The Phytoalexin Resveratrol Ameliorates Ochratoxin A Toxicity in Human Embryonic Kidney (HEK293) Cells

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Abstract

Ochratoxin A (OTA) is a nephrotoxic mycotoxin produced by *Aspergillus* and *Penicillium* fungi. It contaminates human and animal food products, and chronic exposure is associated with renal fibrosis in humans (Balkan endemic nephropathy). Resveratrol, a phytoalexin, possesses anti-cancer and antioxidant properties. We investigated the mechanism of cellular oxidative stress induced by OTA, and the effect of resveratrol in human embryonic kidney (HEK293) cells over 24 and 48 h. Cells were exposed to OTA [IC50 = 1.5 μM (24 h) and 9.4 μM (48 h) determined using MTT assay] and 25 μM resveratrol. Glutathione was quantified by luminometry and gene expression of Nrf2 and OGG1 was determined by qPCR. Protein expression of Nrf2, LonP1, SIRT3, and pSIRT1 was assessed by Western blot, DNA damage (comet assay), and intracellular reactive oxygen species (flow cytometry). At 24 h, resveratrol increased mRNA expression of the DNA repair enzyme, OGG1 ($P < 0.05$), whereas OTA and OTA+resveratrol significantly decreased OGG1 expression ($P < 0.05$). OGG1 expression increased during 48-h exposure to resveratrol and OTA+resveratrol ($P < 0.05$). Comet tail lengths doubled in 48-h OTA-treated cells, whereas at both time periods, OTA+resveratrol yielded shorter comet tails ($P < 0.0001$). During 24- and 48-h exposure, OTA, resveratrol, and OTA+resveratrol significantly decreased mRNA expression of Nrf2 ($P < 0.05$). Luminometry analysis of GSH revealed an increase by OTA+resveratrol for 24 and 48 h ($P < 0.05$ and $P < 0.001$, respectively). Western blot analysis showed decreased Nrf2 protein expression during 24-h exposure, but increased Nrf2 expression during 48 h. LonP1 protein expression increased during 24-h exposure to OTA ($P < 0.05$) and OTA+resveratrol ($P < 0.0011$) and during 48-h exposure to resveratrol ($P < 0.0005$).

KEY WORDS: RESVERATROL; OCHRATOXIN A; OXIDATIVE STRESS; LONP1; HEK293 CELLS

Ochratoxin A (OTA) is a common contaminant of foods, such as grains, fruit, and animal feed [Marin-Kuan et al., 2008]. It is ubiquitously produced by fungal species of *Aspergillus* and *Penicillium*, specifically *Aspergillus ochraceus* [Schilter et al., 2005]. Rural populations in developing countries rely heavily on grains as a staple source of sustenance; many of these communities lack proper storage facilities for harvested grains; this leads to increased fungal contamination and potentially increased production of OTA [Bankole et al., 2006]. OTA was first discovered in South Africa (SA) in 1965 (van der Merwe et al., 1965) and has since been found in food sources around the world [Jonsyn-Ellis, 2012]. Although first discovered in SA, there is still little documented information on OTA mycotoxicosis in SA. Understanding the mechanism of OTA toxicity is particularly important for African countries as grains, such as cassava and maize, contribute to a large part of the daily sustenance for a high percentage of the population [Jonsyn-Ellis, 2012].

Microorganisms in the gastrointestinal tract (GIT) hydrolyze OTA to the less toxic by-product, Ochratoxin α (OTα) [Hohler et al., 1999], which is then excreted via the liver and kidney [Cheng et al., 2013]. Exposure to high levels of OTA has been linked to renal dysfunction and tumourigenesis in humans [Özçelik et al., 2004]. OTA is nephrotoxic and results in destruction of the renal tubular epithelium, causing progressive renal failure [Pfohl-Leszkowicz

and Manderville, 2007]. This disease is particularly prominent in the Balkan regions, such as Bulgaria and Romania, and is known as Balkan endemic nephropathy (BEN) [Pfohl-Leszkowicz and Manderville, 2007]. OTA exerts its toxic effects via a number of mechanisms, the most prominent method is by alteration of the antioxidant potential of the cell [Meyer et al., 2013], resulting in oxidative stress induction, and increased reactive oxygen species (ROS) [Schaaf et al., 2002]. During oxidative stress, nuclear factor-erythroid 2-related factor 2 (Nrf2) dissociates from its inhibitor, Kelch-like ECH-associated protein 1 (Keap-1), translocates into the nucleus, and binds to the antioxidant response element (ARE), resulting in the transcription of proteins associated with the antioxidant defence system [Nguyen et al., 2009]. Therefore, we aimed to investigate the interactions of both OTA and resveratrol with the Nrf2 antioxidant defence system in cultured human kidney cells.

Resveratrol (3, 4′, 5-trihydroxy-trans-stilbene) is a polyphenol found in the skin of injured grapes and some fruit, and is, therefore, commonly present in wine and grape juice [Athar et al., 2007]. Resveratrol, synthesized by plants as a defensive mechanism, is proposed to have antimicrobial, antioxidant, anti-carcinogenic, and anti-inflammatory properties [Athar et al., 2007]. This compound is widely studied and holds great potential in disease treatment. Resveratrol possesses free radical scavenging potential, contributing to its antioxidant capacity [Borra et al., 2005]. The phytoalexin is also known to activate sirtuin 1 (SIRT1), a NAD^+-dependent deacetylase known for its ability to modulate transcription of proteins that augment the oxidative stress response, as well as proteins that promote cell survival in times of stress [Borra et al., 2005; Timmers et al., 2012]. These attributes could contribute to the compound's chemo-preventive ability, as well as the ability to diminish cellular damage due to oxidative stress [Baur and Sinclair, 2006]. Both resveratrol and OTA have been found in grapes; therefore, it is important to have an understanding of the dynamic between these two compounds. OTA exposure is widespread throughout Europe, through the ingestion of grape products, such as wine and juice. This is especially prominent in the grape-producing regions of Italy [Visconti et al., 2008]

The mechanisms of OTA toxicity and the cellular antioxidant response are relatively unknown. This study investigated the cytotoxic effects of OTA on HEK293 cells after 24- and 48-h exposure. Further, the therapeutic effects of resveratrol in both untreated and OTA-treated HEK293 cells were investigated. We believe these results will provide valuable insight into OTA toxicity in human kidney cells, and whether resveratrol can be used as a potential treatment to combat this toxicity.

Materials

Tissue culture consumables were purchased from Whitehead Scientific (Johannesburg, South Africa). The HEK293 cells were obtained from Highveld Biological (Johannesburg, South Africa). Ochratoxin A and resveratrol were purchased from Sigma–Aldrich (St Louis, MO). Methylthiazoltetrazolium (MTT) salt, phosphate buffered saline (PBS) tablets, and agarose were purchased from Capital Laboratory Supplies (Johannesburg, South Africa). The bicinchoninic acid (BCA) assay kit

and tris-chloride were purchased from Sigma–Aldrich. Kits and reagents used for luminometry (GSH-Glo™ Glutathione Assay kit) were purchased from Promega (Madison, USA). Reagents for qPCR were purchased from Bio-Rad (Hercules, CA) and the primer sequences were purchased from Inqaba Biotechnologies (Pretoria, South Africa). Western blotting reagents (Laemmli sample buffer and chemiluminescent reagents) were obtained from Bio-Rad. Primary antibodies (rabbit anti-Nrf2 (8882), rabbit anti-SIRT3 (C73E7), and rabbit anti-pSIRT1 (2314L) were purchased from Cell Signaling Technology (Danvers, MO). Rabbit anti-LonP1 (HPA002192-100UL) was purchased from Sigma-Aldrich (St. Louis, MO) and horse-radish peroxidase (HRP)–conjugated secondary antibodies (anti-rabbit IgG) were purchased from Cell Signaling Technology. Proteins were normalized to anti-β-actin (A3854) from Sigma–Aldrich. All other solvents and salts were purchased from Merck Chemicals (Johannesburg, South Africa). Protein isolation was conducted using Cytobuster™ (Novagen), supplemented with protease inhibitors (Roche 05892791001) and phosphatase inhibitors (04906837001).

Methods

CELL CULTURE

HEK293 cells were cultured in monolayer (10^6 cells per $25\,cm^3$ culture flask). The cells were maintained in complete culture medium [CCM: Dulbecco's minimum essential media (DMEM), 10% fetal calf serum, 1% penstrepfungizone, and 1% L-glutamine] and incubated overnight at 37 °C in a humidified incubator supplied with 5% CO_2. Cells were washed with 0.1 M PBS. Once flasks were confluent, cells were treated, removed by trypsinisation and agitation, and counted using the trypan blue exclusion assay.

OCHRATOXIN A TREATMENTS

A stock solution of 200 μM OTA was prepared in 60% dimethyl sulphoxide (DMSO). Cells were treated with a range (0.25–50 μM) of concentrations to obtain a half maximal inhibitory concentration (IC_{50}) for 24- and 48-h incubation times.

METHYLTHIAZOL TETRAZOLIUM (MTT) ASSAY

Cells were seeded into a 96-well microtiter plate (15,000 cells/well) and allowed to attach overnight. Attached cells were treated with a range of OTA concentrations (0.25–50 μM) for 24 and 48 h. Treatment media was removed and replaced with CCM (100 μl) and 20 μl MTT salt solution (5 mg/ml in PBS). Cells were incubated with the MTT salt solution for 4 h, thereafter the MTT solution was discarded and DMSO (100 μl) was added; the cells were incubated with DMSO for 1 h. Absorbance was read using a Bio-Tek MQx200 spectrophotometer at 595 nm (reference wavelength 655 nm). An inhibitory concentration at which 50% of cell growth was inhibited (IC_{50}) was calculated using GraphPad Prism statistical software (version 5).

RESVERATROL TREATMENTS

Stock solutions of 10 mM resveratrol were prepared in 100% DMSO. Preliminary assays were carried out to determine the optimal resveratrol concentrations for all subsequent treatments. Cells were treated with 25 μM, 50 μM, and 100 μM resveratrol during pre-,

post- and co-OTA treatments for 24 h, respectively. Flow cytometry was used to observe which resveratrol concentration greatly decreased the percentage of intracellular ROS, whereas luminometry was used to detect the levels of intracellular glutathione (GSH), thus indicating which concentration provided a more robust defence against oxidative damage. Based on these results, 25 μM resveratrol was used for all subsequent assays.

CELL PREPARATION FOR ASSAYS

Cells were incubated with the treatments for 24 and 48 h. Treatments included a control (CCM containing the solvent vehicle, DMSO), the IC_{50} values of OTA, a resveratrol control (CCM containing 25 μM resveratrol), and a co-treatment (IC_{50} values of OTA and 25 μM resveratrol in CCM). Cells were removed by trypsinization and agitation, the trypan blue method of cell counting was employed to determine cell viability and number as required per assay performed. The control was used for statistical comparison to all other treatments.

GLUTATHIONE ASSAY

The GSH assay [per GSH-Glo™ glutathione assay protocol (Promega)] was used to determine the concentration of GSH in HEK293 cells. Cells (10^4 cells/well) were seeded in a luminometry plate in triplicate. Standards (0–50–μM) were added in triplicate to generate a standard curve of known GSH concentrations. The GSH-Glo™ reagent (50 μl) was added to each well; the plate was agitated for 30 s, and incubated in the dark (RT, 30 min). The luciferin detection reagent (100 μl) was added to each sample and incubated as previously described (RT, 15 min). The plates were read on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, CA). The GSH concentrations were determined by extrapolation from the standard curve.

SINGLE CELL GEL ELECTROPHORESIS (SCGE) ASSAY

Cells were incubated with the treatments for 24 and 48 h, thereafter cells were removed by trypsinization and agitation, then counted; 15,000 cells were removed and resuspended in 25 μl PBS. Low melting point agarose (LMPA) was used to prepare the gels. The first gel layer (700 μl 2% LMPA) was formed on a frosted microscope slide, covered with a coverslip, and incubated at 4 °C (10 min). Thereafter, the coverslip was removed, a second gel layer (including 1.5 μl GR red dye, 25 μl cell suspension (10^4 cells) in 175 μl 1% LMPA) was added, coverslips were placed over slides, and incubated as above. Coverslips were removed and a third layer was added (1% LMPA, 200 μl) onto the second. Once the third layer solidified, coverslips were removed, slides were submerged in an ice-cold cell lysing solution [10 mM Tris (pH 10), 1% Triton X-100, 2.5 M NaCl, 100 mM EDTA, and 10% DMSO), and incubated in the dark (4 °C, 1 h). The lysing solution was removed, slides were placed in an electrophoresis tank filled with electrophoresis buffer (300 mM NaOH and 1 mM Na$_2$EDTA), and allowed to equilibrate for 20 min. The electrophoresis tank was sealed and a constant voltage was applied (25 V, 35 min). After electrophoresis, slides were washed thrice (5 min each) with neutralization buffer (0.4 M Tris, pH 7.4). Slides were viewed using an Olympus IX5I inverted fluorescent microscope (510–560 nm excitation, 590 nm emission filters) using analySIS Image Processing Software (Novell). Approximately 50 comets per treatment (three replicates) were counted and analyzed by measuring tail length (μm).

RNA EXTRACTION

RNA was isolated following an in-house protocol using Trizol. Trizol reagent (500 μl) was added to each flask and incubated (4 °C, 10 min). Cells were removed from flasks, transferred to 1.5 ml eppendorfs, and stored in Trizol at -80 °C overnight. Chloroform (100 μl) was added to thawed samples and incubated (RT, 3 min). Thereafter, cell suspensions were centrifuged (12,000g, 4 °C, 15 min), the aqueous phase removed, 250 μl isopropanol was added, and samples were left overnight at -80 °C. Samples were thawed and centrifuged (12,000g, 4 °C, 20 min). The supernatant was discarded, the pellet was retained and washed with 75% cold ethanol (500 μl), and centrifuged (7,400g, 4 °C, 15 min). Ethanol was removed and samples were allowed to air dry. The pellet was resuspended in nuclease-free water (15 μl) and incubated (RT, 3 min). The RNA was quantified using the Nanodrop2000 spectropho-tometer (Thermo-Scientific) and the A$_{260}$/A$_{280}$ ratio was used to assess the RNA integrity. The concentration of RNA was standardized to 2,500 ng/μl and used to prepare cDNA using the iScript cDNA synthesis Kit (Bio-Rad) as per manufacturer's instructions (4 μl 5× iScript reaction mix, 1 μl iScript reverse transcriptase, 11 μl nuclease-free water, and 4 μl of each RNA sample).

QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

Gene expression was analyzed using the iScript SYBR Green PCR kit (Bio-Rad), according to the manufacturer's instructions. The final reaction volume totaled 25 μl (12.5 μl SYBR Green, 1 μl forward primer, 1 μl reverse primer, 9 μl nuclease-free water, and 1.5 μl cDNA sample). All primers were obtained from Inqaba Biotec. The mRNA expressions of oxoguanine glycosylase 1 (OGG1) (forward 5′-GCATCGTACTCTAGCCTCCAC-3′; reverse 5′-AGGACTTTGCTCCCTC-CAC-3′) and Nrf2 (forward 5′-AGTGGATCTGCCAACTACTC-3′; reverse 5′-CATCTACAAACGGGAATGTCTG-3′) were investigated. This assay was carried out using three replicates per treatment and β-actin (forward 5′-TGACGGGGTCACCCACACTGTGCCCAT-3′; reverse 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′) as a house-keeping gene. The initial denaturation occurred at 95 °C (4 min). This was followed by 37 cycles of denaturation (95 °C; 15 s), annealing (40 sec; OGG1−60 °C; Nrf2−58 °C), and extension (72 °C; 30 s). The method described by Livak and Schmittgen was employed to determine the changes in relative mRNA expression, where $2^{-\Delta\Delta Ct}$ represents the fold change observed in mRNA expression [Livak and Schmittgen, 2001].

SDS –POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS–PAGE) AND WESTERN BLOTTING

Crude protein was isolated from cells using Cytobuster™ (Novagen) supplemented with protease inhibitors (Roche 05892791001) and phosphatase inhibitors (04906837001). Cytobuster reagent (250 μl) was added to flasks following treatments; the cells were scraped and transferred to a 1.5 ml tube and incubated on ice (10 min). The cell solution was centrifuged (10,000g, 4 °C, 5min). The supernatant was used for protein quantification using the bicinchoninic acid (BCA) assay. Bovine serum albumin (BSA) standards (0–1 mg/ml) were prepared. Samples and standards (25 μl) were pipetted into a 96-well

microtiter plate in duplicate. The BCA reagent was prepared (198 μl BCA: 4 μl CuSO$_4$ per reaction) and 200 μl of this working solution was added to each well. After incubation (37 °C, 30 min), the absorbance was read on a Bio-Tek MQx200 spectrophotometer at 562 nm. A standard curve was constructed and proteins were standardized to 1 mg/ml. Laemmli buffer [dH2O, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue] was added to each sample (1:1, total volume 200 μl), and heated to 100 °C (5 min). Samples were separated on SDS polyacrylamide gel (7.5% resolving gel and 4% stacking gel) for 1 h at 150 V, and transferred onto nitrocellulose membranes using the Transblot$^®$ Turbo^{TM} Transfer system (Bio-Rad). Membranes were blocked with 3% BSA in Tris-buffered saline [TTBS, 25 mM Tris (pH 7.6) 150 mM NaCl, 0.05% Tween 20] for 30 min, and incubated with a primary antibody (1:1,000, 3% BSA in TTBS at 4 °C overnight). Antibodies used were anti-Nrf2 (8882), anti-phospo SIRT1 (2314L), anti-SIRT3 (C73E7), and anti-LonP1 (HPA002192-100UL). Protein concentrations were standardized to anti-β-actin (Sigma–Aldrich A3854). After incubation, the primary antibody was removed, membranes were washed thrice with TTBS (10 min), and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (SantaCruz Biotechnology, Inc.) in 3% BSA (1:10,000) for 1 h. Following incubation, membranes were washed with TTBS. Clarity Western ECL Substrate (Bio-Rad) (400 μl) was added to the membranes and images were captured using a gel documentation system (UviTech Alliance 2.7). The membranes were then quenched with hydrogen peroxide, incubated in a blocking solution (3% BSA, 1 h, RT), rinsed twice in TTBS, and probed with HRP-conjugated anti-β-actin (house-keeping protein) (Sigma). Densitometry analysis was performed using the UviTech Analysis software and protein expression was read as relative band intensity (Arbillaga et al., 2007). Protein expression was reported as RBI of the protein of interest divided by the RBI of the loading control.

FLOW CYTOMETRY – INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS)

Treated cells were removed and counted, 500,000 cells were required per treatment. Cells were incubated in phenol red-free media, supplemented with 10% FCS and 10 μM dichlorodihydrofluorescein diacetate (DCFDA), in the dark (45 min, 37 °C). Thereafter, cells were rinsed thrice with 0.1 M PBS and centrifuged (400g, RT, 5 min), then suspended in 150 μl 0.1 M PBS and analyzed using a BD Accuri^{TM} flow cytometer. Live cells were gated and analyzed using CFlow Plus Software (BD Biosciences).

STATISTICAL ANALYSIS

Statistical analyses were performed using a one-way analysis of variance (ANOVA) test and the Students' t-test, using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). All data are expressed as mean ± standard error. All assays were run in triplicate and differences were considered statistically significant at values of $^*P < 0.05$.

Results

MTT ASSAY

The MTT assay was used to determine the IC$_{50}$ value of OTA during 24-h incubation (1.5 μM) and 48-h incubation (9.4 μM) in HEK293 cells (Fig. 1). The IC$_{50}$ values were used for all subsequent assays.

RESVERATROL DECREASES INTRACELLULAR ROS PRODUCTION DURING 24–H EXPOSURE

Flow cytometry was used to measure intracellular ROS production in HEK293 cells. Resveratrol significantly reduced intracellular ROS in the kidney cells from 47.7% to 20.2% (Fig. 2A; $P < 0.0001$). Both the OTA (25.3%) and OTA+resveratrol (28.9%) treatments significantly reduced ROS when compared to control cells (47.7%) (Fig. 2; $P = 0.0048$). The opposite was observed during the 48-h exposure, where all treatments increased the intracellular ROS when compared to control cells. A significant increase was observed when cells were exposed to OTA+resveratrol over 48 h (Fig. 2; $P = 0.0002$).

ASSESSMENT OF DNA DAMAGE AND REPAIR

OTA-treated cells (24 h) had significantly longer comet tails (44.82 ± 1.516 μm; $P < 0.0001$) as compared to the control cells (Fig. 3B). In contrast, resveratrol significantly decreased comet tail lengths compared to all other treatments (11.69 ± 0.695 μm; $P < 0.0001$). Interestingly, cells co-treated with OTA+resveratrol also showed significantly decreased comet tail lengths as compared
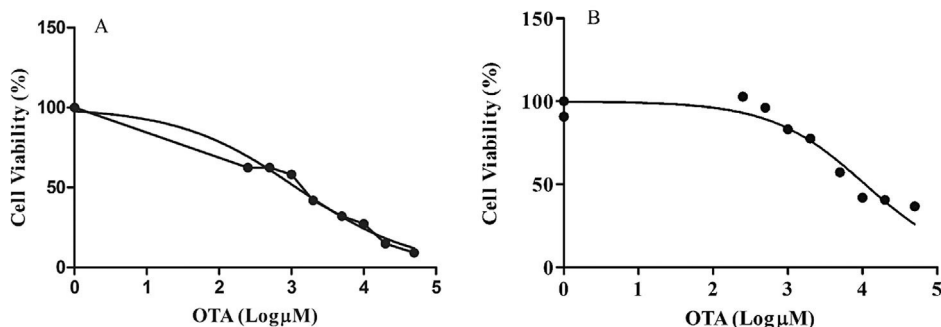

Fig. 1. Percentage viability of cells exposed to OTA over (A) 24 h and (B) 48 h. An IC$_{50}$ of 1.5 μM (24 h) and 9.4 μM (48 h) was calculated from the dose–response curves.
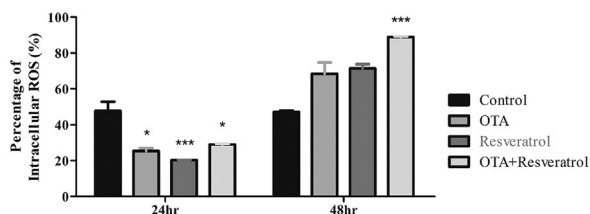
Fig. 2. Assessment of the percentage of intracellular ROS in cells exposed to OTA and resveratrol over 24 and 48 h. Resveratrol significantly decreased the percentage of intracellular ROS ([***]$P < 0.0001$), whereas OTA and OTA+resveratrol treatments decreased the values of ROS when compared to control cells during 24-h exposure ([*]$P = 0.0048$). Upon 48-h exposure, OTA+resveratrol significantly increased the ROS values ([***]$P = 0.0002$), whereas the OTA and resveratrol treatments also increased intracellular ROS.
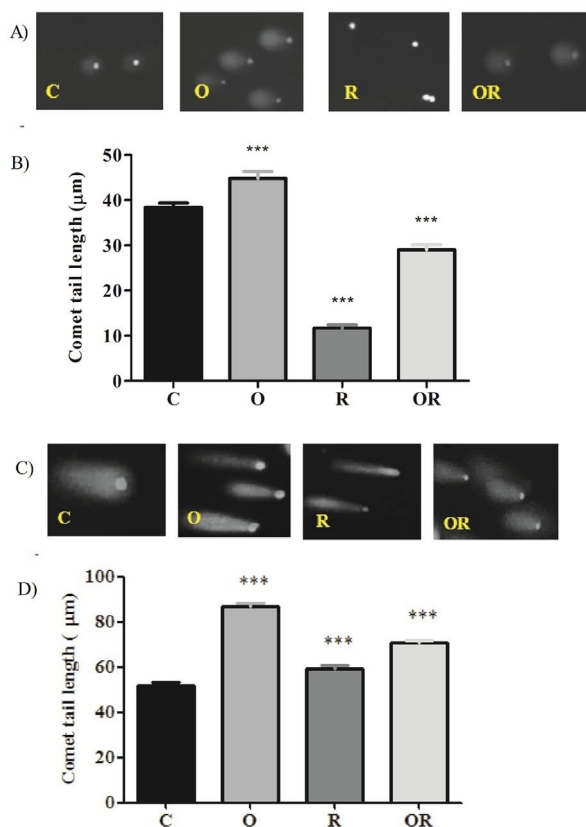

Fig. 3. Assessment of DNA damage showing images of (A) comet tails during 24-h exposure, (B) the measurement of comet tail lengths during 24-h exposure, (C) comet tails during 48-h exposure, and (D) the measurement of comet tail lengths during 48-h exposure. OTA increased comet tail lengths during both exposure time periods ([***]$P < 0.0001$). Comet tail lengths doubled during the 48-h exposure. The OTA+Resveratrol co-treatment significantly decreased lengths compared to cells only treated with OTA ([***]$P < 0.0001$).

to cells only exposed to OTA (Fig. 3B; $29.03 \pm 1.110\,\mu m$ vs. $44.82 \pm 1.516\,\mu m$; $P < 0.0001$). These observations were similar for the 48-h exposure period; however, the comet tails almost doubled in length. OTA-treated cells (48 h) had significantly longer comet tails ($86.67\,\mu m$) as compared to the 24-h treatment ($44.82\,\mu m$) (Fig. 3D). In addition, the comet tails were brighter and longer in OTA-treated cells, indicating that more DNA activity and increased DNA strand breaks occurred with increased exposure. The co-treatment (OTA+resveratrol) significantly decreased comet tail lengths compared to OTA-treated cells (Fig. 3D; $70.72 \pm 1.030\,\mu m$ vs. $86.67 \pm 1.694\,\mu m$; $P < 0.0001$).

MEASUREMENT OF GSH CONCENTRATIONS

Intracellular concentration of GSH was determined using luminometry. GSH concentrations in HEK293 cells were significantly increased by OTA+resveratrol treatment as compared to OTA-treated cells ($P < 0.05$); however, this concentration doubled after 48-h exposure ($P < 0.001$) (Fig. 4). The co-treatment of cells with OTA+resveratrol yields increased concentrations of GSH than in cells exposed to OTA alone. Generally, the GSH concentrations are far higher over 48-h exposure times than 24-h exposures (Fig. 4). The concentration of GSH was seen to decrease in OTA-treated cells compared to controls, thus indicating that longer exposure to OTA could deplete the antioxidant defence of the cells, allowing ROS to overrun the cell.

QPCR ANALYSIS OF ANTIOXIDANT RESPONSE

The mRNA expression of Nrf2 and OGG1 was assessed by qPCR. The expression of Nrf2, an antioxidant defence regulator, was significantly decreased by OTA, resveratrol, and OTA+resveratrol after 24-h exposure (Fig. 5A; $P < 0.05$); Nrf2 expression decreased further after 48 h (Fig. 5B). Resveratrol increased the expression of OGG1 ($P < 0.05$), whereas the co-treatment significantly decreased its expression after 24 h (Fig. 5C; $P < 0.05$). The co-treatment, however, significantly increased OGG1 expression when measured after 48 h (Fig. 5D; $P < 0.05$).

WESTERN BLOTTING

The protein expression of Nrf2, LonP1, SIRT3, and pSIRT1 was determined using Western blot. Nrf2 protein expression was
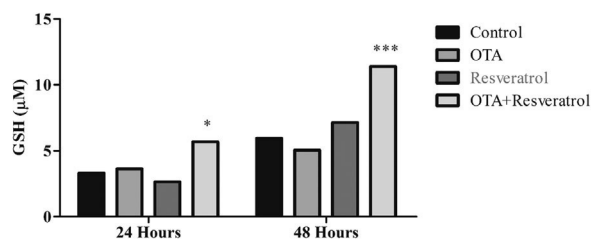

Fig. 4. Concentrations of GSH in HEK293 cells after exposure to OTA and resveratrol. The OTA+resveratrol co-treatment increased the concentrations of GSH during the 24-h exposure compared to GSH present in OTA-treated cells ([*]$P < 0.05$). The co-treatment also increased GSH during the 48-h exposure ([***]$P < 0.001$).
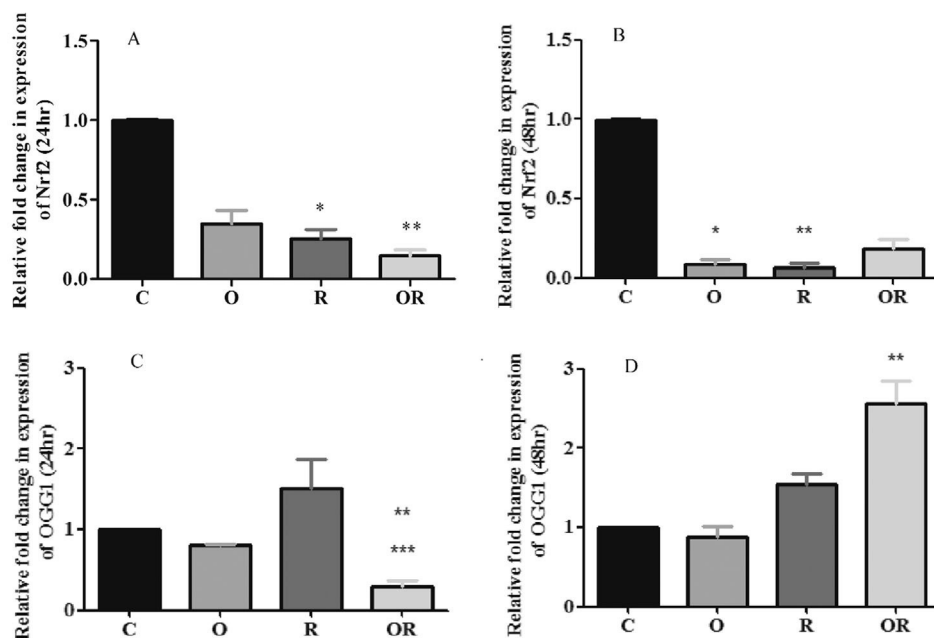
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Fig. 5. Analysis of the mRNA expression of genes associated with cellular protection. Nrf2 expression was seen to decrease upon exposure to OTA, resveratrol (*$P < 0.05$), and OTA+Resveratrol (**$P = 0.001$) for 24 h. (B) The expression of Nrf2 was seen to decrease further when exposed to OTA (*$P < 0.05$) and resveratrol (**$P = 0.0021$) for 48 h. The DNA glycosylase enzyme, OGG1, was observed to increase during 24-h resveratrol exposure and significantly decrease during exposure to OTA+resveratrol (**$P < 0.05$) for 24 h. (C) OGG1 expression was significantly decreased during OTA+resveratrol exposure compared to resveratrol-treated cells (***$P = 0.0002$).(D) Recovery of OGG1 expression was observed during 48-h exposure to OTA+resveratrol (**$P < 0.05$).

decreased by all treatments after 24-h exposure, significantly by OTA+resveratrol ($P < 0.05$), but increased by OTA ($P < 0.05$) and resveratrol ($P < 0.0008$) after 48 h. An inverse relationship was found to exist between SIRT3 and LonP1 after 24 h, SIRT3 decreased and LonP1 increased. Resveratrol increased SIRT3 ($P < 0.05$) and LonP1 ($P = 0.0005$) expression (48 h), and pSIRT1 at both time periods ($P = 0.0002$ and $P = 0.0054$, respectively) (Fig. 6).

Discussion

OTA is a mycotoxin that frequently contaminates a wide range of foods. Once ingested, OTA exerts its toxic effects via several pathways. One such pathway in the kidney is increased oxidative stress, resulting in DNA damage and progressive renal failure in individuals who chronically ingest foods contaminated with high concentrations of OTA [Pfohl-Leszkowicz and Manderville, 2007]. OTA poses a health risk to populations in developing countries, as they depend largely on agricultural crops often heavily contaminated with fungi, such as *Aspergillus* and *Penicillium* [Castegnaro et al., 2006]. OTA has been identified as an etiological agent in BEN [Castegnaro et al., 2006]. The exact mechanism of OTA toxicity is still relatively unknown, although many mechanisms have been proposed. The general trend of reduced antioxidant defense capacity has been documented in different cell lines, such as the normal rat kidney (NRK) epithelial cells [Schilter et al., 2005; Cavin et al., 2007].

In this study, we provide insight into the biochemical changes induced by OTA in human embryonic kidney (HEK293) cells.

Resveratrol, a phytoalexin, possesses antioxidant characteristics, chemopreventive potential, and has been proposed as a treatment for illnesses, such as diabetes, cancer, and cardiovascular disease [Rossi et al., 2012]. Our study determined whether resveratrol could reduce/ prevent OTA-induced cytotoxicity in kidney cells. Our aim was to determine whether resveratrol could prevent cytotoxicity induced by OTA in kidney cells. Under normal conditions, ROS regulates intracellular signaling; however, under conditions of excessive ROS production, intracellular signaling is compromised and will result in cellular damage [Wang et al., 2013]. Excessive oxidative stress induces cytotoxicity and alters cellular functioning [Weydert and Cullen, 2010]. Lipids, proteins, and DNA are susceptible to ROS attack and subsequent damage [Marin-Kuan et al., 2011].OTA decreased intracellular ROS (as determined by FACS), but still induced single strand DNA breaks (comet assay). Exposure to OTA (24 h, 1.5 μM) resulted in significant DNA strand breaks in HEK293 cells, suggesting that OTA is genotoxic. In comparison to control cells, OTA significantly increased the lengths of comet tails by 16.8%, whereas resveratrol significantly decreased comet tail lengths by 69.5% (Fig. 3). Resveratrol also significantly reduced OTA genotoxicity as the co-treatment decreased DNA tail lengths (35.2%). In addition, the intracellular ROS was the lowest in resveratrol-treated cells (20.2%). When DNA damage occurs, the cell activates DNA repair enzymes, such as OGG1–a DNA glycosylase
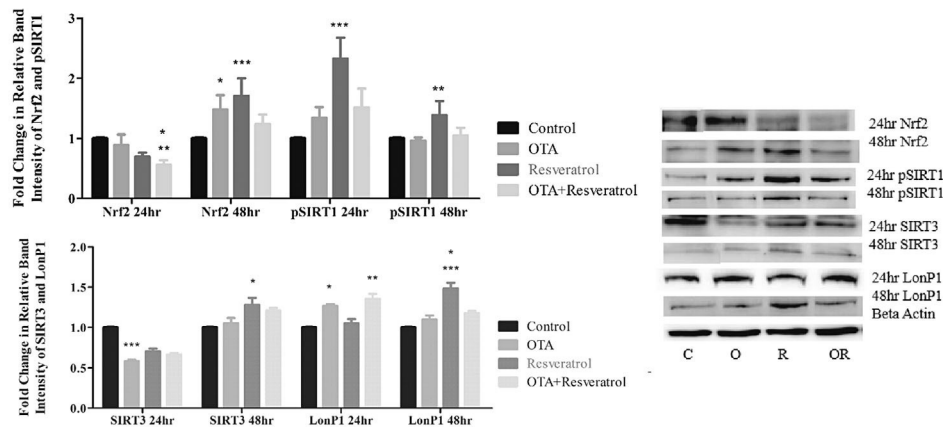
Fig. 6. Western blot images and relative fold change in protein expression of Nrf2, pSIRT1, SIRT3, and LonP1 over 24 and 48 h, in response to OTA and resveratrol exposure. Resveratrol decreases Nrf2 expression during 24–h exposure, but increases Nrf2 during 48–h exposure ($^{***}P < 0.0008$). OTA significantly increased Nrf2 expression during 48–h exposure ($^{*}P < 0.05$). The OTA+resveratrol co–treatment significantly decreased Nrf2 expression during 24–h exposure ($^{*}P < 0.05$). Resveratrol significantly increased the expression of pSIRT1 during 24–h exposure ($^{***}P = 0.0002$) and 48–h exposure ($^{**}P = 0.0054$). SIRT3 expression decreased in all treatments during 24–h exposure with a significant decrease during OTA exposure ($^{***}P = 0.0003$). The opposite was observed during 48–h exposure, all treatments increased SIRT3 expression with significance in the resveratrol treatment ($^{*}P < 0.05$). LonP1 expression exhibits the inverse of SIRT3 expression, with an increase in LonP1 expression during 24–h exposure to OTA ($^{*}P < 0.05$) and OTA+Resveratrol ($^{**}P = 0.0011$). LonP1 expression increased significantly upon 48–h exposure to resveratrol ($^{***}P = 0.0005$).

enzyme, which removes 8-oxoguanine base lesions, that could result in mutations and carcinogenesis if left to accumulate [Cheng et al., 2013]. The OTA decreased expression of OGG1 and increased DNA tail lengths. This suggests that OGGI enzyme activity is compromised and as a consequence, base lesions persist. However, resveratrol increased OGG1 expression in kidney cells, resulting in significantly shorter DNA tail lengths, whereas the co-treatment, although inducing low expression of OGG1, significantly decreased DNA tail lengths. In the co-treatment, resveratrol afforded a protective function, due to its antioxidant properties, by decreasing intracellular ROS and increasing OGG1 expression. This may explain its cyto-protective and potential anti-cancer property. The 48-h ROS results differ, in that all treatments show increased intracellular ROS (Fig. 2) and a concomitant significant increase in OGG1 expression and shorter comet tails. This suggests that resveratrol aided the cells to survive in response to OTA toxicity.

Palma et al. (2007) showed that OTA induced DNA damage by increasing the production of free radicals. Our data are in agreement with this study as demonstrated by the comet and flow cytometry ROS assays.

GSH, a low molecular weight thiol found abundantly in cells, is a potent endogenous antioxidant [Wu et al., 2004]. GSH depletion indicates an increase in ROS and oxidative stress. OTA severely depleted GSH concentrations ($5.06\,\mu M$) in kidney cells, whereas resveratrol increased GSH ($2.65\,\mu M$ at 24 h; $7.14\,\mu M$ at 48 h). Further in the co-treated cells, resveratrol significantly increased GSH from $5.68\,\mu M$ (24 h) to $11.39\,\mu M$ (48 h). These results indicate that resveratrol increases the antioxidant capacity of the cell over longer periods of time by increasing GSH synthesis. In addition, resveratrol clearly shows its potent antioxidant capacity by dampening the effects of OTA induced toxicity in kidney cells.

At both 24- and 48-h treatment periods, Nrf2 mRNA expression was significantly decreased. Nrf2 expression was lowest at 24 h but higher at 48 h in the co-treated cells. Although resveratrol induced low expression of Nrf2 compared to other treatments, the concentrations of GSH were significantly increased and DNA tail lengths were shorter. Taken together, our data show that resveratrol is a potent antioxidant that can function independently in the cell without influence from Nrf2. OTA induced a non-significant increase in Nrf2 expression with increased DNA damage, high ROS levels, and lower GSH concentrations.

Western blot protein analysis revealed changes in Nrf2 expression over the two treatment periods; at 24 h, all treatments decreased Nrf2 expression, wheres at 48 h, Nrf2 expression was increased. The decreased Nrf2 expression strongly suggests that resveratrol itself mediates the antioxidant defense response, in keeping with its antioxidant properties [Momchilova et al., 2014]. The protein and mRNA expression of Nrf2 at 24 h is in agreement; however, these results are different at 48 h. The mRNA expression of Nrf2 is significantly decreased, but the protein expression is significantly increased. This result could be due to alterations in post-translational modifications or the increased expression of LonP1. LonP1, a protease, is known for its regulatory function and could assist in degrading potentially compromised proteins [Bota et al., 2002].

SIRT3 has been identified as a regulator of LonP1 expression; increased SIRT3 decreases LonP1 [Gibellini et al., 2014]. This inverse relationship is clearly evident at 24 h−decreased SIRT3 and increased LonP1 expression. A significant increase in LonP1 and decreased SIRT3 expression was observed in OTA and co-treated kidney cells. The increased expression of LonP1 is triggered by increased oxidative stress to combat accumulation of defective

proteins. This mitochondrial protease could have acted in a compensatory manner by degrading proteins damaged by oxidative stress [Zhang et al., 2014].

SIRT1, a NAD$^+$-dependent deacetylase, is activated by resveratrol. In our study, resveratrol increased SIRT1 expression in cells at both 24 and 48 h. Activated SIRT1 by phosphorylation influences many processes, such as DNA repair and cellular metabolism, thereby impacting the cellular stress response. Phosphorylated SIRT1 increases nuclear localization of the protein, as well as increased enzymatic activity [Sasaki et al., 2008]. The stress protection pathway is mediated by SIRT1, increased phosphorylation would influence this function, and induce possible deacetylation of stress proteins, such as p53 and PGC-1α, causing cellular protection and survival [Nasrin et al., 2009].

These data provide insight into the behavior of OTA, as well as the therapeutic potential of resveratrol. We are proposing that short-term effects of OTA contribute to its toxicity. The cells experience an increase in the antioxidant response due to the recognition of a cytotoxic environment; however, it is not possible to sustain an increased protective response while DNA damage occurs. This is evidenced by the 48-h data, as the time period progresses, so too does the extent of damage inflicted on the cell by the toxin. Our data suggest that OTA exhausts the cell's antioxidant reservoir during acute exposure, and thus the cell is left vulnerable as the exposure period continues. Taken together, theses data suggest that resveratrol could be a potential treatment for OTA toxicity, as well as a possible therapy for other oxidative stress-inducing toxins. Regarding the information on DNA damage prevention, this research also attests to the chemo-preventive potential of resveratrol.

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