Protective effect of carnosic acid against acrylamide-induced toxicity in RPE cells

Albalawi, Aishah Eis S; Alhasani, Reem Hasaballah A.; Biswas, Lincoln; Reilly, James; Shu, Xinhua

Published in:
Food and Chemical Toxicology

DOI:
[10.1016/j.fct.2017.01.026](https://doi.org/10.1016/j.fct.2017.01.026)

Publication date:
2017

Document Version
Author accepted manuscript

[Link to publication in ResearchOnline](#)

Citation for published version (Harvard):

Albalawi, AES, Alhasani, RHA, Biswas, L, Reilly, J & Shu, X 2017, 'Protective effect of carnosic acid against acrylamide-induced toxicity in RPE cells', *Food and Chemical Toxicology*, vol. 108, no. Part B, pp. 543-553.
<https://doi.org/10.1016/j.fct.2017.01.026>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

If you believe that this document breaches copyright please view our takedown policy at <https://edshare.gcu.ac.uk/id/eprint/5179> for details of how to contact us.

Protective effect of carnosic acid against acrylamide-induced toxicity in RPE Cells

Aishah Albalawi^{1,2}, Reem Hasaballah A Alhasani^{1,3}, Lincoln Biswas¹, James Reilly¹, Xinhua Shu¹

1 Department of Life Sciences, Glasgow Caledonian University, Cowcaddens Rd, G4 0BA, Glasgow, UK

2 Department of Biology, University of Tabuk, Tabuk, Saudi Arabia

3 Department of Biology, Umm Al-Qura University, Makkah, Saudi Arabia

Correspondence author

Dr Xinhua Shu, Department of Life Sciences, Glasgow Caledonian University, Glasgow G4 0BA, UK. Tel: 0044 141 4418763; Email: Xinhua.Shu@gcu.ac.uk

Abstract

Acrylamide is a substance that can be neurotoxic in humans and experimental animals. It is formed at different rates in starchy foods cooked at temperatures above 120°C as a result of interaction between monosaccharides and the amino acid asparagine. Carnosic acid accounts for over 90% of the antioxidant properties of rosemary extract and is a powerful inhibitor of lipid peroxidation in microsomal and liposomal systems. Carnosic acid has been shown to protect against oxidative and inflammatory effects. In order to investigate the protective properties of carnosic acid against acrylamide-induced toxicity in human retinal pigment epithelium (RPE) cells, ARPE-19 cells were pre-treated with 10µM CA for 24h followed by treatment with acrylamide (0.7 or 1 mM) for 24h. ARPE-19 cells pre-treated with 10µM carnosic acid showed significantly increased cell viability and decreased cell death rate when compared to ARPE-19 cells treated with acrylamide alone. Activities of SOD and catalase and the level of GSH and expression of NRF2 and a number of anti-oxidant genes were significantly decreased in ARPE-19 cells, while there were significant increases in ROS and MDA; pre-treatment with carnosic acid significantly counteracted these changes. Our results suggest that carnosic acid protected RPE cells from acrylamide-induced toxicity.

Key words Acrylamide, carnosic acid, neurotoxicity, oxidative damage, retinal pigment epithelium cells

1. Introduction

Acrylamide (Fig. 1E) is a highly water-soluble white substance with a solid crystalline form (Becalski et al., 2003; Exon, 2006). The main application of acrylamide and polyacrylamides is in the manufacture of plastics and in the welding of waterpipes, although they are also used in the production of paper and textiles. In addition, acrylamide monomer is used in the preparation of polyacrylamide electrophoresis gels in research and clinical diagnosis labs (Dybing et al., 2005). Acrylamide is also formed in starchy foods such as fried potatoes, bread, cookies, and coffee heated to temperatures above 120°C (Tareke et al., 2002; Kahkeshani et al., 2015). Generally, the formation of acrylamide during cooking at high temperatures is due to the reaction between the amino acid asparagine and a carbonyl-containing source (Zyzak et al., 2003). Jakobsen et al. (2016) estimated that the mean dietary intake of acrylamide in the Danish population is 0.27 µg/kg bw/day (females) and 0.36 µg/kg bw/day (males). Two earlier studies estimated that the Dutch population had a slightly higher mean acrylamide exposure of 0.48 (Konings et al., 2003) and 0.5 (Boon et al., 2005) µg/kg bw/day. Similar to that of the Dutch population, the US population had an estimated mean acrylamide exposure of 0.44 µg/kg bw/day (Doerge et al., 2008). The major contribution to dietary intake of acrylamide is French-fries /fried potatoes (Boon et al., 2005; Doerge et al., 2008; Dybing and Sanner, 2003; Jakobsen et al., 2016; Svensson et al., 2003). Acrylamide is classified as a potential carcinogen, one possible reason being its genotoxicity (Dobrovolsky et al., 2016; EFSA, 2015; Meil et al., 2008; Mei et al., 2010). Rodents with oral exposure to acrylamide experienced tumours in various organs (EFSA, 2015) while humans exposed to acrylamide have an increased risk of developing cancer (EFSA, 2015).

Acrylamide was identified as a neurotoxin about 60 years ago (Kuperman, 1958). Humans and laboratory animals exposed to acrylamide exhibited neurotoxicity characterised by ataxia, weight loss, skeletal muscle weakness, and distal swelling and degeneration of axons in the peripheral and central nervous systems (LoPachin and Decaprio, 2005; El-Tantawi, 2007). The retina is a part of the central nervous system and contains three main

layers: photoreceptor layer, inner nuclear layer and ganglion cell layer (Patnaik et al, 2015). The retina is responsible for receiving light and converting it to neural signals that are passed to the brain for visual processing. Early studies showed that chronic exposure of monkeys (*Macaca nemestrina*) to acrylamide led to significantly reduced visual function (Merigan et al., 1985) and degeneration of ganglion cells, although the inner nuclear and photoreceptor layers were preserved (Eskin et al., 1985; Eskin and Merigan, 1986). Maternal exposure of rat to acrylamide caused massive structural abnormalities in outer and inner nuclear layers and degeneration of ganglion cells in retinas of offspring at age 7 and 14 days (Sakr et al., 2011). Adult rats administered with acrylamide (15 mg/kg body weight) for 28 days exhibited a significant reduction in amplitude of electroretinogram a and b waves (Ali et al., 2014).

Carnosic acid (Fig. 1E) is a phenolic diterpene extracted from rosemary (*Rosmarinus officinalis*) and has shown antioxidative activity (Aruoma et al., 1992; Wu et al., 2015), anti-inflammatory activity (Lin et al., 2014), anticarcinogenic activity (Cortese et al., 2016; Russo et al., 2009), and antibacterial activity (Vázquez et al., 2016). Carnosic acid is characterized by its lipid solubility and its expulsion of free radicals of oxygen, hydroxyl, and lipid peroxy. Therefore, it prevents the oxidation of lipids and the rupture of biological membranes (Aruoma et al., 1992; Haraguchi et al., 1995). It has been reported that carnosic acid prevents the accumulation of the protein cysteine and reduces meta-fat oxidation, thus slowing the rate of free radical formation and thereby protecting cells from damage (Poeckel et al., 2008; Sozio et al., 2008). Additionally, carnosic acid has been reported to induce the transcriptional activation of antioxidant Nrf2/ARE pathway, which may be involved in the protective effects of carnosic acid in chronic neurodegenerative conditions like Parkinson's disease (Bahri et al., 2016).

Retinal pigment epithelium (RPE) cells lie beneath the photoreceptor layer in the retina and maintain the function of photoreceptors by helping to renew photoreceptor outer segments and by providing nutrition to photoreceptors (Strauss, 2005). In the current study we investigated the toxicity of acrylamide to RPE cells and evaluated the protective effects of carnosic acid against acrylamide-induced toxicity.

2. Materials and methods

2.1 Cell Culture

Adult human retinal pigment epithelium ARPE-19 cells (ATCC® CRL-2302™) were grown in Dulbecco's Modified Eagle Medium (DMEM/F12, Lonza, UK) comprising 2.4 mM L-Glutamine, 17.5mM glucose, and supplemented with 10% fetal bovine serum (FBS, Lonza), 100µg/mL streptomycin, and 100 units/mL penicillin (Lonza, UK) and 0.26% sodium bicarbonate (Sigma).

2.2 Cell viability

ARPE-19 cells were seeded in a clear 6-well tissue culture plate (Greiner Bio One, UK) for 24 hours at a density of 3×10^5 cells/well. To test cytoprotective effects of carnosic acid to acrylamide-induced cell death, cells were pretreated with 10µM of carnosic acid (Cat. Number C0609, purity>91%, Sigma) dissolved in Dimethyl sulfoxide (DMSO, Sigma) for 24 hours in serum-free medium. Cells were then treated with acrylamide (0.7 and 1mM) for 24 hours. Cells were stained with 0.2% crystal violet (Sigma, UK). The absorbance was measured at 570nm in a microplate spectrophotometer Epoch reader (Biotech, UK). The percentage of viable cells was determined using the following formula:

$$\% \text{ of viable cells} = \frac{[(\text{absorbance of untreated cells} - \text{absorbance of treated cells}) / \text{absorbance of untreated cells}] \times 100.}$$

2.3 Measurement of reactive oxygen species (ROS)

ARPE-19 cells (25,000/well) were seeded in clear-bottomed black 96-well tissue culture plates (Greiner Bio One, UK) and cultured for 24 hours. Cells were then treated with carnosic acid (10µM), while control cells were treated with 0.1% DMSO alone for 24 hours. Cells were washed twice with PBS (200 µl/well), then treated with acrylamide (0.7 and 1mM) for 24 hours. Total ROS was measured using the 6-Carboxy-2',7'-Dichlorofluorescein diacetate (DCFH-DA) (Sigma, UK) according to the manufacturer's guidelines. Briefly, a stock solution of 10 mM of DCFH-DA was prepared in DMSO, then cells were incubated with 10 µM DCFH-

DA in PBS (150µl/well) and incubated for 30 minutes in 5% CO₂ incubator at 37°C. The fluorescence was measured at 485 nm (excitation) and 525 nm (emission) using a Fluostar Optima microplate reader (BMG-labtech).

2.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using Trizol Reagent (Sigma, UK) according to the manufacturer's protocol. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, UK). The quantification of gene expression was detected by qRT-PCR assay using a Platinum® SYBR® Green QPCR SuperMix-UDG w/ROX kit (Invitrogen, UK). The relative change in gene expression was determined according to $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen, 2001) in which gene expression was normalized to expression of the housekeeping gene, β -actin, in the treated and control samples. The sequences of primers used in qRT-PCR are listed in Table 1.

2.5. Measurement of catalase (CAT) and superoxide dismutase (SOD) activities

CAT and SOD activities were detected in ARPE-19 cells using the SOD Detection Kit (OxiSelect Superoxide Dismutase Activity Assay Kit; Cell Biolabs, STA-340) and the OxiSelect Catalase Activity Assay Kit (Cell Biolabs, STA-341), according to the manufacturer's instructions.

2.6 Quantification of glutathion and malondialdehyde

Quantitation of glutathion (GSH) and malondialdehyde (MDA) in treated and control ARPE-19 cells was measured using the total glutathion (GSSG/GSH) assay kit (Cell Biolabs, STA-312) and TBARS Assay kit (for MDA) (Cell Biolabs, STA-330) according to the manufacturer's instructions.

2.7 Western blotting assay

Control and treated ARPE-19 cells were lysed with ice-cold RIPA lysis buffer (Thermo Scientific), the lysates were collected and centrifuged, and the supernatants were stored at -80°C until required. Protein concentration was determined using Precision Red Advanced Protein Assay reagent (Cytoskeleton, Inc. Cat. # ADV02-A) according to the manufacturer's guidelines. Proteins were run in SDS-PAGE and transferred to nitrocellulose membrane (Amersham Biosciences). The membrane was blocked by incubation in 5% milk powder in Tris-Buffered Saline-Tween 20 buffer for 1 hour at room temperature, followed by incubation with primary antibodies (GAPDH and NRF2, 1:1000 dilution) overnight at 4°C. The membrane was washed and incubated with secondary antibodies (1:10000 dilution). The signals were detected using the LI-COR Odyssey FC Imaging System and the signal intensity was analyzed by Image Studio™ Lite analysis software (LI-COR).

2.8 Detection of cell death

Cell death was detected using DeadEnd™ fluorometric TUNEL assay kit (Promega) following the manufacturer's instructions. Briefly, treated and control ARPE1-9 cells were fixed with 4% PFA for 20 minutes at 4°C and washed with PBS once, followed by permeabilisation with 0.2% Triton X-100 in PBS for 5 min. Cells were labelled with rTDT reaction mix for one hour at 37°C and the reaction was stopped with 2X SSC. Cells were rinsed with PBS and mounted using Vectashield mounting media containing DAPI (Vector laboratories). Images were captured using ZEISS LSM 800 confocal microscopy. To quantify cell death, the number of TUNEL positive cells in 300 cells was counted from three individual samples (100 cells in each sample).

2.9 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 6 software. All data were obtained from three independent experiments using one-way ANOVA test. Data are presented as means ± standard error.

3. Results

3.1 Acrylamide treatment resulted in decreased RPE cell viability that was significantly counteracted by carnosic acid

To investigate the effect of acrylamide-induced toxicity in RPE cells, cell viability was assessed by crystal violet staining (Fig. 1D). Cell viability of ARPE-19 cells was significantly decreased to 65.55%, 50.05%, and 36.37% of untreated levels following exposure to 0.7, 1.0, or 2.0mM acrylamide respectively (Fig. 1A). ARPE-19 exposed to acrylamide at 0.37 and 0.5mM showed no significant reduction in cell viability. Prior to investigating the protective effect of carnosic acid on acrylamide exposure, we assessed cell viability of ARPE-19 cells treated with carnosic acid at 10, 20, 30, 40, 50, and 60 μ M and found that cell viability was significantly decreased when treated with carnosic acid of 40, 50 or 60 μ M but was not significantly changed when treated with carnosic acid at 10, 20 or 30 μ M (Fig. 1B). Consequently, acrylamide at concentrations of 0.7 and 1.0mM and carnosic acid at concentration of 10 μ M were used for subsequent experiments. ARPE-19 cells first treated with carnosic acid (10 μ M) for 24 h and then exposed to 0.7 or 1mM acrylamide for 24 h demonstrated significantly increased cell viability by 12.21% and 14.25% respectively when compared with cells treated with acrylamide alone (Fig. 1 C, D).

3.2 Carnosic acid protects against acrylamide-induced cell death in RPE cells

We examined whether reduced cell viability is associated with apoptotic cell death. We used TUNEL staining to assess the cell death (Fig. 2A) and found that there was a significantly increased cell death rate in ARPE-19 cells exposed to 0.7 and 1mM acrylamide when compared to untreated control cells and that pre-treatment with carnosic acid led to significant reduction in cell death induced by acrylamide (Fig. 2B).

We also measured the expression of cell death related genes, caspase-3 and caspase-9 (Fig. 3). Acrylamide treatment (0.7 and 1mM) resulted in significantly increased expression of caspase-3 and caspase-9 genes when compared to the control ARPE-19 cells. Expression

of caspase-3 and caspase-9 was significantly decreased in ARPE-19 cells pre-treated with carnosic acid (10 μ M) when compared to cells treated with acrylamide alone (Fig. 3).

3.3 Carnosic acid suppressed acrylamide-induced reactive oxygen species (ROS) production

We investigated whether acrylamide treatment can increase intracellular ROS in ARPE-19 cells. As shown in Fig. 4, cells treated with 0.7mM and 1mM acrylamide exhibited notably increased ROS by 32.02% and 34.83% respectively compared with untreated control cells. Cells pre-treated with carnosic acid (10 μ M) then treated with acrylamide (0.7 and 1mM) showed significantly reduced ROS by 15.02% and 17.33% respectively when compared with cells treated with acrylamide alone.

3.4 Carnosic acid treatment reverses acrylamide-induced changes in the expression of antioxidant genes

Acrylamide-induced ROS production is possibly due to cellular antioxidant imbalance, so we examined expression of antioxidant genes in ARPE-19 cells exposed to acrylamide. We found expression of antioxidant genes was decreased in acrylamide-treated cells compared to untreated cells (Fig. 5). Compared to the control group, expression of SOD1 gene was decreased by 50.24% following 0.7mM acrylamide treatment and by 56.58% following 1mM acrylamide treatment; GPX1 gene expression was decreased by 29.47% and 37.66% respectively; CAT gene expression was decreased by 47.80% and 52.27% respectively; NQO-1 gene expression was decreased by 33.67% and 53.04% respectively (although in the former case the difference was not statistically significant); and GCLM gene expression was decreased by 27.34% and 36.31% respectively (although in both cases the difference was not statistically significant). ARPE-19 cells pre-treated with carnosic acid (10 μ M) showed notably increased expression of these genes compared to cells treated with acrylamide alone: SOD1 expression was increased by 542.6% (pre-treatment with carnosic acid followed by treatment with acrylamide (0.7mM)) and 577.84% (pre-treatment with carnosic

acid followed by treatment with acrylamide (1.0mM)); GPX1 expression was increased by 384.2% and 304.13% respectively; CAT expression was increased by 457.66% and 418.39% respectively; NQO-1 expression was increased by 352.93% and 429.3% respectively; and GCLM expression was increased by 343.1% and 353.27% respectively (Fig.5).

We also investigated the effects of carnosic acid on SOD and CAT activities. As shown in Fig. 6, ARPE-19 cells treated with acrylamide at 0.7 or 1.0mM had SOD activities that were significantly reduced by 60.75% and 82.26% respectively, and for CAT by 23.86% and 48.6% respectively, when compared to untreated control cells. Pre-treatment with carnosic acid at 10 μ M resulted in significantly increased activities of SOD by 512.1% following treatment with acrylamide (0.7mM) and by 665.35% following treatment with acrylamide (1.0mM) and increased activity of CAT by 33.68% and 19.49% respectively, when compared to cells treated with acrylamide alone (Fig. 6A and B).

3.5 Carnosic acid treatment reverses acrylamide-induced changes in the production of glutathione and malondialdehyde

Acrylamide treatment can cause decreased glutathione (GSH) and increased malondialdehyde (MDA) in cell lines and in animal models (Kahkeshani *et al.*, 2015). We investigated the levels of GSH and MDA in acrylamide-treated ARPE-19 cells and found that GSH was significantly decreased by 37.39% (0.7mM acrylamide treatment) and 65.02% (1mM acrylamide treatment) compared to the untreated cells. ARPE-19 cells pre-treated with carnosic acid (10 μ M) had significantly increased GSH by 247.24% (0.7 mM acrylamide treatment) and 261.26% (1.0 mM acrylamide treatment), when compared to cells treated solely with acrylamide (Fig. 6C).

Production of MDA in ARPE-19 cells treated with acrylamide at 0.7 or 1.0mM was increased by 22.8% and 27.42% respectively, when compared to the untreated control cells. Pre-treatment with carnosic acid (10 μ M) led to decreased levels of MDA by 14.99% (0.7mM acrylamide treatment) and 17.22% (1.0mM acrylamide treatment) when compared to cells treated with acrylamide alone (Fig. 6D).

3.6 Carnosic acid reverses acrylamide-induced changes in the expression of *NRF2* gene

NRF2 is known to play an important role in protection against oxidative stress by enhancing the expression of antioxidant enzyme genes (Kobayashi and Yamamoto, 2006). Knockdown of NRF2 in PC12 cells further decreased the generation of GSH and increased MDA production induced by acrylamide (Pan *et al.*, 2016). Firstly we examined expression of NRF2 gene by qRT-PCR (Fig. 7A) and found acrylamide exposure (0.7 or 1mM) caused significantly decreased expression of NRF2 by 45.50% and 51.43% respectively, when compared to the untreated control cells. However, pre-treatment with carnosic acid (10µM) induced a significant increase in NRF2 expression by 432.65% (0.7mM acrylamide treatment) and 433.18% (1.0mM acrylamide treatment), when compared to cells treated solely with acrylamide (Fig. 7B). Secondly we examined NRF2 protein level by western blotting using anti-NRF2 antibody (Fig. 7C) and found the level of NRF2 protein was significantly decreased by 65.15% and 82.68% in ARPE-19 cells treated with acrylamide at 0.7 and 1.0mM respectively, when compared to untreated control cells. ARPE-19 cells, pre-treated with carnosic acid (10 µM) and then treated with acrylamide (0.7 or 1.0mM), had a significant increase in NRF2 protein by 423.33% and 629.21% respectively, compared to cells treated solely with acrylamide (Fig. 7D).

Discussion

Humans can be chronically exposed to acrylamide through the intake of certain foods (such as bread, cereals, potato chips and crisps, and coffee) cooked or heated at high temperatures. Workers exposed occupationally to acrylamide exhibited peripheral and central neuropathies (Pennisi *et al.*, 2013). Tunnel workers exposed to N-methylolacrylamide and acrylamide experienced decreased light sensitivity and colour discrimination (Goffeng *et al.*, 2008a). Electroretinographic assessment of these workers showed that cone photoreceptor function was significantly affected (Goffeng *et al.*, 2008b). Adult rodents and primates exposed to acrylamide showed visual function defects (Merigan

et al., 1985; Ali et al., 2014). Following maternal exposure to acrylamide or acrylamide-containing fried potato chips, rat offspring showed morphologically abnormal retinal pigment epithelium (RPE) cells, photoreceptors and ganglion cells (El-Sayyad et al., 2011; Sakr et al., 2011). However, information about acrylamide-induced toxicity in RPE cells and the effects of carnosic acid on acrylamide-induced toxicity is very limited.

Our current study used ARPE-19 cells as an *in vitro* model to assess the toxicity of acrylamide and evaluate the protective effects of 24 hours of pre-treatment with carnosic acid. Dietary polyphenols can undergo degradation in cell culture conditions (Xiao and Högger, 2014) but carnosic acid in ethanolic solutions is fairly stable, degrading by less than 20% after 24 hours (Zhang et al., 2012). Previous studies have shown that acrylamide exposure caused significantly decreased cell viability and increased ROS production in neurally-associated cell lines such as PC12, SH-SY5Y, U-1240 MG, U-87 MG and U-251 MG cells (Chen et al., 2009; Chen et al., 2013; Lee et al., 2014; Mehri et al., 2012; Pan et al., 2016; Sumizawa and Igisu, 2007). Acrylamide also inhibited the differentiation and proliferation of SH-SY5Y and U-1240 MG cells (Chen and Chou, 2015) and promoted endothelial cell (HUVEC) ageing (Sellier et al., 2015). Our results also demonstrated that acrylamide exposure resulted in dose-dependent decreased cell viability (Fig. 1A) and increased ROS production (Fig. 4). Acrylamide-induced cell death was thought to be caspase-3 dependent; elevated caspase-3 activities have been reported in acrylamide-exposed SH-SY5Y, PC12 and Caco-2 cells (Pan et al., 2016; Rodríguez-Ramiro et al., 2011; Sumizawa and Igisu, 2007). A recent study showed that acrylamide-induced cell death was mediated by ER stress and associated eIF2 α -ATF4-CHOP signalling pathway (Komoike & Matsuoka, 2016). Our data showed that acrylamide exposure caused marked cell death in RPE cells, possibly through the caspase-3 dependent pathway (Figs. 2 and 3) although we cannot exclude the involvement of other cell death pathways in acrylamide-treated RPE cells.

Increased ROS production in acrylamide-exposed cells is due to the disruption of redox homeostasis. Acrylamide exposure resulted in significantly decreased GSH level and

antioxidant enzyme activities in *in vitro* mammalian cells and in *in vivo* animal models (Ali et al., 2014; Kahkeshani et al., 2015; Lakshmi et al., 2012; Mehri et al., 2015; Pan et al., 2016; Rodríguez-Ramiro et al., 2011). MDA, a marker for lipid peroxidation and oxidative stress, was also overproduced in acrylamide-exposed cells and animals (Ali et al, 2014; Mehri et al, 2015; Pan et al, 2016). We too found that RPE cells exposed to acrylamide had notably decreased GSH and antioxidant enzyme (SOD and catalase) activities, and MDA was also significantly increased (Fig. 6), suggesting acrylamide treatment caused the redox imbalance in RPE cells.

Carnosic acid has shown a protective role in cancer, vascular diseases, neurodegenerative diseases, metabolic disorders and organ injuries (Bahri et al., 2016). Carnosic acid protected retinal cells from H₂O₂-induced oxidative damage *in vitro* and from light-induced retinal degeneration *in vivo* (Rezaie et al., 2012); Administration of carnosic acid in rd10 mouse model of retinitis pigmentosa suppressed photoreceptor degeneration (Kang et al., 2016). NRF2 has been involved in carnosic acid-mediated neuroprotection (Kang et al., 2016; Rezaie et al., 2012; Satoh et al., 2008). Carnosic acid treatment resulted in NRF2 upregulation in cortical neurons, retinal cells and tissues (Kang et al., 2016; Rezaie et al., 2012; Satoh et al., 2008). NRF2 signal pathway also mediated acrylamide-induced apoptosis in PC12 cells (Pan et al., 2016). Our results demonstrated that acrylamide exposure downregulated NRF2 expression in RPE cells and that carnosic acid treatment reversed this effect (Fig. 7). Acrylamide exposure also downregulated the expression of NRF-targeting antioxidant genes (SOD1, GPX1, catalase, NQO1 and GCLM) (Fig. 5); again, carnosic acid treatment was able to reverse these effects.

In summary, our results suggest that carnosic acid can protect RPE cells from acrylamide-induced oxidative damages through NRF2 signalling pathway and has a potential for neuroprotection against acrylamide-induced retinal toxicity in humans.

Acknowledgement

AA and RHA are supported by PhD scholarships from Saudi Arabia Government. We would like to thank the Rosetrees Trust, the Glasgow Children's Hospital Charity and the Visual Research Trust for supporting this work.

References

- Ali, M.A., Aly, E.M., Elawady, A.I., 2014. Effectiveness of selenium on acrylamide toxicity to retina. *Int. J. Ophthalmol.* 7(4), 614-620.
- Aruoma, O., Halliwell, B., Aeschbach, R., Löliger, J., 1992. Antioxidant and pro-oxidant properties of active rosemary constituents: carnosol and carnosic acid. *Xenobiotica*. 22(2), 257-268.
- Bahri, S., Jameleddine, S., Shlyonsky, V., 2016. Relevance of carnosic acid to the treatment of several health disorders: Molecular targets and mechanisms. *Biomed. Pharmacother.* 84, 569-582.
- Boon, P.E., de Mul, A., van der Voet, H., van Donkersgoed, G., Brette, M., van Klaveren, J.D., 2005. Calculations of dietary exposure to acrylamide. *Mutat. Res. Toxicol. Environ. Mutagen* 580, 143e155.
- Becalski, A., Lau, B.P., Lewis, D., Seaman, S.W., 2003. Acrylamide in foods: occurrence, sources, and modeling. *J. Agric. Food Chem.*, 51(3), 802-808.
- Chen, J.H., Wu, K.Y., Chiu, IM., Tsou, T.C., Chou, C.C., 2009. Acrylamide-induced astrogliotic and apoptotic responses in human astrocytoma cells. *Toxicol. In Vitro* 23(5), 855-861.
- Chen, J.H., Yang, C.H., Wang, Y.S., Lee, J.G., Cheng, C.H., Chou, C.C., 2013. Acrylamide-induced mitochondria collapse and apoptosis in human astrocytoma cells. *Food Chem. Toxicol.* 51, 446-452.
- Chen, J.H., Chou, C.C., 2015. Acrylamide inhibits cellular differentiation of human neuroblastoma and glioblastoma cells. *Food Chem. Toxicol.* 82, 27-35.

391 Cortese, K., Daga, A., Monticone, M., Tavella, S., Stefanelli, A., Aiello, C., Bisio, A., Bellese,
 392 G., Castagnola, P., 2016. Carnosic acid induces proteasomal degradation of Cyclin B1, RB
 393 and SOX2 along with cell growth arrest and apoptosis in GBM cells. *Phytomedicine* 23(7),
 394 679-685.

395 Dobrovolsky, V.N., Pacheco-Martinez, M.M., McDaniel, L.P., Pearce, M.G., Ding, W., 2016.
 396 In vivo genotoxicity assessment of acrylamide and glycidyl methacrylate. *Food Chem.*
 397 *Toxicol.* 87, 120-127.

398 Doerge, D.R., Young, J.F., Chen, J.J., DiNovi, M.J., Henry, S.H., 2008. Using dietary
 399 exposure and physiologically based pharmacokinetic/pharmacodynamic modeling in human
 400 risk extrapolations for acrylamide toxicity. *J. Agric. Food Chem.* 56, 6031-6038.

401 Dybing, E., Farmer, P.B., Andersen, M., Fennell, T.R., Lalljie, S.P., Müller, D.J., Olin, S.,
 402 Petersen, B.J., Schlatter, J., Scholz, G., Scimeca, J.A., Slimani, N., Törnqvist, M., Tuijelaars,
 403 S., Verger, P., 2005. Human exposure and internal dose assessments of acrylamide in food.
 404 *Food Chem. Toxicol.* 43(3), 365-410.

405 EFSA, 2015. Scientific opinion on acrylamide in food. *EFSA J.* 13, 4104.

406 El-Sayyad, H.I., Sakr, S.A., Badawy, G.M., Afify, H.S., 2011. Hazardous effects of fried
 407 potato chips on the development of retina in albino rats. *Asian Pac. J. Trop. Biomed.* 1(4),
 408 253-260.

409 El-Tantavi, H.G.M., 2007. The protective role of ginger (*Zingiber officinale*) against
 410 acrylamide induced neurotoxicity in mice. *Egypt. J. Histol.* 30, 325–336

411 Eskin, T.A., Lapham, L.W., Maurissen, J.P., Merigan, W.H., 1985. Acrylamide effects on the
 412 macaque visual system. II. Retinogeniculate morphology. *Invest. Ophthalmol. Vis. Sci.* 26(3),
 413 317-329.

414 Eskin, T.A., Merigan, W.H., 1986. Selective acrylamide-induced degeneration of color
 415 opponent ganglion cells in macaques. *Brain Res.* 378(2), 379-384.

416 Exon, J. H., 2006. A review of the toxicology of acrylamide. *J Toxicol Environ Health B Crit*
 417 *Rev.* 9(5), 397-412.

418 Goffeng, L.O., Heier, M.S., Kjuus, H., Sjöholm, H., Sørensen, K.A., Skaug, V., 2008a. Nerve
 419 conduction, visual evoked responses and electroretinography in tunnel workers previously
 420 exposed to acrylamide and N-methylolacrylamide containing grouting agents. *Neurotoxicol*
 421 *Teratol.* 30(3),186-194.

422 Goffeng, L.O., Kjuus, H., Heier, M.S., Alvestrand, M., Ulvestad, B., Skaug, V., 2008b. Colour
 423 vision and light sensitivity in tunnel workers previously exposed to acrylamide and N-
 424 methylolacrylamide containing grouting agents. *Neurotoxicology* 29(1), 31-39.

425 Haraguchi, H., Saito, T., Okamura, N., Yagi, A., 1995. Inhibition of lipid peroxidation and
 426 superoxide generation by diterpenoids from *Rosmarinus officinalis*. *Planta Med.* 61(4), 333-
 427 336.

428 Jakobsen, L.S., Granby, K., Knudsen, V.K., Nauta, M., Pires, S.M., Poulsen, M., 2016.
 429 Burden of disease of dietary exposure to acrylamide in Denmark. *Food Chem. Toxicol.* 90,
 430 151-159.

431 Kahkeshani, N., Saeidnia, S., Abdollahi, M., 2015. Role of antioxidants and phytochemicals
 432 on acrylamide mitigation from food and reducing its toxicity. *J. Food Sci. Technol.* 52(6),
 433 3169-3186.

434 Kang, K., Tarchick, M.J., Yu, X., Beight, C., Bu, P., Yu, M., 2016. Carnosic acid slows
 435 photoreceptor degeneration in the Pde6b(rd10) mouse model of retinitis pigmentosa. *Sci.*
 436 *Rep.* 6, 22632.

437 Kobayashi, M., & Yamamoto, M., 2006. Nrf2-Keap1 regulation of cellular defense
 438 mechanisms against electrophiles and reactive oxygen species. *Adv. Enzyme Regul.* 46,113-
 439 140.

440 Komoike, Y., Matsuoka, M., 2016. Endoplasmic reticulum stress-mediated
 441 neuronal apoptosis by acrylamide exposure. *Toxicol. Appl. Pharmacol.* 310, 68-77.

442 Konings, E.J.M., Baars, A.J., Van Klaveren, J.D., Spanjer, M.C., Rensen, P.M., Hiemstra, M.,
 443 Van Kooij, J.A., Peters, P.W.J., 2003. Acrylamide exposure from foods of the Dutch
 444 population and an assessment of the consequent risks. *Food Chem. Toxicol.* 41, 1569-1579.

445 Kuperman, A.S., 1958. Effects of acrylamide on the central nervous system of the cat. J.
 446 Pharmacol. Exp. Ther. 123, 180-192.
 447 Lakshmi, D., Gopinath, K., Jayanthi, G., Anjum, S., Prakash, D., Sudhandiran, G., 2012.
 448 Ameliorating effect of fish oil on acrylamide induced oxidative stress and
 449 neuronal apoptosis in cerebral cortex. Neurochem. Res. 37(9), 1859-1867.
 450 Lee, J.G., Wang, Y.S., Chou, C.C., 2014. Acrylamide-induced apoptosis in rat primary
 451 astrocytes and human astrocytoma cell lines. Toxicol. In Vitro 28(4), 562-570.
 452 Lin, C.Y., Chen, J.H., Fu, R.H., Tsai, C.W., 2014. Induction of Pi form of glutathione S-
 453 transferase by carnosic acid is mediated through PI3K/Akt/NF- κ B pathway and protects
 454 against neurotoxicity. Chem. Res. Toxicol. 27(11), 1958-1966.
 455 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
 456 quantitative PCR and the 2^{(-Delta Delta C(T))} Method. Methods 25(4), 402-408.
 457 Lopachin, R.M., Decaprio, A.P., 2005. Protein adduct formation as a molecular mechanism in
 458 neurotoxicity. Toxicol. Sci. 86(2), 214–225.
 459 Mehri, S., Abnous, K., Khooei, A., Mousavi, S.H., Shariaty, V.M., Hosseinzadeh, H., 2015.
 460 Crocin reduced acrylamide-induced neurotoxicity in Wistar rat through inhibition of oxidative
 461 stress. Iran J. Basic Med. Sci. 18(9), 902-908.
 462 Mehri, S., Abnous, K., Mousavi, S.H., Shariaty, V.M., Hosseinzadeh, H., 2012.
 463 Neuroprotective effect of crocin on acrylamide-induced cytotoxicity in PC12 cells. Cell Mol.
 464 Neurobiol. 32(2), 227-235.
 465 Mei, N., Hu, J., Churchwell, M.I., Guo, L., Moore, M.M., Doerge, D.R., Chen, T., 2008.
 466 Genotoxic effects of acrylamide and glycidamide in mouse lymphoma cells. Food Chem.
 467 Toxicol. 46, 628-636.
 468 Mei, N., McDaniel, L.P., Dobrovolsky, V.N., Guo, X., Shaddock, J.G., Mittelstaedt, R.A.,
 469 Azuma, M., Shelton, S.D., McGarrity, L.J., Doerge, D.R., Heflich, R.H., 2010. The
 470 genotoxicity of acrylamide and glycidamide in big blue rats. Toxicol. Sci. 115, 412-421.

471 Merigan, W.H., Barkdoll, E., Maurissen, J.P., Eskin, T.A., Lapham, L.W., 1985.
 472 Acrylamide effects on the macaque visual system. I. Psychophysics and electrophysiology.
 473 Invest. Ophthalmol. Vis. Sci. 26(3), 309-316.
 474 Pan, X., Yan, D., Wang, D., Wu, X., Zhao, W., Lu, Q., Yan, H., 2016. Mitochondrion-
 475 mediated apoptosis induced by acrylamide is regulated by a balance between Nrf2
 476 antioxidant and MAPK signaling pathways in PC12 cells. Mol Neurobiol, [Epub ahead of
 477 print].
 478 Patnaik, S.R., Raghupathy, R.K., Zhang, X., Mansfield, D., Shu, X., 2015. The role of RPGR
 479 and its interacting proteins in ciliopathies. J. Ophthalmol. 2015, 414781.
 480 Parzefall, W., 2008. Minireview on the toxicity of dietary acrylamide. Food Chem. Toxicol.
 481 46(4), 1360–1364.
 482 Poeckel, D., Greiner, C., Verhoff, M., Rau, O., Tausch, L., Hörnig, C., Steinhilber,
 483 D., Schubert-Zsilavecz, M., Werz, O., 2008.. Carnosic acid and carnosol potently inhibit
 484 human 5-lipoxygenase and suppress pro-inflammatory responses of stimulated human
 485 polymorphonuclear leukocytes. Biochem. Pharmacol., 76(1), 91–97.
 486 Pennisi, M., Malaguarnera, G., Puglisi, V., Vinciguerra, L., Vacante, M., Malaguarnera, M.,
 487 2013. Neurotoxicity of acrylamide in exposed workers. Int. J. Environ. Res. Public Health 10,
 488 3843-3854.
 489 Rezaie, T., McKercher, S.R., Kosaka, K., Seki, M., Wheeler, L., Viswanath, V., Chun, T.,
 490 Joshi, R., Valencia, M., Sasaki, S., Tozawa, T., Satoh, T., Lipton, S.A., 2012. Protective
 491 effect of carnosic acid, a pro-electrophilic compound, in models of oxidative stress and light-
 492 induced retinal degeneration. Invest. Ophthalmol. Vis. Sci. 53(12), 7847–7854.
 493 Rodríguez-Ramiro, I., Ramos, S., Bravo, L., Goya, L., Martín, M.Á., 2011. Procyanidin B2
 494 and a cocoa polyphenolic extract inhibit acrylamide-induced apoptosis in human Caco-2 cells
 495 by preventing oxidative stress and activation of JNK pathway. J. Nutr. Biochem. 22(12):1186-
 496 1194.

497 Russo, A., Lombardo, L., Troncoso, N., Garbarino, J., Cardile, V., 2009. *Rosmarinus*
 498 *officinalis* extract inhibits human melanoma cell growth. *Nat. Prod. Commun.* 4(12), 1707-
 499 1710.

500 Sakr, S.A., Badawy, G.M., El-sayyad, H.I., Afify, H.S., 2011. Adverse effects of acrylamide
 501 on the developing retina of albino rats. *J. Basic Appl. Sci. Res.* 1(7), 706–712.

502 Satoh, T., Kosaka, K., Itoh, K., Kobayashi, A., Yamamoto, M., Shimojo, Y., Kitajima, C., Cui,
 503 J., Kamins, J., Okamoto, S., Izumi, M., Shirasawa, T., Lipton, S.A., 2008. Carnosic acid, a
 504 catechol-type electrophilic compound, protects neurons both in vitro and in vivo through
 505 activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1. *J.*
 506 *Neurochem.* 104(4), 1116-1131.

507 Sellier, C., Boulanger, E., Maladry, F., Tessier, F.J., Lorenzi, R., Nevière, R., Desreumaux,
 508 P., Beuscart, J.B., Puisieux, F., Grossin, N., 2015. Acrylamide induces accelerated
 509 endothelial aging in a human cell model. *Food Chem. Toxicol.* 83, 140-145.

510 Sozio, P., Iannitelli, A., Cerasa, L.S., Cacciatore, I., Cornacchia, C., Giorgioni, G., Ricciutelli,
 511 M., Nasuti, C., Cantalamessa, F., Di Stefano, A., 2008. New L-dopa codrugs as potential
 512 antiparkinson agents. *Arch Pharm. (Weinheim)* 341(7), 412-417.

513 Strauss, O., 2005. The retinal pigment epithelium in visual function. *Physiol. Rev.* 85(3), 845-
 514 881.

515 Sumizawa, T., Igisu, H., 2007. Apoptosis induced by acrylamide in SH-SY5Y cells. *Arch*
 516 *Toxicol.* 81(4), 279-282.

517 Tareke, E., Rydberg, P., Ericksson, S., Törnqvist, M., 2002. Analysis of acrylamide, a
 518 carcinogen formed in heated foodstuffs. *J. Agric. Food Chem.* 50, 4998-5006.

519 Vázquez, N.M., Fiorilli, G., Cáceres Guido, P.A., Moreno, S., 2016. Carnosic acid acts
 520 synergistically with gentamicin in killing methicillin-resistant *Staphylococcus aureus* clinical
 521 isolates. *Phytomedicine* 23(12),1337-1343.

522 Wu, C.R., Tsai, C.W., Chang, S.W., Lin, C.Y., Huang, L.C., Tsai, C.W.. 2015. Carnosic acid
 523 protects against 6-hydroxydopamine-induced neurotoxicity in in vivo and in vitro model of

524 Parkinson's disease: involvement of antioxidative enzymes induction. Chem. Biol. Interact.
525 225, 40–46.

526 Xiao, J., Högger, P., 2015. Stability of dietary polyphenols under the cell culture conditions:
527 avoiding erroneous conclusions. J. Agric. Food Chem. 63(5),1547-1557.

528 Zhang, Y., Smuts, J.P., Doddiba, E., Rangarajan, R., Lang, J.C., Armstrong, D.W., 2012.
529 Degradation study of carnosic acid, carnosol, rosmarinic acid, and rosemary extract
530 (Rosmarinus officinalis L.) assessed using HPLC. J. Agric. Food Chem., 60(36), 9305-9314.

531 Zyzak, D.V., Sanders, R.A., Stojanovic, M., Tallmadge, D.H., Eberhart, B.L., Ewald, D.K.,
532 Gruber, D.C., Morsch, T.R., Strothers, M.A., Rizzi, G.P., Villagran, M.D., 2003.

533 Acrylamide formation mechanism in heated foods. J. Agric. Food Chem. 51(16), 4782-4787.

534

535

536

537

538

539

540

541

542

543

544

545

546

547

548

549

550

551

552 **Table 1 The primers used for qRT-PCR**

Gene	Forward primers 5'-3'	Reverse primers 5'-3	Annealing temperature °C	PCR product	Reference
NRF2	AGTGGATCTGCCAACTACTC	CATCTACAAACGGGAATGTCTG	56.5	106	(Clements et al., 2006)
GPX 1	AGTCCACCGTGTATGCCTTC	CTCCTGGTGTCCGAAGTAT	57.0	218	Designed in lab
SOD 1	AGGGCATCATCAATTTGAG	CATTGCCCAAGTCTCCAAC	55.0	217	Designed in lab
CAT	ATCTCGTTGGAAATAACACC	AGAAACCTGATGCAGAGACT	57.5	161	Designed in lab
NQO-1	CCTCTATGCCATGAAGTT	TATAAGCCAGAACAGACTC	48.6	107	Qaisiya et al,2013
GCLM	GCCATAGGTACCTCTGATC	CTTGACAGACAACATACTGTC	51.2	487	Rezaie et al,2012
CASPASE 3	AACTGGACTGTGGCATTG	ACCAGGTGCTGTGGAGTA	54.3	107	Zhou et al., 2008
CASPASE 9	ATGGACGAAGCGGATCGGCGGCTCC	GCACCACTGGGGTAAGGTTTTCTAG	64	331	Ceruti et al., 2005
β-ACTIN	TCCACGAAACTACCTTCAACTC	GTCATACTCCTGCTTGCTGAT	57.5	269	Designed in lab

553

554

555

556

557

558

559

560

561

562

563

564

565

566

567

568

569

570

Figure legends

Figure 1 The effects of acrylamide (ACR) and carnosic acid (CA) on cell viability. (A) Dose-dependent decreases in cell viability in ARPE-19 cells treated with ACR at different concentrations. (B) Dose-dependent decreases in cell viability in ARPE-19 cells treated with CA at different concentrations. (C) Microscopic images of ARPE-19 cells exposed to ACR and / or CA. (D) CA pre-treatment in ARPE-19 cells reversed ACR-caused toxic effects. All data are presented as the means $\pm$ standard error of three independent experiments. Statistical significance was analysed using on-way ANOVA test. UT, untreated control cells; ns, no significance; **P <0.01, ***P<0.001, ****P<0.0001. (E) Structure of acrylamide and carnosic acid.

Figure 2 Significant increases of apoptosis in ACR-exposed ARPE-19 cells detected by a TUNEL assay. (A) Nuclei of apoptotic cells were stained in green. Cells treated with DNase were used as positive control for apoptosis. ARPE-19 cells were exposed to 0.7 or 1mM of ACR only, or 0.7 mM ACR + 10 μ M CA, or 1 mM ACR + 10 μ M CA for 24 hours then stained with TUNEL reagents and DAPI to detect apoptotic cells. (B) Quantification of apoptotic cell number in 300 cells (in percentage). All data are presented as the means $\pm$ standard error of three independent experiments. Statistical significance of apoptotic cells between each group was analysed using one-way ANOVA test. **P<0.01, ***P<0.001, ****P<0.0001.

Figure 3 ACR exposure led to notably increased expression of caspase 3 and 9, pre-treatment with CA reversed the effects. (A) Agarose gel electrophoresis of qRT-PCR products. (B) Expression levels of caspase 3 in ARPE-19 cells treated with ACR only for 24 h or pre-treated with CA then treated with ACR for 24 h were measured by qRT-PCR. (C) Expression levels of caspase 9 in ARPE-19 cells treated with ACR only for 24 h or pretreated with CA then treated with ACR for 24 h were measured by qRT-PCR. All data are presented as the means $\pm$ standard error of three independent experiments. Statistical significance of

expression of caspase3 and 9 was analysed using one-way ANOVA test. UT, untreated control cells; ns, no significance; * $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 4 Pre-treatment with CA suppressed ACR-induced ROS production. ARPE-19 cells were exposed to ACR only or pre-treated with CA then treated with ACR. ROS production was measured using DCFH-DA staining. All data are presented as the means $\pm$ standard error of three independent experiments. Statistical significance was analysed using one way ANOVA test. UT, untreated control cells; *** $P < 0.001$, **** $P < 0.0001$.

Figure 5 ACR exposure led to notably decreased expression of antioxidant genes and pre-treatment with CA reversed the effects. (A) Agarose gel electrophoresis of qRT-PCR products. Expression levels of SOD1 (B), GPX1 (C), catalase (D), NQO-1 (E) and GCLM in ARPE-19 cells treated with ACR only for 24 h, or pre-treated with CA then treated with ACR for 24 h, were measured by qRT-PCR. All data are presented as the means $\pm$ standard error of three independent experiments. Statistical significance of expression of these anti-oxidant genes was analysed using one way ANOVA test UT, untreated control cells; ns, no significance; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 6 CA treatment reversed ACR-induced effects on the activities of SOD and catalase, the level of GSH and MDA. Activities of SOD (A) and catalase (B) were significantly decreased in ARPE-19 cells challenged with ACR only for 24 h, and notably increased when pre-treated with CA. (C) The generation of GSH was significantly decreased in ARPE-19 cells challenged with ACR only for 24 h, and notably increased when pre-treated with CA. (D) MDA production was significantly increased in ARPE-19 cells challenged with ACR only for 24 h, and notably decreased when pre-treated with CA. All data are presented as the means $\pm$ standard error of three independent experiments. Statistical significance was analysed using one-way ANOVA test. UT, untreated control cells. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$, **** $P < 0.0001$).

Figure 7 ACR exposure downregulated the expression of NRF2 and pre-treatment with CA reversed the effects. (A) Agarose gel electrophoresis of qRT-PCR products. (B) Expression levels of NRF2 in ARPE-19 cells treated with ACR only for 24 h or pre-treated with CA then treated with ACR for 24 h were measured by qRT-PCR. (C) Protein levels of NRF2 in ARPE-19 cells treated with ACR only for 24 h or pre-treated with CA then treated with ACR for 24 h were examined by western blotting. (D) Quantification of NRF2 protein levels normalized with GAPDH protein. All data are presented as the means $\pm$ standard error of three independent experiments. Statistical significance was analysed using one-way ANOVA test. UT, untreated control cells; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 2

672 **Figure 3**

673
674
675
676
677
678
679
680
681
682
683
684
685
686

687 **Figure 4**

688
689
690
691
692
693
694
695
696
697
698

Figure 6

711 **Figure 7**

712

713

714

715

716