, and oxidative stress markers.

2. Materials and Methods

2.1 Volcanic ash collection and characterization

Volcanic ash samples were collected in the urban area of Villa la Angostura in the province of Neuquén, Argentina. Pueyehue – Cordón Caulle volcanic complex (PCCVC) ash samples were collected in September 2011 and Calbuco samples in April 2015. Each of the ash samples was sieved through a 0.5 mm mesh metal sieve to remove the coarse dust component.

Scanning Electron Microscopy and energy dispersive X-ray spectroscopy (EDX) were employed to analyze particle morphology, size, and chemical composition. For scanning electron microscopy observations, ash particles were coated with platinum (Pt) using a sputter coater (Cressington Scientific Instruments). Imaging was performed with a Carl Zeiss NTS- Supra 40 Scanning Electron

Microscopy (Germany) and for chemical composition a coupled EDX dispersion detection unit was used to collect elemental spectra (Oxford Instruments, INCA program, England).

2.2 IOBA-NHC cell culture

The normal human conjunctival epithelium cell line (IOBA-NHC) was kindly provided by Yolanda Diebold, Ph.D. (University Institute of Applied Ophthalmobiology, University of Valladolid, Valladolid, Spain, (Diebold et al., 2003)). Cells were subcultured once a week in order to maintain the cell line. IOBA-NHC (passages 75-80) were grown in 24 well plates (Jet Biofil, Guangzhou, China) in 1 mL of culture medium per well at a seeding density of 2 x 10^5 cells/well. The medium consisted of Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% fetal bovine serum (Internegocios, Mercedes, Buenos Aires, Argentina), 2 ng/ mL epidermal growth factor (EGF), 5 µg/ mL hydrocortisone (Sigma, St. Louis, MO), 1 µg/ mL bovine pancreas insulin (Sigma), 50 U/ mL penicillin, 50 µg/ mL streptomycin, and 2 mg/ mL amphotericin B. Cells were incubated in a humid atmosphere of 37°C with 5% CO_2 and reached 90-100% confluence after 24 hours. All experiments were carried out under these conditions except for the MTT assay, which is detailed in section 2.4.

2.3 Exposure of cell cultures to volcanic ash

An ash suspension of 1000 µg/ mL from PCCVC or Calbuco eruptions was performed by weighing ash in 15 mL plastic tubes (Jet Biofil, Guangzhou, China), adding complete culture medium, and sonicating in an ultrasonic bath (Ultrasonic Cleaner; Testlab, Bernal Oeste, Buenos Aires, Argentina) for 15 minutes. Particles did not aggregate. Suspensions of 50, 100, and 500 µg/ mL were then made from the 1000 µg/ mL suspension and brought to final volume with complete culture medium. Finally, all the suspensions (50-1000 µg/ mL) were sonicated for 15 minutes.

Cells were exposed to a suspension of 100 µg/ mL of diesel exhaust particles (DEP) (Laks et al., 2008) in order to show the responsiveness of the cell system. Suspensions were prepared weighing DEP, adding complete culture medium, and sonicating in an ultrasonic bath for 30 minutes as described by our group (Lasagni Vitar et al., 2018, 2015; Tau et al., 2013)

Conjunctival cell monolayers were exposed to 1 mL ash suspensions, DEP as positive control and complete culture media as experimental control, for 24 hours.

2.4 Proliferation evaluation

Cell proliferation was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described elsewhere (Berridge et al., 2005; Meerloo et al., 2011; Molinari et al., 2005). MTT is a yellowish aqueous solution, which is reduced by dehydrogenases, and reducing agents present in metabolically active cells, to insoluble violet-blue formazan crystals. IOBA-NHC cells were plated at 3 X 10^4 cells per well in a 24-well culture plate for 24 hours prior to treatment. Culture medium was removed and volcanic ash suspensions from PCCVC or Calbuco (50, 100, 500, and 1000 µg/ mL) were added for 24 hours. Medium from control or exposed cells was removed from cell cultures, washed twice with PBS 1X, and then 0.5 mL of fresh complete growth media supplemented with 50 µL MTT (4 mg/ mL) was added for 3 hours. Immediately after incubation, 10% SDS was added to stop the MTT reaction and to solubilize the formazan precipitate. Finally, the lysate of each well was homogenized and centrifuged at 10,000g in order to eliminate remaining ash particles. The absorbance of each supernatant was measured at 550 nm in a plate reader (iMark Microplate Absorbance Reader; Bio-Rad, Hercules, CA).

2.5 Imaging of cell cultures

IOBA cells with ash treatment or culture media as control were imaged by light microscopy to evaluate any changes in cell morphology. Cell monolayers were grown over 10 mm glass covers and exposed to ash particles or culture media as control. Cover slips were washed twice with PBS 1X and fixed in 100% methanol. Cell monolayers were stained with Papanicolaou technique (PAP). Briefly the following steps were performed in sequence: 96°alcohol for 2 minutes, 70° alcohol for 2 minutes, distilled water for 30 seconds, haematoxylin (Harris-Biopack, Argentina) for 5 minutes, tap water 2 minutes, 70° alcohol for 30 seconds, Orange G solution (OG6 s/PAPANICOLAOU-Biopack, Argentina)

for 3 minutes, 96° alcohol for 30 seconds, EA 36 (Papanicolaous' EA 36-Biopack, Argentina) for 5 minutes, 96° alcohol for 4 minutes, 100° alcohol for 1 minute and xylene for 30 seconds. DPX mounting media (Biopack, Argentina) was added directly to the cell monolayers and glass cover slip were mount over slides. Images were captured at 400X magnification using a light microscope (Olympus CX21FS2, Tokyo, Japan).

2.6 Cell viability and apoptosis evaluation

The percentage of apoptotic and necrotic cells was determined by flow cytometry (FACSCalibur; BD Biosciences) using the Annexin V–FITC and propidium iodide (PI) kit (BD Biosciences, Franklin Lakes, NJ). Cell monolayers were washed twice with 1 mL of PBS and trypsinized (250 µL per well for 2-5 minutes, 37°C, 5% CO_2). Trypsin was neutralized with culture media and cells were centrifuged at 1,000g for 5 minutes. A density of 2.5-3 X 10^5 cells were placed in cytometer tubes, washed twice with cold PBS, and resuspended in 100 µL Annexin V binding buffer. Then, 5 µL Annexin V–FITC and 5 µL PI were added to each tube. The tubes were gently vortexed and incubated for 15 minutes in the dark. Finally, 400 µL Annexin V binding buffer was added to each tube, and the flow cytometric analysis was performed (FACSCalibur; BD Biosciences, argon laser 488 nm). The values obtained were analyzed by Cyflogic 1.2.1 (CyFlo Ltd., Turku, Finland). Annexin V binds to negatively charged phospholipid surfaces with a higher specificity for phosphatidylserine (PS) than most other phospholipids. PS is an internal plasma membrane phospholipid that in the early apoptotic cascade is exposed on the outer layer of the plasma membrane, before the cells become permeable to PI. Therefore, early apoptotic cells are Annexin V–FITC positive and PI negative, and late apoptotic or necrotic cells are both, Annexin V–FITC and PI positive. Viable cells are Annexin V–FITC and PI negative.

2.7 Cell lysate preparation

Experimental control, PCCVC and Calbuco cell lysates were obtained scraping off the monolayers from the culture plate with 100 µL of lysis buffer (50 mMTrisHCl, 150 mMNaCl, 1 mM protease inhibitor

(Sigma), 2 mM EDTA, 1% Triton X-100) and then sonicating in cold water for 10 minutes (whole lysate). Finally, the whole lysates were centrifuged at 1,000g for 10 minutes in order to obtain the cell lysates. Proteins in supernatants were determined by Lowry's method (Lowry, 1951).

2.8 Evaluation of MUC1 protein expression by western blot

Cell lysates (40 µg of proteins/lane) were resolved on 7.5 % acrylamide SDS-PAGE gels. Proteins were transferred to PVDF membranes and blocked for 1 hour in 5 % nonfat dry milk in PBS with 13 % NaN_3 and hybridized overnight with anti-MUC1 (1:200; sc-7313, Santa Cruz Biotechnology) or anti-actin (Calbiochem, CP01). Membranes were washed three times in PBS, and secondary detection was performed using 1:5,000 dilutions of HRP-conjugated anti-mouse antibody (Cappel). Membranes were washed three times, and chemiluminescent detection was done using ECL (Thermo Scientific). Bands were quantified using Image J program (1.50i, Wayne Rasband, National Institutes of Health, USA).

2.9 Determination of pro-inflammatory cytokines

Cytokine secretion was determined from supernatants collected after ash exposure. In order to eliminate remaining ash particles, supernatants were centrifuged at 10,000g for 10 minutes at 4°C. Enzime-linked immunosorbent assays (ELISA) kits (BD Biosciences) were used to perform the quantification of IL-6 (No. 2645KI) and IL-8 (No. 2654KI) following the manufacturer's protocol.

2.10 Intracellular ROS production

Cell monolayers were washed with 1 ml of PBS and trypsinized as described in section *2.6.* Cells (2.5 x 10^5 cells/ mL) were loaded with 5 µM 2′,7′-dichlorofluorescein (DCF) diacetate and analyzed by flow cytometry. DCF diacetate passively diffuses into cells, where it is de-esterified by intracellular sterases and readily oxidized to the green-fluorescent product DCF upon its reaction with oxidant species. This measurement indicates intracellular generation of oxidizing species. After a 30 minutes incubation in

the dark at 37 °C, 20,000 events per sample were acquired in a FACSCalibur; BD Biosciences equipped with a 488 nm argon laser. The cell population was gated based on light scattering properties. DCF signal was analyzed in the FL-1 channel with Cyflogic 1.2.1 (CyFlo Ltd., Turku, Finland), and quantified as median fluorescence intensities (MFI)(Lasagni Vitar et al., 2015). The results were expressed as mean fold of increase over control cells.

2.11 Indirect measurement of nitric oxide (NO) production

The concentration of NO in the cell culture supernatants was determined by the accumulation of nitrite (NO_2^-). The nitrite concentration was measured using a spectrophotometric method based on the Griess reaction (Ding et al., 1988). Cell culture supernatants were centrifuged as described in section *2.9*. A sample of 400 µL were mixed with 1% sulfanilamide and 0.1% naphthyl-ethylenediamine (400 µL), which were allowed to react at room temperature for 10 minutes. The nitrite concentration was determined by measuring the absorbance at 550 nm in comparison with standard solutions of sodium nitrite at different concentration. The results were expressed as nmol/ mg protein.

2.12 Antioxidant enzymes activity

2.12.1 Superoxide dismutase (SOD)

A colorimetric assay was used based on the inhibition of adrenochrome formation rate at 37 °C due to the addition of increasing amounts of cells lysate in a reaction medium consisting in 1 mM epinephrine and 50 mM glycine buffer (pH= 10.50). Measurements were performed at 480 nm in a Hitachi U-2000 Spectrophotometer (Hitachi Ltd., Chiyoda, Tokyo, Japan). Enzymatic activity was expressed as USOD/ mg protein. One SOD unit was defined as the volume of sample needed to inhibit adrenochrome formation rate by 50% (Misra and Fridovich, 1972).

2.12.2 Glutathione S-transferase (GST)

Glutathione S-transferase activity was measured using 1 chloro– 2,4 dinitrobenzene (CDNB), which forms GS-dinitrobenzene (GS-DNB) in the presence of glutathione, reaction catalyzed by GST provided by the cell lysate. This adduct absorbs at 340 nm. Briefly, cells lysate (50 µL) was mixed in the reaction medium consisted of phosphate buffer (pH= 6.50), 10 mM glutathione (GSH), and 20 mM CDNB. Results were expressed as mUGST/mg protein. One GST unit was defined as the amount of enzyme that catalyzes the formation of 1 µmol GS-DNB per minute at 30 °C (Habig et al., 1974).

2.13 Reduced glutathione (GSH) levels

Cells lysate samples (100 µL) were mixed with 1 M $HClO_4$ 2 mM EDTA (1:1) and centrifuged at 20,000g for 20 minutes at 4 °C. Supernatants were filtered through 0.22 µm cellulose acetate membranes (Corning Inc., NY, US) and frozen at −80 °C until use. HPLC analysis was performed in a Perkin Elmer LC 250 liquid chromatography (Perkin Elmer, Waltham, MA, US), equipped with a Perkin Elmer LC ISS 200 advanced sample processor and a Coulochem II (ESA, Bedford, MA, US) electrochemical detector. A Supelcosil LC-18 (250 × 4.6 mm ID, 5 µm particle size) column protected by a Supelguard (20 × 4.6 mm ID) precolumn (Supelco, Bellfonte, PA, US) was used for sample separation. GSH was eluted at a flow rate of 1.2 mL/min with 20 mM sodium phosphate (pH= 2.70), and electrochemically detected at an applied oxidation potential of +0.800 V. A calibration curve with GSH standard was performed. Results were expressed as nmol/1x10^6 cells (Rodriguez-Ariza et al., 1994).

2.14 Oxidative damage to macromolecules

2.14.1 Protein oxidation

Cell content of carbonyl groups from oxidatively modified proteins were detected in cell lysate with 10 mM 2,4-dinitrophenylhydrazine (DNPH), which leads the formation of a stable 2,4-

dinitrophenylhydrazone product (DNP) that is soluble in 6 M guanidine. The DNP absorbs ultraviolet light so that the total carbonyl content of a protein can be quantified by a spectrophotometric assay at 370 nm (Levine et al., 1990). Results were expressed as nmol/ mg protein.

2.14.2 Lipid oxidation

Oxidative damage to lipids was determined as thiobarbituric acid reactive substances (TBARS), using a fluorometric assay (Yagi, 1976). Briefly, whole lysate samples (100 µL) were incubated with 200 µL of 0.1 N HCl, 30 µL of 10% (w/v) phosphotungsticacid, and 100 µL of 0.7% (w/v) 2- thiobarbituric acid (TBA) in a dry bath. After 60 minutes, TBARS were extracted in 500 µL of n-butanol and centrifuged at 1000 g for 10 minutes. The fluorescence of the butanolic layer was measured in a Perkin Elmer LS 55 Fluorescence Spectrometer (Perkin Elmer, Waltham, MA, US) at 515 nm (excitation) and 553 nm (emission). A calibration curve was performed using 1,1,3,3-tetramethoxypropane (MDA) as standard. Results were expressed as nmol MDA/ mg protein.

2.15 Statistical Analysis

All data were expressed as mean ± standard error of mean (SEM) of at least 3 independent experiments. A t-student or one-way ANOVA test and Dunnett's as a post hoc test were used for statistical analysis using GraphPad PRISM software (San Diego, CA, USA). Tests were considered significant when $p<0.05$.

3. Results

3.1 Characterization of ash particles

Ash samples from PCCVC and Calbuco eruptions collected in Villa la Angostura were analyzed by scanning electron microscopy/EDX in order to characterize particles size and elemental composition.

Particle size from PCCVC ash samples is heterogeneous ranging from less than 10 µm to 100 µm (Fig. 1a). Scanning electron microscopy images in Figure 1b, shows that Calbuco samples present mainly blocky particles of 100-300 µm and small particles of less than 10 µm. Ash oxide composition analyzed by EDX confirmed that PCCVC particles contain more SiO_2 , Na_2O, and K_2O (70% vs. 56%, 6% vs. 3%, and 2.9% vs. 0.5% respectively, $p<0.05$) and less FeO, CaO, and MgO (5% vs. 9%, 2% vs. 10%, 0,3% vs. 5.6% respectively, $p<0.01$) when compared to Calbuco ash particles (Fig. 1c).

3.2 IOBA-NHC proliferation

We examined the effect of volcanic ash particles (50, 100, 500, and 1000 µg/ mL) on cell proliferation by MTT assay. IOBA-NHC proliferation significantly decreased 26% and 29% after the exposure to 500 and 1000 µg/ mL of PCCVC ash respectively in comparison with control ($p<0.01$). Calbuco particles did not induce a significant reduction of MTT values (Fig. 2).

3.3 IOBA-NHC morphology evaluation

IOBA-NHC were stained with PAP technique in order to evaluate cell morphology. Cells treated with ash particles (PCCVC or Calbuco) did not show any alterations in its morphology comparing with control. The cells showed the typical polygonal morphology, the presence of nuclei with intensely stained nucleoli, and some mitotic figures (Fig. 3).

3.4 *Viability and apoptosis assessment*

Cell viability, apoptosis, and necrosis were assessed by flow cytometry using the Anexin V/PI kit in cells exposed to 500 and 1000 µg/ mL ash particles concentration. IOBA-NHC viability was over 95% in all groups and apoptotic or necrotic percentages also remained unaltered when compared to control (Table 1).

Table 1. IOBA-NHC viability and apoptosis percentages

Cells	Control	PCCVC 500	PCCVC 1000	Calbuco 500	Calbuco 1000	DEP 100
Viable	97.3 ± 0.1	94.0 ± 1.0	95.0 ± 0.6	97.1 ± 0.7	96.8 ± 0.1	68.0 ± 3.0*
Early Apoptotic	0.7 ± 0.1	1.2 ± 1.0	1.2 ± 0.2	0.4 ± 0.1	0.7 ± 0.1	7.0 ± 2.0*
Late apoptotic and necrotic	1.4 ± 0.1	4.0 ± 2.0	3.2 ± 0.7	2.0 ± 0.6	2.0 ± 0.2	22.0 ± 3.0*

Values are expressed as mean ± SEM. Results are mean values of three independent experiments.

**p<0.05* compared with control by One way ANOVA followed by Dunnett's post hoc test.

3.5 MUC1 protein expression

We decided to evaluate the expression of the transmembrane protein MUC1 by western blot. Figure 4a1 shows that the expression of MUC1 diminished in cells lysates previously exposed to the higher concentrations of PCCVC ash (500 and 1000 µg/ mL). When the relative protein expression was calculated (Fig. 4b) we observed a decrease of 60% and 78% in MUC1 expression in PCCVC 500 and PCCVC 1000 groups, respectively (p<0.05). Lower concentrations of PCCVC ash (100 µg/ mL) or the highest concentration assayed for Calbuco ash (1000 µg/ mL) as is shown in Figure 4a2 did not alter the expression of MUC1 in IOBA-NHC cells.

3.6 Secretion of pro-inflammatory cytokines

The exposure of IOBA-NHC to the higher concentrations of PCCVC particles (500-1000 µg/ mL) resulted in a significant increased (p<0.05) of IL-6 and IL-8 release compared to the control group. Whereas the exposure of IOBA-NHC only at the highest concentration of Calbuco particles (1000 µg/ mL) used was able to increase (p<0.05) the release of IL-8 when compared to the control group (Fig. 5a-b).

3.7 Evaluation of ROS and RNS production

In order to evaluate ROS and RNS, we exposed IOBA-NHC to a low (50 µg/ mL) and a high (1000µg/ mL) concentration of ash particles. The intracellular generation of oxidizing species was determined with DCF-DA probe by flow cytometry. As shown in figure 6a cells were selected based on light-scattering properties (side scatter [SSC] versus forward scatter [FSC]) and 20,000 events per sample were acquired. Figure 6b shows that overlaid histograms of PCCVC groups displayed an increase in FL-1 DCF compared with the control group while, histograms of Calbuco groups, coincide with the control group histogram (Fig. 6c). The MFI of DCF increased 25% and 19% in cells exposed to 50 and 1000 µg/ mL of PCCVC ash, respectively (p<0.01, Fig. 6d). The increase in DCF oxidation indicates a rise of the intracellular ROS production in PCCVC groups. On the contrary, DCF fluorescence intensity of Calbuco groups did not differ significantly from control.

Nitrite level was used to assess NO production, as this is its final metabolite. An increase of nitrite levels was observed in PCCVC groups exposed to either 50 µg/ mL (183%, p<0.01) or 1000 µg/ mL (143%, p<0.01) comparing with control. Calbuco groups show a slight increase on nitrite concentration, still no significant with respect to the control group (Fig. 7).

3.8 Assessment of antioxidant defenses

The activity of antioxidant enzymes is shown in Table 2. SOD is a key enzyme in the detoxification of superoxide anion. In our experimental model, we found an increase in SOD activity for the cells incubated with 50 and 1000 µg/ mL PCCVC ash in comparison with the control group (29% and 39%, respectively, p<0.01). Whereas, we observed only an increase in this enzyme activity at the highest concentration of Calbuco ash employed (1000 µg/ mL, p< 0.05).

GSH, the principal intracellular low molecular weight antioxidant, was found increased in cells under PCCVC ash treatment, regardless the concentration used. No changes were observed in GSH levels

for the Calbuco treated groups when compared to controls (Table 2). GST activity remained unchanged in all the groups studied.

Table 2. Antioxidant defenses

	GSH (nmol/1 x 10^6 cells)	**SOD (U/mg protein)**	**GST (mU/mg protein)**
Control	2.06 ± 0.04	4.10 ± 0.06	7.00 ± 0.60
PCCVC 50	2.53 ± 0.08*	5.30 ± 0.30**	6.30 ± 0.50
PCCVC 1000	2.60 ± 0.10*	5.70 ± 0.06 ***	7.00 ± 1.00
Calbuco 50	2.30 ± 0.10	4.20 ± 0.20	5.20 ± 0.80
Calbuco 1000	2.50 ± 0.10	5.10 ± 0.20*	5.70 ± 0.40
DEP 100	1.70 ± 0.20**	10.3 ± 0.70***	12.0 ± 0.70*

Values are expressed as mean ± SEM. Results are mean values of three independent experiments. *$*p<0.05$ $**p<0.01$ $***p<0.001$* compared with control by One way ANOVA followed by Dunnett's post hoc test.

3.9 Protein and lipid oxidation

In order to assess protein oxidative damage, we measured carbonyl groups, one of the protein oxidation products. Higher levels of carbonyls were found in IOBA-NHC cells exposed to 1000 µg/ mL of PCCVC ash (*$p<0.05$*). No significant differences were observed in carbonyl content in cells cultured with the same concentration of Calbuco ash or with the lowest concentration of PCCVC particles (50 µg/ mL). Oxidative damage to lipids was determined as lipoperoxidation by TBARS. TBARS levels in cells with ash treatment were similar to control, therefore no oxidative damage to lipids was proven under these experimental conditions (Table 3).

Table 3. Protein and lipid oxidative damage markers

	Carbonyl content (nmol/mg protein)	TBARS (pmol MDA/ mg protein)
Control	4.9 ± 0.5	701.3 ± 53.9
PCCVC 50	4.3 ± 2.0	673.7 ± 37.4
PCCVC 1000	10.3 ± 2.0 *	784.7 ± 178.4
Calbuco 1000	9.1 ± 3.0	772.7 ± 130.9

Values are expressed as mean ± SEM. Results are mean values of three independent experiments.*$p<0.05$ compared with control by One way ANOVA followed by Dunnett's post hoc test.

4 Discussion

Ash produced during volcanic eruptions or remobilized from ashfall deposits could pose a hazard to the ocular surface as it is constantly exposed to environmental particles. The aim of this study was to evaluate the biological effect of volcanic ash particles on a human conjunctival cell line (IOBA-NHC). For this purpose, we collected ash particles in Villa la Angostura, a village that was severely affected by ash dispersal after the latest eruptions of the PCCVC (2011) and Calbuco volcano (2015). Ash particles from these volcanoes vary in size, composition, and morphology. We found that PCCVC ash particles range from less than 10 µm to 100 µm while, Calbuco particles present two defined size populations: one ranging from 100-300 µm and the other one including 10 µm or less than 10 µm particles. Regarding the chemical composition, PCCVC ash presented more SiO_2, Na_2O, and K_2O and less FeO, CaO, and MgO than Calbuco particles. These results are consistent with previous studies where PCCVC ash collected in Villa la Angostura showed a predominantly rhyolitic composition with oxide percentages similar to those found in this study (Botto et al., 2013; Canafoglia et al., 2012). Several studies found that Calbuco ash particles present a basaltic-andesitic composition and a bimodal grain size distribution with a population of 100-300 µm particles and fine ash of less than 10 µm in samples located between 80 and 150 km from the vent

(Reckziegel et al., 2016; Romero et al., 2016). Villa la Angostura is located at 100 km from Calbuco volcano and Scanning Electron Microscopy results from our samples reflect this tendency as we observe similar grain size populations to those mentioned above.

The Argentine Mining Geological Service (SEGEMAR) carried out a systematic monitoring of PM_{10} (particulate matter of aerodynamic diameter ≤ 10 μm) for 10 months starting in September 2011. The level of breathable aerosols was very high from September 2011 to January 2012 being considered of high to moderate risk for the human health (Elissondo et al., 2016). As no data was found regarding the concentration of total suspended particles in Villa la Angostura after the PCCVC eruption or the Calbuco eruption, we could not estimate the range of potential eye exposure concentrations. In consequence, we decided to perform our studies using urban particulate matter concentrations (50, 100, 500, and 1000 μg/ mL) similar to those used in other *in vitro* ocular surface experiments (Fujishima et al., 2013; Gao et al., 2016; Lasagni Vitar et al., 2018, 2015; Tau et al., 2013). The SEGEMAR team measured PM_{10} at an outdoor station during September 2011 and found that according to values established by law 24.585 (Argentine Environmental law on standard air quality, water and soil levels) the daily exposure limit (150 μg/ m^3) was highly exceeded. Some values reached peaks over 300 μg/ m^3 (Elissondo et al., 2016). We have not found published information regarding the air volume in contact with the ocular surface and in addition to a lack of information of the total suspended particles present after the eruption; it is hard to predict the real ash concentration that the ocular surface might have experienced. In addition, concentrations may also depend on weather conditions such as strong winds and absence of rainfall that would intensify ash resuspension. People's lifestyle would also influence on the final concentration estimated. Even though we are unable of calculating an exposure concentration, we believe that, as the higher peaks of PM_{10} found were around 300 μg/ m^3 , even considering the percentage of particles over 10 μm of diameter, this study would mimic to an overload situation, as we have used concentrations of greater orders of magnitude.

In the present study, we first evaluated if PCCVC or Calbuco ash particles affected IOBA-NHC cell proliferation and viability. Previous studies performed with volcanic ash from diverse sources on respiratory

cells demonstrated that ash particles induced a cytotoxic effect diminishing cell proliferation but cell viability remained unchanged (Damby et al., 2016; Wilson et al., 2000). Our results show that PCCVC ash at the two higher concentrations (500-1000 µg/mL) used in this study were able to reduced cell proliferation without affecting cell viability. In addition, cell morphology was not altered when cells were exposed to Calbuco or PCCVC particles. IOBA-NHC presented the typical polygonal morphology, the presence of nuclei with intensely stained nucleoli, and some mitotic figures as described by Diebold et al (2003).

Conjunctival cells play a central role in inflammatory processes and the release of several cytokines/chemokines contribute to inflammation (Contreras-Ruiz et al., 2013; Ghasemi, 2017). This study shows that the pro-inflammatory cytokine, IL-6 and the neutrophil chemoattractant IL-8, elicited increased levels in conjunctival epithelial cells exposed to high levels of PCCVC ash. The highest concentration of Calbuco ash induced only an increase on IL-8 levels. Lower concentrations of ash particles (50-100 µg/mL) did not promote a pro-inflammatory response. These result are consistent with other acute ash exposition studies performed on lung epithelial cells where no pro-inflammatory response was observed for volcanic ash particle concentrations of 50 or 100 µg/mL (Damby et al., 2016, 2013; Horwell et al., 2013).

In order to preserve vital cellular functions, it is necessary to maintain a continuous balance between pro-oxidant and antioxidant species. An increase in the pro-oxidant species such as ROS and RNS over the antioxidant defenses, result in oxidative/nitrosative stress (Bandyopadhyay et al., 1999; Kohen and Nyska, 2002; Rahal et al., 2014). Environmental pollution is considered a source of pro-oxidant species (Kohen and Nyska, 2002; Rahal et al., 2014). Previous studies have suggested that volcanic ash particles have also oxidative potential due to transition metal ions such as iron which is capable of generating free radicals (Cullen et al., 2002; C. J. Horwell et al., 2003a; Horwell, 2007). In this study we found a significant increase in ROS and RNS in conjunctival epithelial cells exposed to PCCVC ash at both concentrations evaluated.

It has been addressed that NO could play a central role in the development of the inflammatory response induced by a large variety of noxae. These biological responses include a direct effect where NO acts as an oxidant specie against the noxae and an indirect effect where it induces and regulates the inflammatory

response by immune cells (Erdinest et al., 2015). Therefore, NO is considered an important intracellular messenger which in turn could be used as an extracellular indicator of the intracellular redox status.

A well-established antioxidant system is essential to counterbalance the action of increased pro-oxidant species (Valko et al., 2007) as ROS and RNS could damage biological targets such as lipids, DNA, and proteins (Birben et al., 2012; Kohen and Nyska, 2002; Valko et al., 2007). We found in PCCVC groups an increase in SOD enzymatic activity. This enzyme catalyzes the superoxide anion dismutation to H2O2, an essential component in the signal transduction pathways that involve cell survival (Trachootham et al., 2008). The highest SOD level corresponded to PCCVC 1000 group where an inflammatory response mediated by IL-6 and IL-8 was confirmed. It has been demonstrated that SOD activity is positively modulated under a pro inflammatory environment (Fattman et al., 2003). Non-enzymatic antioxidants also play an important role in regulating the cell redox balance. GSH, the major low-molecular-weight thiol of the cell, is essential in order to maintain redox homeostasis as well as it is for several cell processes such as the regulation of cell proliferation and apoptosis, immune response, and detoxification (Lu, 2009; Shelly, C., Lu, 2014). GSH also act as a scavenger of hydroxyl radical, the initiator of lipid peroxidation chain reaction that is generated by the combination of H2O2 with iron or cupper metal ions by Fenton-Haber Weiss oxidative damage in the pro-oxidant environment triggered by ash particles at concentrations and exposure times evaluated in this study. However, we detected protein carbonylation in conjunctival cells exposed to 1000 µg/mL of PCCVC ash. It should be pointed out that low PCCVC dose induced oxidative stress where SOD activity and GSH levels were able to provoke an adaptive response, not impairing overall cell function and proliferation. On the contrary, PCCVC at the highest dose used in this study induced oxidative stress, but the antioxidant defenses were not sufficient to prevent protein oxidation and the inflammatory response, leading to reduced proliferation.

Conjunctival and corneal epithelial cells among other components produce mucins to protect and maintain the health of the ocular surface. Mucins are large and highly glycosylated glycoproteins that trap and remove debris and harmful pathogens from the external environment and facilitate lubrication of the ocular surface (Ablamowicz and Nichols, 2016; Dartt, 2004; Mantelli and Argüeso, 2008). We decided to evaluate the mucin MUC1, which is the membrane-associated mucin (MAM) most ubiquitously expressed by both

corneal and conjunctival epithelia (Govindarajan and Gipson, 2010) and is normally present on IOBA-NHC cells (Diebold et al., 2003). We found that the higher concentrations of PCCVC ash provoked a decrease on MUC1 expression. The differential MUC1 expression observed could be due to several factors including altered mucin gene transcription, mRNA translation to protein and/or post-translational protein modification (Dartt, 2004; Hodges and Dartt, 2013). It has been described that oxidative stress could produce a decrease in mucin expression in conjunctiva, deteriorating the tear film stability (Kojima et al., 2015). Moreover, an *in vitro* study on corneal epithelial cells showed that IL-6 downregulates MUC1 cellular protein without modifying mRNA expression (Albertsmeyer et al., 2010). In accordance with this hypothesis, we found an increase in IL-6 and IL-8 as well as a redox imbalance on conjunctival epithelial cells exposed to PCCVC ash.

According to Gipson and co-workers (Gipson et al., 2014), a decrease in the expression of MUC1 in corneal epithelial cells enhances its barrier function as it leaves a more uniform barrier formed by MUC16, which presents a larger ectodomain and is more glycosylated. As MUC-16 is also expressed in conjunctival epithelial cells, the decrease in the expression of MUC1 could be a conjunctival cell adaptive response to protect the ocular surface forming a more uniform barrier composed basically by MUC16. Further studies analyzing MUC16 should be performed in order to reveal its barrier function under our experimental conditions.

Our findings demonstrate that PCCVC particles induce a greater cytotoxic response on conjunctival cells in culture than Calbuco particles. We consider that some of these effects could be related to differences in particle size and composition between PCCVC and Calbuco ash. Calbuco ash presents a larger particle size range than PCCVC ash therefore, at an equal ash concentration less Calbuco particles would be present in the cell cultures. Consequently, there would be lesser Calbuco particles IOBA-NHC cells contact. It should also be considered that IOBA-NHC are capable of incorporating particles (Lasagni Vitar et al., 2015). Thus, it is possible that the ash effects we detected are at least in part due to the entrapped small-size ash. Additionally, the toxic effects could be also because of the soluble metals released into the culture medium, especially if ash particles are too big to be taken up by cells. This situation could explain the fact

that although Calbuco particles present more FeO than PCCVC particles, a greater oxidative environment was found in cells exposed to PCCVC ash. Further studies should be performed using centrifuged ash suspension and exposing the cells to supernatant only or employing similar ash particles sizes, in order to elucidate if the effects revealed in this study are in fact due mainly to the differential ash composition or particle size.

In addition, as the ocular surface is exposed directly to the environment, we believe that it would be interesting to study IOBA cells ash exposure using the novel nebulization method performed by Tomašek et al. (Tomasek et al., 2016). This method represents a more realistic scenario of ash exposure as volcanic ash is applied in its dry state directly onto the air-liquid interface of the cell culture.

To our knowledge, this is the first study that shows the cytotoxic effects of volcanic ash from different origin, size, and composition, on human conjunctival epithelial cells. Our results could provide a better understanding of the ocular symptoms manifested by people living near volcanic areas.

5 Conclusion

In summary, our results demonstrate that PCCVC but not Calbuco ash particles are cytotoxic for human conjunctival epithelial cells viewed as a decrease in cell proliferation; a differential expression of the transmembrane mucin MUC1; a pro-inflammatory response mediated by IL-6 and IL-8; and an imbalance of the redox environment leading to protein oxidative damage.

As we did not evaluate the mechanical damage that ash particles could provoke to the ocular surface, we believe that it would be necessary to perform experiments *in vivo* as well as epidemiological studies to accomplish a better understanding of the overall effects of volcanic ash onto the ocular surface.

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Figure legends

Figure 1. Representative Scanning Electron Microscopy images of PCCVC (a) and Calbuco (b) ash samples collected in Villa la Angostura. (c) Ash oxide composition of PCCVC and Calbuco ash estimated after EDX elemental analysis. Results are expressed as mean values ± SEM of total weight percentage of at least three ash samples. , *p<0.05, ** p<0.01, *** p<0.001 by t-test.

Figure 2. IOBA-NHC proliferation after the exposure to PCCVC or Calbuco measured by the MTT assay. Experimental control: cells cultured in culture media and positive control: cells exposed to 100 µg/ mL of DEP. Results are mean values ± SEM of n=3 independent experiments. **p<0.01 ***p<0.001 compared with control by One way ANOVA followed by Dunnett's post hoc test.

Figure 3. Cell morphology analyzed by light microscopy. (a) Control cells, (b) and (c) cells treated with PCCVC 500 and 1000 µg/ mL respectively. In (d) and (e) cells were exposed to Calbuco 500 and 1000 µg/ mL respectively. Black arrows show volcanic particles while hollow arrows show mitotic figures. Images are representative of all samples. Magnification of all images: 400x

Figure 4. Western analysis of cell lysates exposed to PCCVC or Calbuco ash. Control: cells in culture media. In (a1) lane 1 is the control group, lanes 2-4 are lysates from cells exposed to PCCVC ash (100, 500 and 1000 µg/ mL) while in (a2) line 1 is the control group and lane 2 lysate from cells exposed to Calbuco ash (1000 µg/ mL). Bands of approximately 200 kDa correspond to MUC1 and Actin (45 kDa) which was used as housekeeping. (b) Bars represent the relative protein level calculated by the ratio of MUC1/Actin. Data are expressed as the mean ± SEM of 3-5 independent experiments. * p<0.05 **p<0.01 compared with control by One way ANOVA followed by Dunnett's post hoc test.

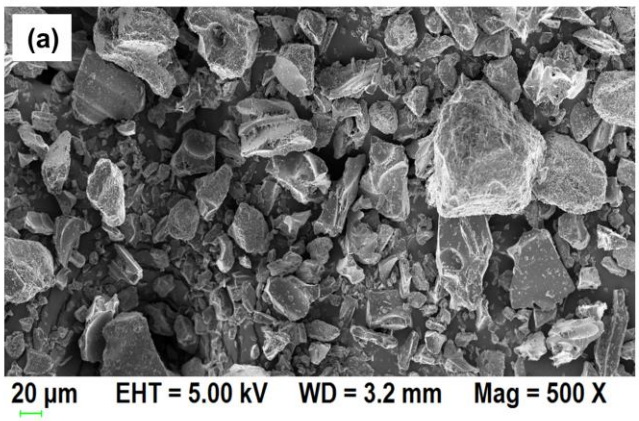
Figure 5. IL-6 (a) and IL-8 (b) released in supernatants of IOBA-NHC cells. Experimental control: cells cultured in culture media and positive control: cells exposed to 100 µg/ mL of DEP. Results are mean values ± SEM of n=3-5 independent experiments. *p<0.05 **p<0.01 ***P<0,001 compared with control by ANOVA followed by Dunnett's post hoc test.

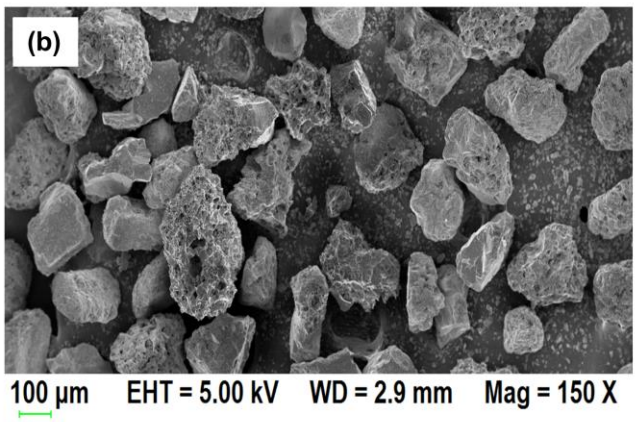
Figure 6. ROS production of IOBA-NHC. Experimental control: cells cultured in culture media and positive control: cells exposed to 100 µg/ mL of DEP. (a) Cells were selected based on light-scattering properties (SSC versus FSC) and 20,000 events per sample were acquired. (b) Overlaid histograms of PCCVC groups displayed an increase in FL-1 DCF compared with the experimental control group. (c) Histograms of Calbuco groups coincide with the control group histogram. (d) Dichlorofluorescein (DCF) fluorescence quantification. Results are expressed as the fold of increase; mean ± SEM of n=3 independent experiments. ** $p<0.01$ compared with control by One way ANOVA followed by Dunnett's post hoc test.

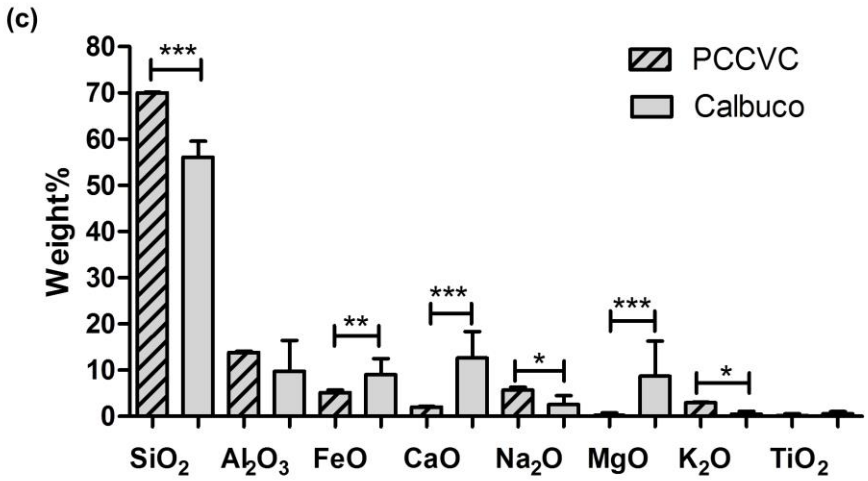
Figure 7. Nitrite concentration in cell culture supernatants of IOBA-NHC. Experimental control: cells cultured in culture media and positive control: cells exposed to 100 µg/ mL of DEP. Results are mean values ± SEM of n=3 independent experiments. ** $p<0.01$ *** $p<0.001$ compared with control by One way ANOVA followed by Dunnett's post hoc test.

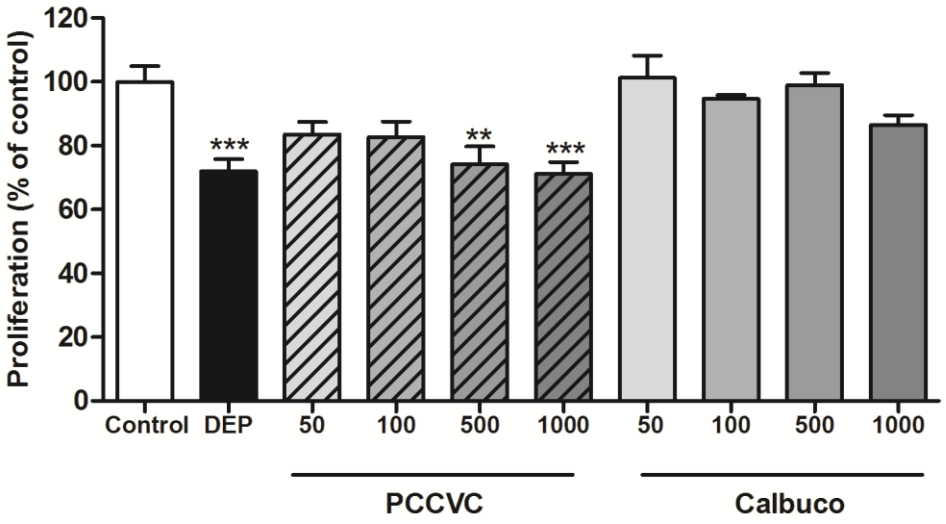
Highlights

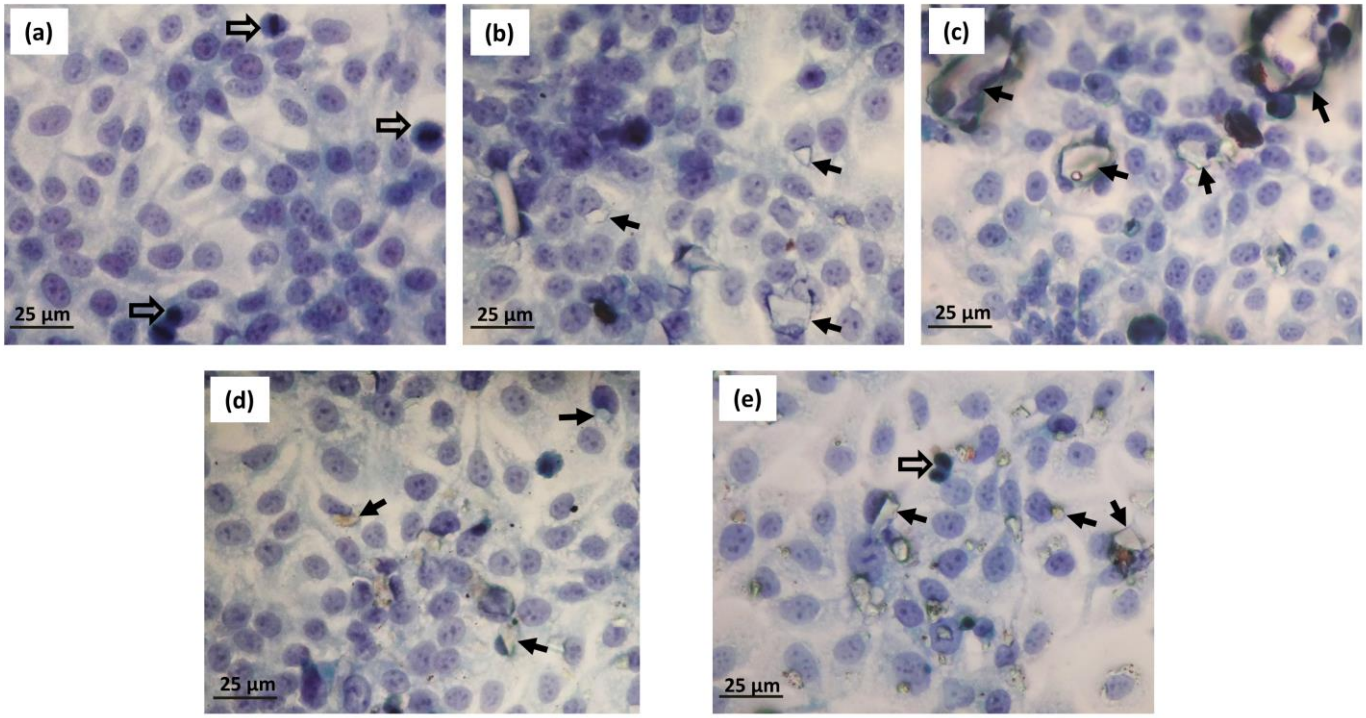
- Volcanic ash induces a redox imbalance on conjunctival epithelial cells
- Volcanic ash triggers a pro-inflammatory response mediated by IL-6 and IL-8
- MUC1 expression decreased on conjunctival cells exposed to volcanic ash

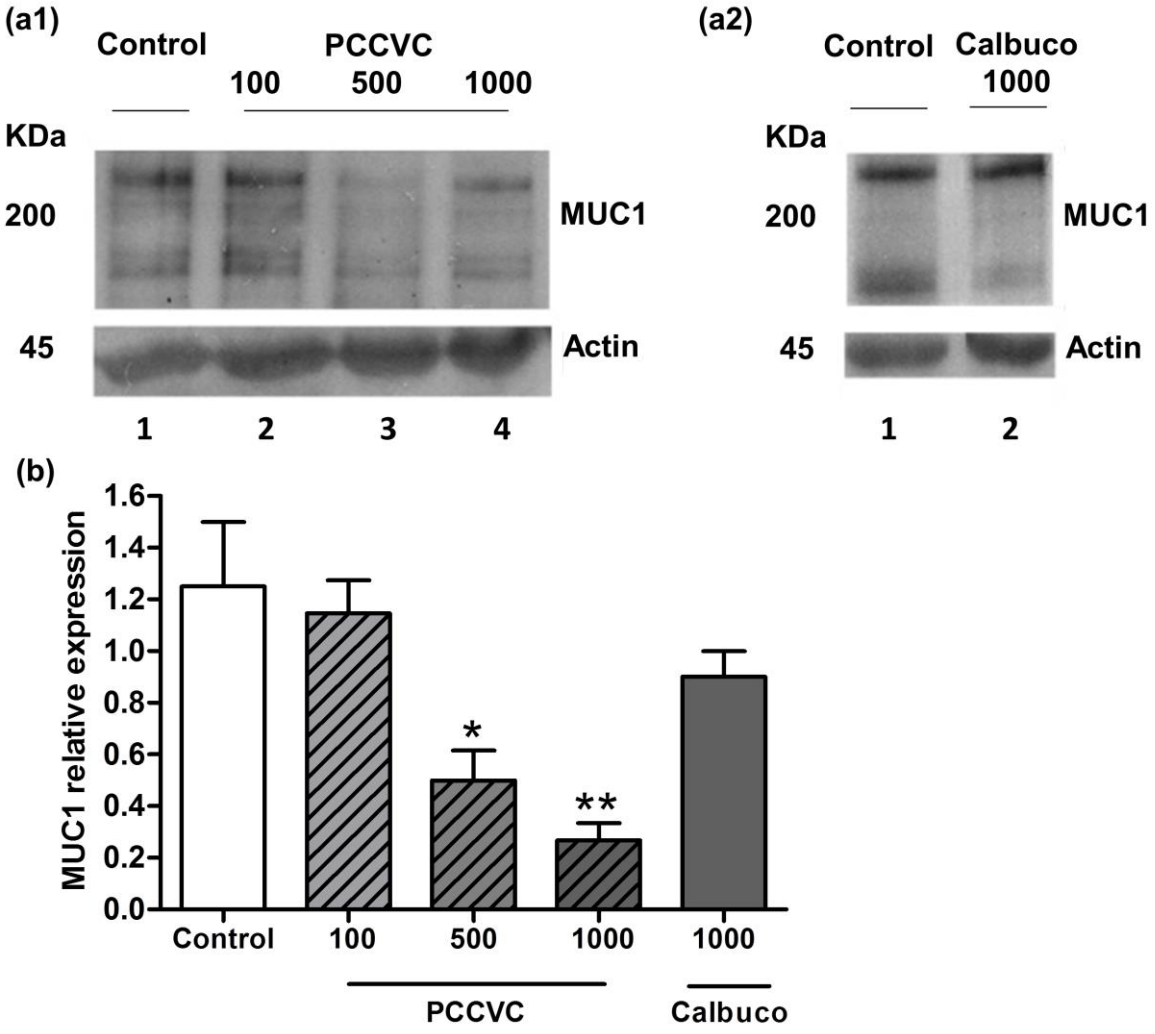

(a)
20 µm EHT = 5.00 kV WD = 3.2 mm Mag = 500 X

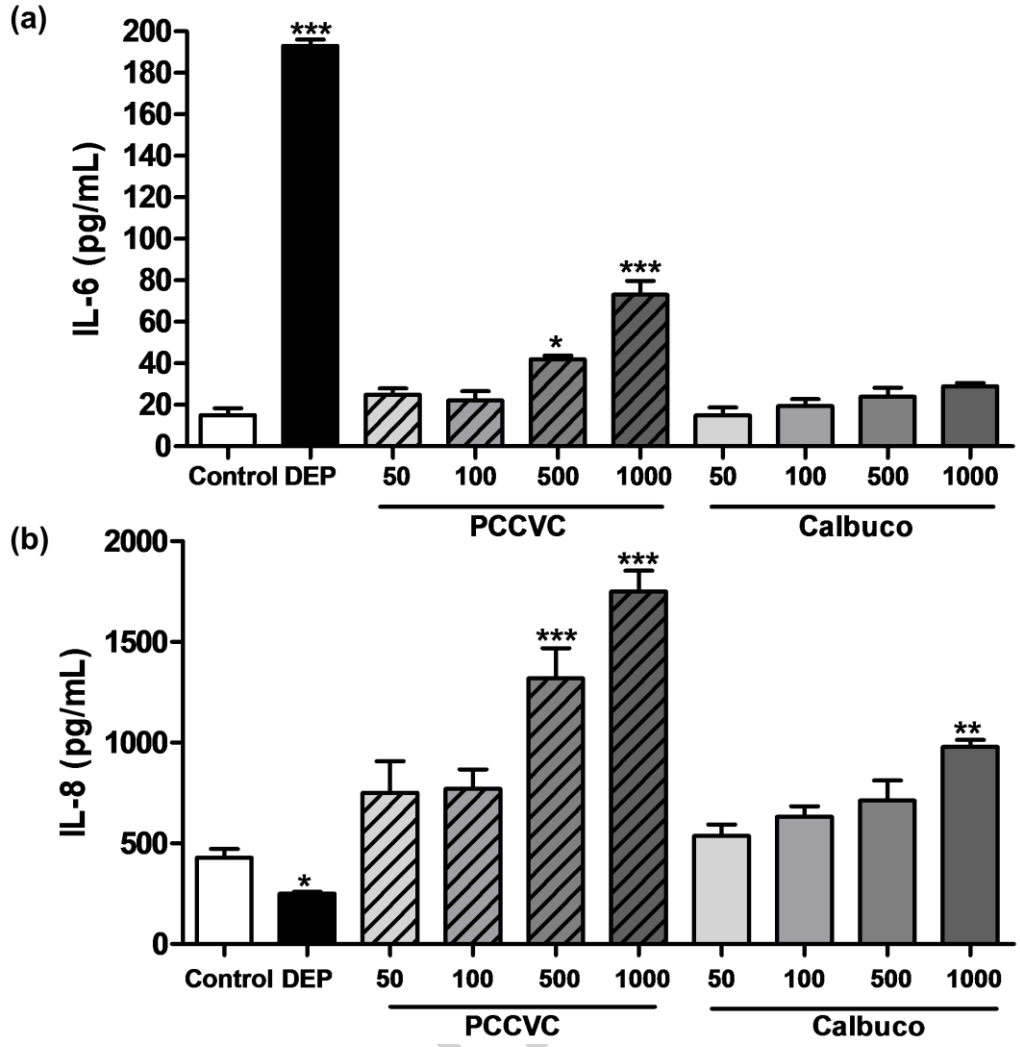

(b)
100 µm EHT = 5.00 kV WD = 2.9 mm Mag = 150 X

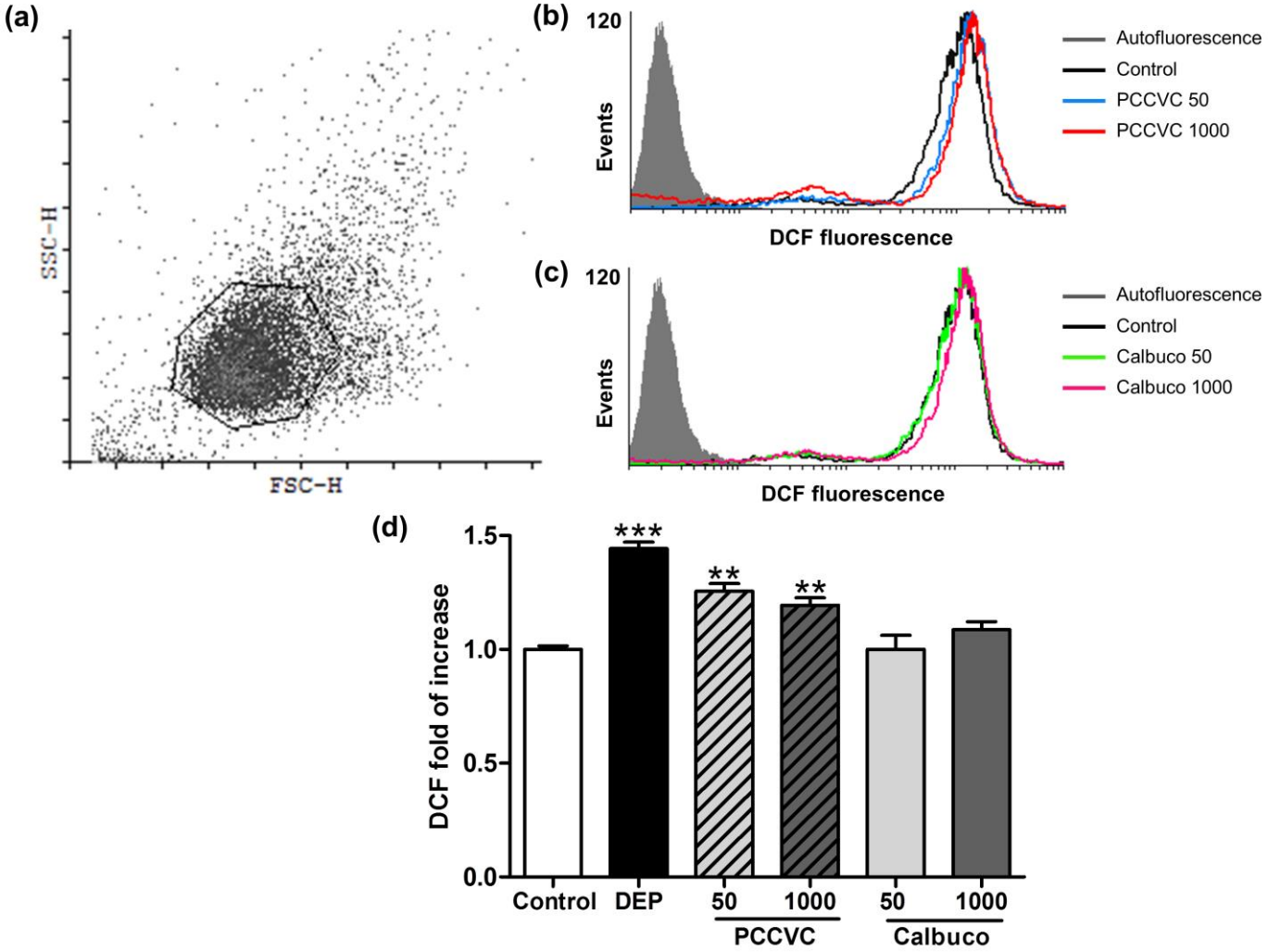

(c)
PCCVC
Calbuco
Weight%
80
70
60
50
40
30
20
10
0
***
**
***
*
***
*
SiO2
Al2O3
FeO
CaO
Na2O
MgO
K2O
TiO2

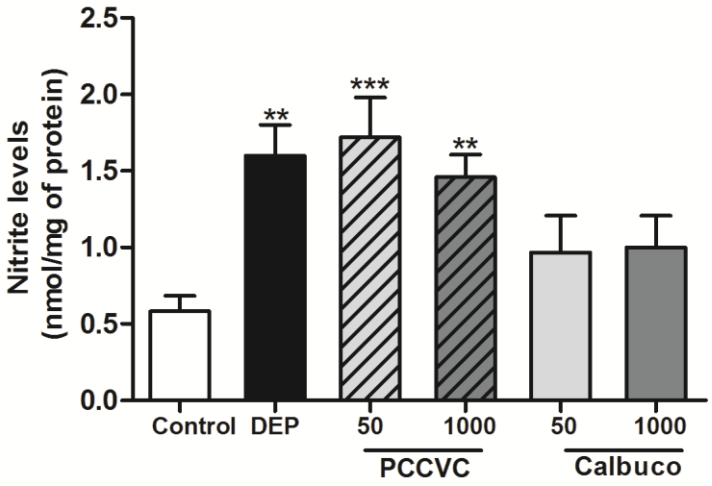

Proliferation (% of control)
120
100
80
60
40
20
0
***
**
***
Control
DEP
50
100
500
1000
50
100
500
1000
PCCVC
Calbuco






(a)
IL-6 (pg/mL)
200
180
160
140
120
100
80
60
40
20
0
***
*
***
Control DEP
50
100
500
1000
50
100
500
1000
PCCVC
Calbuco
(b)
IL-8 (pg/mL)
2000
1500
1000
500
0
*
***
***
**
Control DEP
50
100
500
1000
50
100
500
1000
PCCVC
Calbuco


(a)
SSC-H
FSC-H
(b)
120
Events
DCF fluorescence
Autofluorescence
Control
PCCVC 50
PCCVC 1000
(c)
120
Events
DCF fluorescence
Autofluorescence
Control
Calbuco 50
Calbuco 1000
(d)
DCF fold of increase
1.5
1.0
0.5
0.0
***
**
**
Control
DEP
50
1000
PCCVC
50
1000
Calbuco


Nitrite levels
(nmol/mg of protein)
2.5
2.0
1.5
1.0
0.5
0.0
**
***
**
Control
DEP
50
1000
PCCVC
50
1000
Calbuco