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Published in:
Food and Chemical Toxicology

DOI:
10.1016/j.fct.2017.01.026

Publication date:
2017

Document Version
Peer reviewed version

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

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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 peroxyl. 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:

% of viable cells = [(absorbance of untreated cells - absorbance of treated cells) / absorbance of untreated cells] ×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′-Dichlorofluorescin 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% CO_2 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 60μM and found that cell viability was significantly decreased when treated with carnosic acid of 40, 50 or 60μM but was not significantly changed when treated with carnosic acid at 10, 20 or 30μM (Fig. 1B). Consequently, acrylamide at concentrations of 0.7 and 1.0mM and carnosic acid at concentration of 10μM were used for subsequent experiments. ARPE-19 cells first treated with carnosic acid (10μM) 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 (10μM) 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 (10μM) 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 (10μM) 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 10μM 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 (10μM) 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 μM) 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 eIF2α-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 H_2O_2-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 NFR-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 suported 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.

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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	CTCCTGGTGTCCGAACTGAT	57.0	218	Designed in lab
SOD 1	AGGGCATCATCAATTTCGAG	CATTGCCCAAGTCTCCAAC	55.0	217	Designed in lab
CAT	ATCTCGTTGGAAATAACACC	AGAAACCTGATGCAGAGACT	57.5	161	Designed in lab
NQO-1	CCTCTATGCCATGAACTT	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

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571 **Figure legends**

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

581

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

590

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

598 expression of caspase3 and 9 was analysed using one-way ANOVA test. UT, untreated
599 control cells; ns, no significance; *P>0.05, **P<0.01, ***P<0.001, ****P<0.0001.
600
601 **Figure 4** Pre-treatment with CA suppressed ACR-induced ROS production. ARPE-19 cells
602 were exposed to ACR only or pre-treated with CA then treated with ACR. ROS production
603 was measured using DCFH-DA staining. All data are presented as the means± standard
604 error of three independent experiments. Statistical significance was analysed using on way
605 ANOVA test. UT, untreated control cells; ***P<0.001, ****P<0.0001.
606
607 **Figure 5** ACR exposure led to notably decreased expression of antioxidant genes and pre-
608 treatment with CA reversed the effects. (A) Agarose gel electrophoresis of qRT-PCR
609 products. Expression levels of SOD1 (B), GPX1 (C), catalase (D), NQO-1 (E) and GCLM in
610 ARPE-19 cells treated with ACR only for 24 h, or pre-treated with CA then treated with ACR
611 for 24 h, were measured by qRT-PCR. All data are presented as the means± standard error
612 of three independent experiments. Statistical significance of expression of these anti-oxidant
613 genes was analysed using one way ANOVA test UT, untreated control cells; ns, no
614 significance; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
615
616 **Figure 6** CA treatment reversed ACR-induced effects on the activities of SOD and catalase,
617 the level of GSH and MDA. Activities of SOD (A) and catalase (B) were significantly
618 decreased in ARPE-19 cells challenged with ACR only for 24 h, and notably increased when
619 pre-treated with CA. (C) The generation of GSH was significantly decreased in ARPE-19
620 cells challenged with ACR only for 24 h, and notably increased when pre-treated with CA. (D)
621 MDA production was significantly increased in ARPE-19 cells challenged with ACR only for
622 24 h, and notably decreased when pre-treated with CA. All data are presented as the
623 means± standard error of three independent experiments. Statistical significance was
624 analysed using one-way ANOVA test. UT, untreated control cells. *P<0.05, **P<0.001,
625 ***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± 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.

654 **Figure 1**

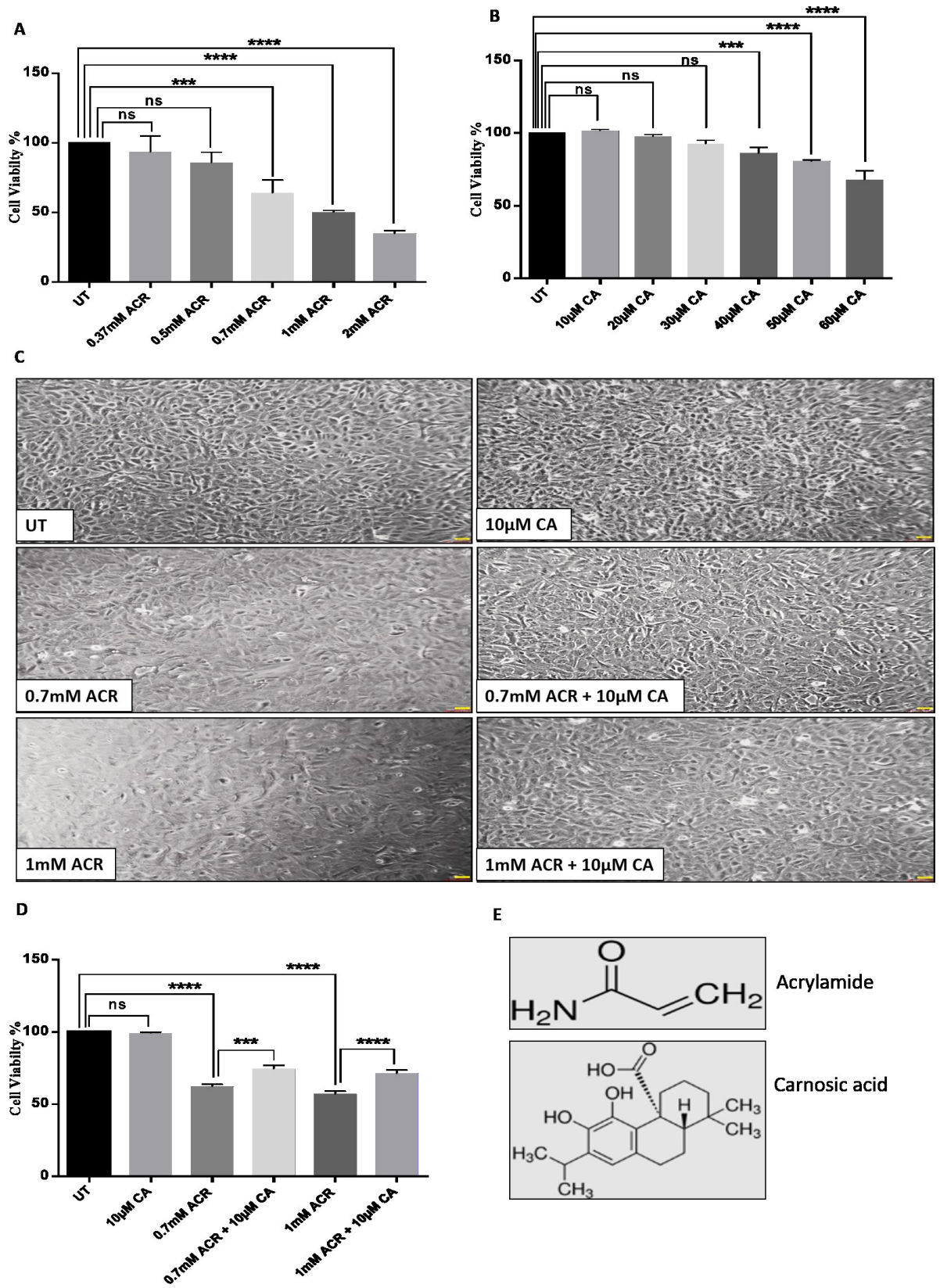

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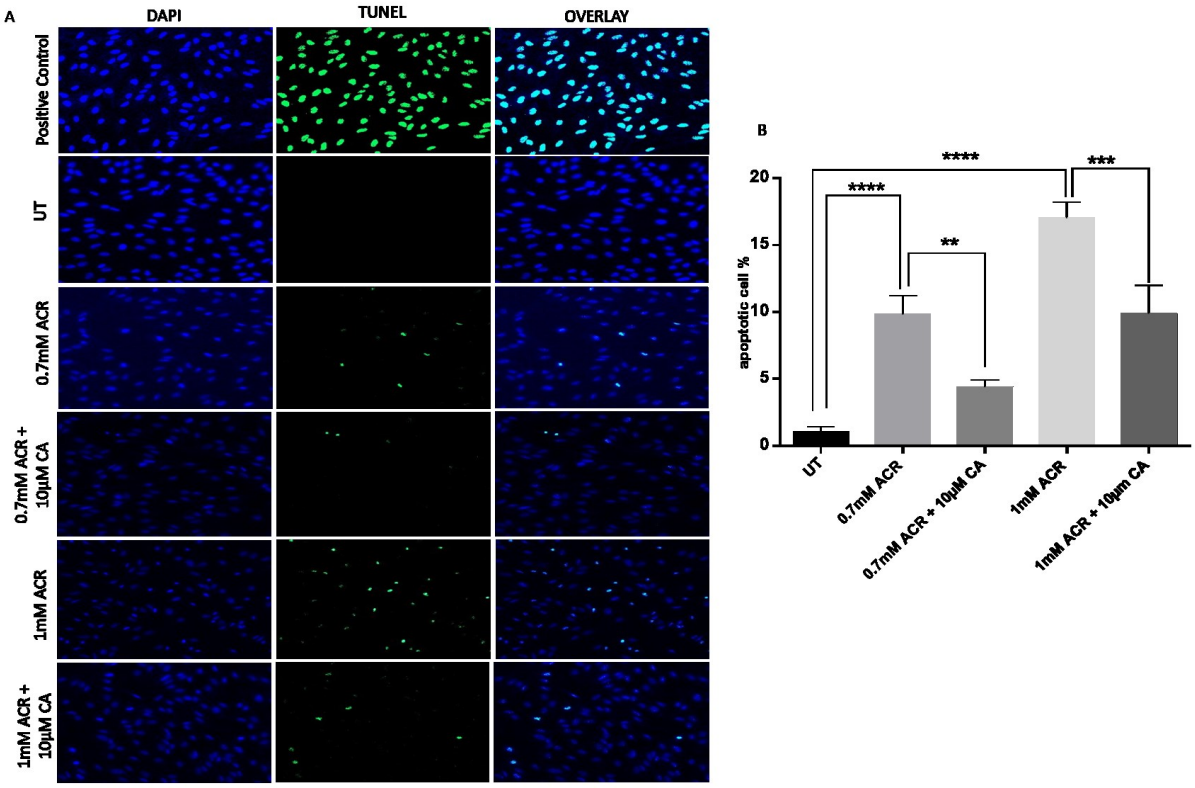
Figure 2


A
DAPI
TUNEL
OVERLAY
Positive Control
UT
0.7mM ACR
0.7mM ACR + 10µM CA
1mM ACR
1mM ACR + 10µM CA
B
apoptotic cell %
0
5
10
15
20
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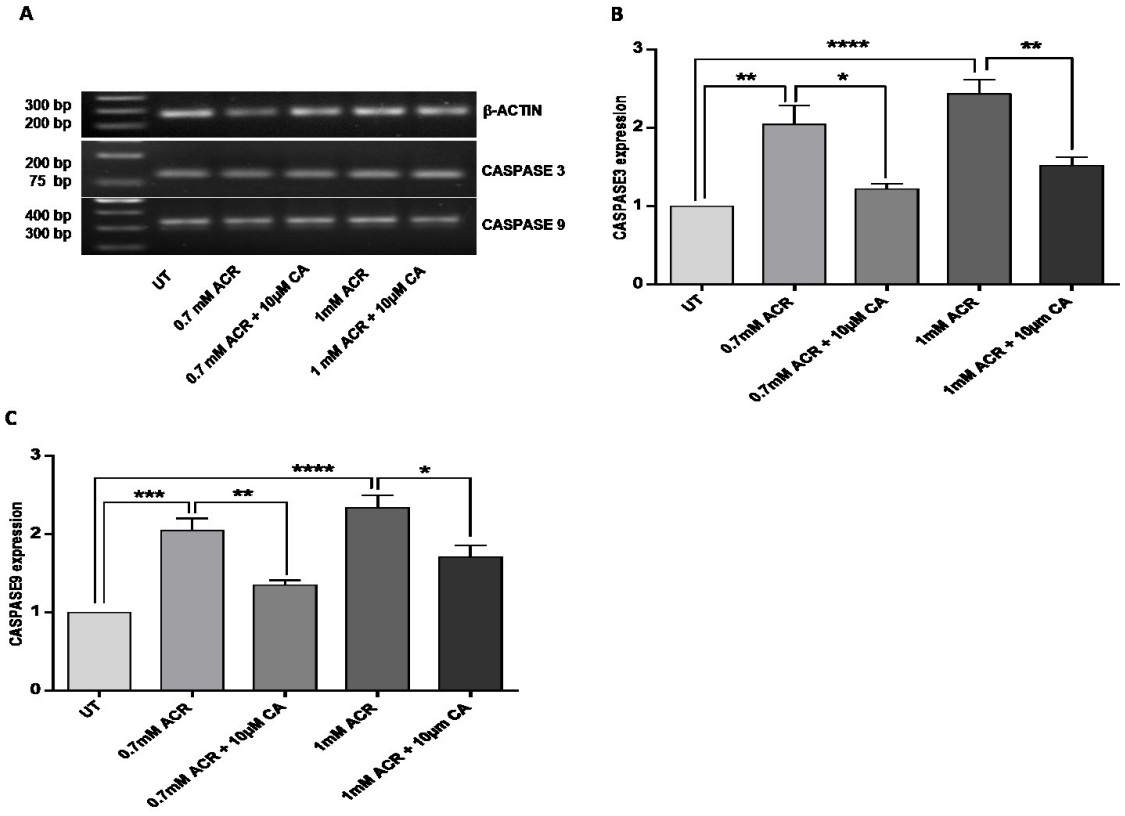
**
UT
0.7mM ACR
0.7mM ACR + 10µM CA
1mM ACR
1mM ACR + 10µm CA

672 **Figure 3**



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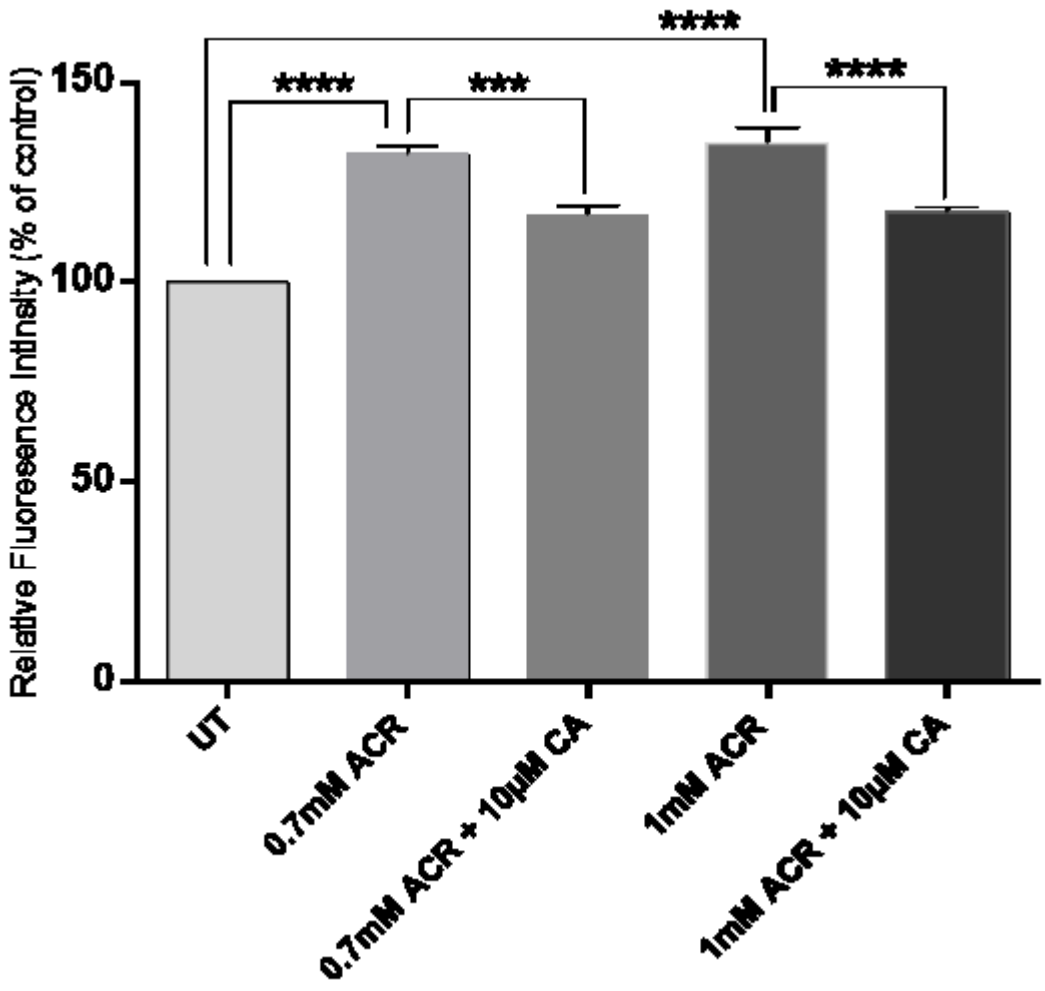
687 **Figure 4**



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699 **Figure 5**

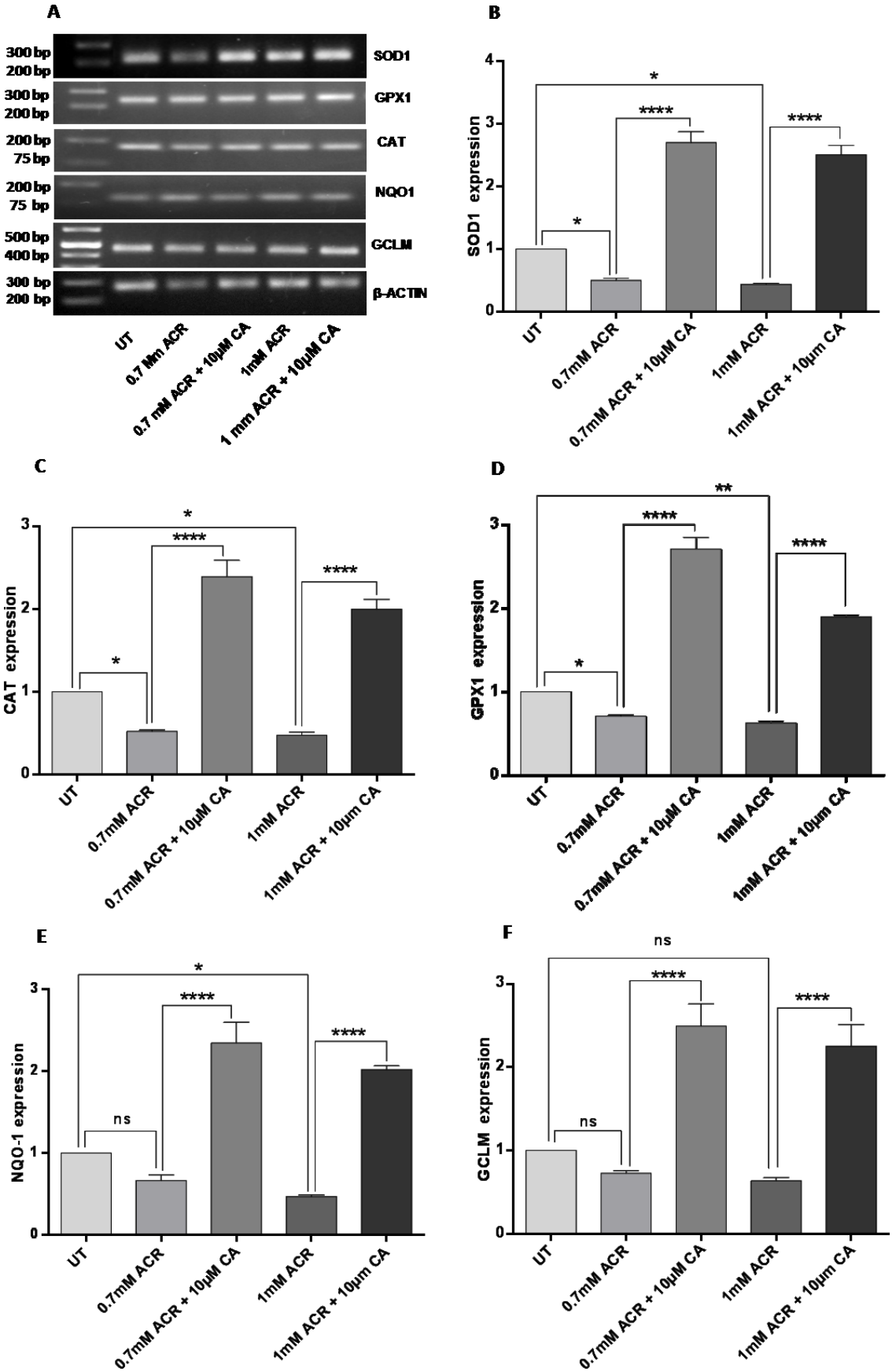

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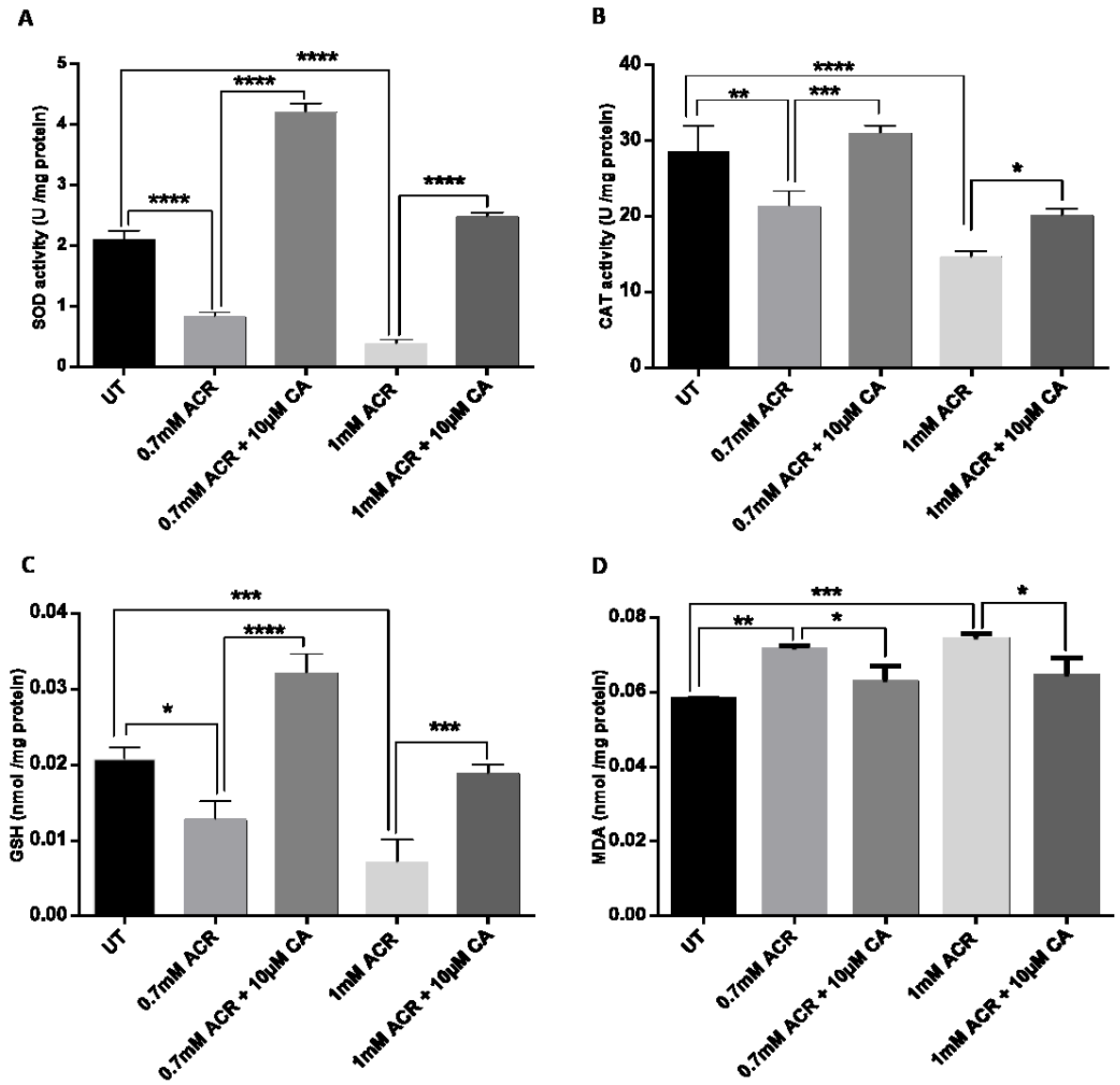
Figure 6


A
SOD activity (U /mg protein)
0
1
2
3
4
5
****
****
****
****
UT
0.7mM ACR
0.7mM ACR + 10μM CA
1mM ACR
1mM ACR + 10μM CA
B
CAT activity (U /mg protein)
0
10
20
30
40
****
**
***
*
UT
0.7mM ACR
0.7mM ACR + 10μM CA
1mM ACR
1mM ACR + 10μM CA
C
GSH (nmol /mg protein)
0.00
0.01
0.02
0.03
0.04
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*
****
***
UT
0.7mM ACR
0.7mM ACR + 10μM CA
1mM ACR
1mM ACR + 10μM CA
D
MDA (nmol /mg protein)
0.00
0.02
0.04
0.06
0.08
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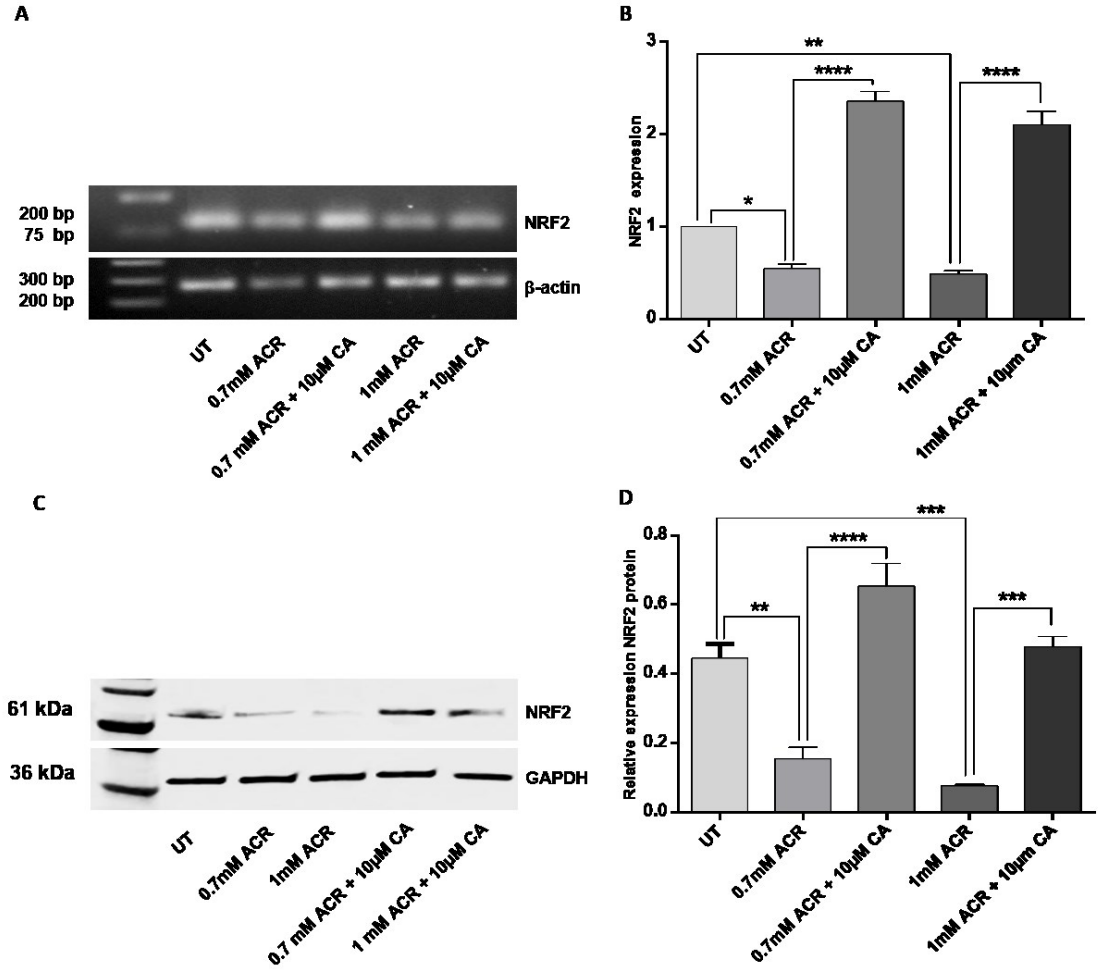
UT
0.7mM ACR
0.7mM ACR + 10μM CA
1mM ACR
1mM ACR + 10μM CA

711 **Figure 7**



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