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Toxicity of a seafood toxin, domoic acid, in the retina via modulation of the NRF2 and NF-κB pathways

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ABSTRACT: Domoic acid (DA), a biotoxin, is produced by several species of marine dinoflagellate and diatom during harmful algal bloom events. DA is a neurotoxin, in humans and non-human primates, oral exposure to DA results in gastrointestinal effects, while DA at higher doses leads to neurological symptoms, seizures and memory deficiency. Exposure of humans to DA occurs mainly through consumption of contaminated seafoods containing an accumulation of the toxin. Previously, it was unclear if DA can have toxic effects on the retina. We assessed the toxicity of DA in human retinal cells (ARPE-19) and in zebrafish embryos. DA significantly lowered ARPE-19 cell viability dose-dependently, and decreased anti-oxidative capacity, increased inflammation, and promoted cell death, possibly through modulating the NRF2 and NF-κB signalling pathways. Zebrafish embryos exposed to DA for four days from one day post fertilization (dpf) had an increase in mortality and a decrease in both hatching and heartbeat rate and exhibited morphological abnormalities. DA treatment also significantly downregulated expression of antioxidant genes and upregulated expression of inflammation mediators, as well as causing photoreceptor death in zebrafish embryos. The results demonstrate that consuming seafood containing DA will have potential toxic effects in human retinas.

Keywords: Seafood toxin; domoic acid; human retinal pigment epithelial cells; zebrafish embryos; photoreceptors

1. Introduction

Historically, the first registered case of fatal human poisoning caused by the consumption of mussels was in California in 1927 (Ferrari, 2001). At that time, researchers had identified a relatively strong microalga producing toxins from a mussel. Therefore, it was suspected that this species was associated with the human poisoning, resulting in death by consumption of mussels in that part of the world (Marr et al, 1992). Later, in 1976 in Galicia, toxins from the paralytic shellfish poisoning (PSP) group were identified. Between 1978 and 1982, several poisoning cases were recorded in Europe, all associated with the consumption of bivalve mollusks, the most notable of which occured in 1981 when nearly 5,000 cases were registered (Vale, 2004). Records of these cases could not identify a diarrheic shellfish poisoning (DSP) group in mussels, as liquid

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chromatography analyses were not performed. From 1986, the number of cases of human poisoning was gradually rising, and researchers stated that this was associated with the group of DSP toxins (Tubaro et al., 2003). In 1987 in Canada, domoic acid (DA) was identified for the first time as the cause of poisoning following consumption of mussels (Perl et al., 1990). 99 of the 107 patients who met the case definition for shellfish poisoning completed a questionnaire and reported symptoms that included vomiting (76%), abdominal pain (51%), diarrhoea (42%), headache (43%), and short-term memory loss (25%). 12 of the 19 patients who were hospitalized required intensive care treatment for experiencing, in addition to one or more of the above symptoms, profuse respiratory secretions, blood pressure instability and, in more serious cases, convulsions and coma (Perl et al., 1990).

DA is a biotoxin that is produced mainly by the diatomic algal genus of *Pseudo-nitzschia*, which contains 52 species, 26 of which are considered to be toxigenic (Hasle, 2002; Bates et al., 2018). The most well-known species are *P. nitzschia, P. delicatissima australis, Chondria armata* and *P. nitzschia multiseries*. DA is usually produced during an algal bloom and accumulates in herbivorous fish, zooplankton and shellfish (Silvagni et al., 2005; Tubaro & Hungerford, 2007). The neurotoxic effects of DA result from the interaction with several subtypes of receptors related to ionotropic glutamate in synaptic terminals (Pulido, 2008). Therefore, regions in which these receptors are distributed are typically affected after exposure to DA. Ramsdell & Zabka (2008) demonstrated that DA is an excitatory amino acid that competes for the receptors for glutamic acid in synaptic regions, causing depolarization of neurons and increasing intracellular calcium concentration that results in incessant activation of calcium-sensitive enzymes, with consequent energy depletion, turgidity and cell death. DA exposure causes neurological damage by astrocytosis in the hippocampus, the brain structure responsible for learning and memory (Pulido, 2008). Severe damage in humans could lead to short-term memory loss.

The toxicity effect of DA has also been associated with some functional abnormalities in other organs, such as those of the cardiovascular system (Ramsdell & Zabka, 2008). DA exposure has been shown to cause defects in cardiac development in zebrafish and damage to the heart of rats (Hong et al., 2015; Vieira et al., 2016), while long-term environmental exposure to DA increased the risk of heart disease in southern sea otters (Moriarty et al., 2021). Studies of DA targeting on reproduction indicate that it may affect the fetal development of some mammalian species (Gwaltney-Brant et al., 2007). Although there are no reports of the effects of DA on pregnant women, exposure of some mammals to DA in natural and experimental studies have demonstrated a negative impact on reproduction. For example, California sea lions exposed to DA faced reproductive failure, leading to abortion and premature parturition (Ramsdell & Zabka, 2008). Oral or intraperitoneal administration of DA resulted in accumulation of DA predominantly in animal kidneys (Lefebvre et al., 2007; Funk et al., 2014). DA exposure in mice also caused kidney damage, including tubule cell desquamation, epithelial cell vacuolization and swelling, and cell death (Funk et al., 2014). However, there is limited information related to the toxicity of DA in the retina.

In the current study, the toxic effects of DA were assessed in human retinal pigment epithelial (RPE) cells and in zebrafish embryos, with the underlying toxic mechanisms elucidated using biochemical, molecular biological and histological approaches.

2. Materials and methods

2.1 Cell treatment

ARPE-19 cells (ATCC® CRL- 2302™) were seeded at a density of 3.0×10^4 cells per well in 96-well plates and incubated for 24 hours at 5% CO_2 and 37℃. Confluent cells were exposed to DA in different concentrations (0.0, 0.25, 0.50, 1.0, 2.5, 5.0, 7.5 and 10.0 μmol/L) for 24 hours. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was applied in order to assess the cell viability, according to the manufacturer's instructions. For other experiments, cells were grown in 96-well plates (3.0×10^4 cells/well) or 6-well plates (5.0×10^5 cells/well) for 24 hours then treated with 5 μmol/L DA for 24 hours, followed by individual analyses.

2.2 Zebrafish embryo treatment

All zebrafish work was performed followed the UK Office Animal Care regulations (Project License 70/8697). For initial toxicity assessment, zebrafish embryos at 1 day post fertilization (dpf) were exposed to 0, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 μmol/L of DA till 5 dpf. During the period of the treatment, hatching, heartbeat, morphological abnormality and mortality were monitored daily and recorded. For other experiments, zebrafish embryos were administrated with/without with 5 μmol/L DA from 1 dpf to 5 dpf, then homogenized in buffer containing 0.32 mmol/L sucrose, 20Mm HEPES, 1 mmol/L $MgCl_2$ and 0.5 mmol/L of phenylmethyl sulfonylfluoride (pH 7.4), followed by a centrifugation (15.000 × g for 20 minutes) at 4℃. Supernatants were collected and subjected to individual analyses.

2.3 Reactive oxygen species (ROS) production

To measure production of reactive oxygen species cells (ROS) in ARPE-19 cells, 3.0×10^4 cells/well were cultured for 24 hours in clear-bottomed black 96-well plates then treated with/without 5 μmol/L DA and incubated for 24 hours. The medium was discarded and cells were incubated with DCFH-DA (6-Carboxy-20,70- Dichlorofluorescin diacetate) for 30 minutes. ROS was measured according to the manufacture guidance. In order to measure ROS generation in control or DA-treated zebrafish embryos, 20 μL of supernatant was allocated to each well, to which DCFH-DA solution was added, then incubated for 30 minutes at 37 ℃ in the dark. ROS was measured according to the manufacturer's protocol.

2.4 Measurement of caspase 3/7 activities

Confluent ARPE-19 cells in a 96-well plate were treated with/without 5 μmol/L DA for 24 hours and then equilibrated at room temperature. Following this, 100μl of caspase Glo 3/7 reagent was carefully added to the cultured medium of each well containing treated and untreated cells. The sample was gently mixed on a plate shaker at 300 r/min for 1 minute, followed by incubation for 2 hours at room temperature. Luminescence was measured using a plate-reading luminometer, according to the manufacturer's protocol.

2.5 Measurement of catalase activity and levels of malondialdehyde and glutathione

Catalase (CAT) activity in DA untreated or treated ARPE-19 cells or zebrafish was measured using a CAT kit (Cell Biolabs, STA-341) following the manufacturer's protocol. Levels of malondialdehyde (MDA) and glutathione (GSH) were quantified using, respectively, an MDA kit (Cell Biolabs, STA-312) and a GSH kit (Cell Biolabs, STA-330), according to the manufacturers' instructions.

2.6 Enzyme-linked immunosorbent assay (ELISA)

Presence of secreted cytokines – human IL-1β, IL-6, IL-8 and TNF-α – in the medium of ARPE-19 cells treated with/without 5μmol/L DA was determined using, respectively, human IL-1β Mini ABTS ELISA Development Kit, human IL-6 Mini ABTS ELISA Development Kit, and Human IL-8 Mini ABTS ELISA Development Kit and human TNF-α Mini ABTS ELISA Development Kit (PeproTech), according to the manufacturer's protocol.

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

Total RNA was extracted from ARPE-19 cells or zebrafish embryos treated with/without 5 μmol/L DA using TRI Reagent (Sigma UK). The cDNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific, UK) in accordance with the manufacture's protocol. MRNA level of targeted genes was detected using SYBR Green real-time PCR mix (Thermo Fisher, UK) according to the manufacturer's guidance and calculated using the $2^{-\Delta\Delta CT}$ formula. The primer sequences used in the current study are shown in Tables S1 and S2.

2.8 Western blot

5 μmol/L DA treated/untreated ARPE-19 Cells were lysed with ice-cold cytoplasmic or nuclear extraction buffer (MERCK), according to the manufacturer's protocols. 50μg proteins were separated in SDS-PAGE then transferred to nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 5% (w/v) milk powder, incubated with primary antibody and then secondary antibody. Signals of target proteins were detected using the Image StudioTM Lite analysis software (LI-COR).

2.9 Cell death detection

5 μmol/L DA treated/untreated ARPE-19 Cells on round coverslips were fixed with cold paraformaldehyde (4%, w/v) for 20 minutes, washed with 1xPBS three times and permeabilised with Triton® X- 100 solution in 1 × PBS (0.2%, v/v) for 5 minutes, followed by washing twice with cold 1xPBS. Apoptotic cells were detected using a DeadEndTM fluorometric TUNEL assay kit (Promega) according to the manufacturer's guidelines, and imaged by a ZEISS LSM 800 confocal microscope.

2.10 Cytoskeletal staining

5 μmol/L DA treated/untreated ARPE-19 Cells on round coverslips were fixed with cold 4% Paraformaldehyde for 30 minutes and washed with 1xPBS. The cells were permeabilised with 0.1% Triton® X-100 solution in 1 × PBS for 5 minutes. After washed with 1xPBS, the cells were incubated with 5μg/ml FITC-labelled phalloidin (targeting the F-actin microfilaments, Sigma, UK) at room

temperature for 90 minutes. The nuclei were stained with 0.5 mg/ml DAPI (Sigma, UK) and the cytoskeletal structure was imaged under a confocal microscope.

2.11 Histological analysis

5 μmol/L DA treated/untreated zebrafish embryos were fixed with 4% paraformaldehyde (w/v) and dehydrated with ethanol at 10%, 30%, 50%, 70%, 80%, 90% and 100%. After dehydration, the samples were further submerged in histoclean, embedded in paraffin, then sectioned using a microtome. The sections were stained with haematoxylin and eosin based on a protocol. Fixed Zebrafish embryos were also cryoprotected with 5%, 20% and 30% sucrose in 1 × PBS, embedded in OCT and cut in a cryostat. The cryosections were subjected to immunostaining with incubation with primary and secondary antibodies.

2.12 Data analysis

Data was statistically analysed with the GraphPad Prism software (GraphPad Software Inc. San Diego, CA, version 9.0). The pairwise comparisons were performed using a nonparametric t-test, one-way or two-way ANOVA followed by an appropriate *post hoc* test. The significance was reached when $P < 0.05$.

3. Results

3.1 Effect of DA on RPE cell viability

DA is a neurotoxin widely reported as an ionotropic receptor activator in cell membranes, causing interference with cell function that results in cell swelling and ultimately cell death (Lefebvre & Robertson, 2010). To assess the toxicity of DA on human RPE cells, we used an MTT assay on ARPE-19 cells exposed to DA at various concentrations (0, 0.50, 1.0, 2.5, 5.0, 7.5, 10.0 μmol/L). The result indicated no significant difference in cell viability treated with 0.5 μmol/L DA compared to the untreated cells. However, DA significantly decreased viability dose-dependently, reduced cell viability by 21.7% ($P < 0.05$) in cell treated with 1 μmol/L, by 27.4% ($P < 0.05$) in 2.5 μmol/L, and by 38% ($P < 0.01$) in 5 μmol/L compared to the untreated control cells (Figure S1); it also decreased cell viability by 51.7% ($P < 0.001$) when treated with 7.5 μmol/L and by 73% ($P < 0.0001$) in ARPE-19 cells exposed to 10 μmol/L (Figure S1). Exposure to concentrations of DA of 1μm and above resulted in a significant decrease in cell viability. The concentration of 5μm was more toxic ($P < 0.01$) than the concentrations of 1μm and 2.5μm ($P < 0.05$) and less toxic than the concentrations of 7.5μm and 10μm ($P < 0.001$ and $P < 0.0001$, respectively). Accordingly, we chose the concentration of 5μm for further experiments.

3.2 DA caused oxidative stress in RPE cells

Previous studies have demonstrated that DA exposure results in a significant increase in ROS production in neuron-related cell lines (Gajski et al., 2020). We measured ROS production in ARPE-19 cells treated with/without 5 μmol/L DA for 24 hours. The result showed that ROS level in treated cells was significantly higher by 55.5% ($P < 0.001$) compared to untreated cells (Figure 1A). CAT uses hydrogen peroxide as a substrate that breaks down to maintain an optimal level of the molecule in the cells, an essential mechanism for maintenance of cell function (Nandi et al., 2019). We investigated the effects of DA

on CAT activity in untreated and DA-treated RPE cells. Our data showed that DA caused a significant decrease in CAT activity by 27% ($P < 0.001$) compared to the controls (Figure 1A). Given that GSH has been described as playing an essential role in retinal cell antioxidant homeostasis by scavenging ROS or acting as a critical cofactor for GSH S-transferases and peroxidases (Gajski et al., 2020), we measured its levels in cells exposed to DA for 24h and in those untreated for 24h. Our data showed a decrease by 96% ($P < 0.0001$) in the treated group compared to the untreated control group (Figure 1A). MDA can indicate a cell membrane injury and act as a biomarker of lipid peroxidation. An imbalance in free radicals affects MDA production; therefore, we quantified the MDA level in ARPE-19 cells treated with or without 5 μmol/L DA for 24h. Our results show that the MDA level was significantly increased by 37% ($P < 0.001$) compared to the untreated control group (Figure 1A).

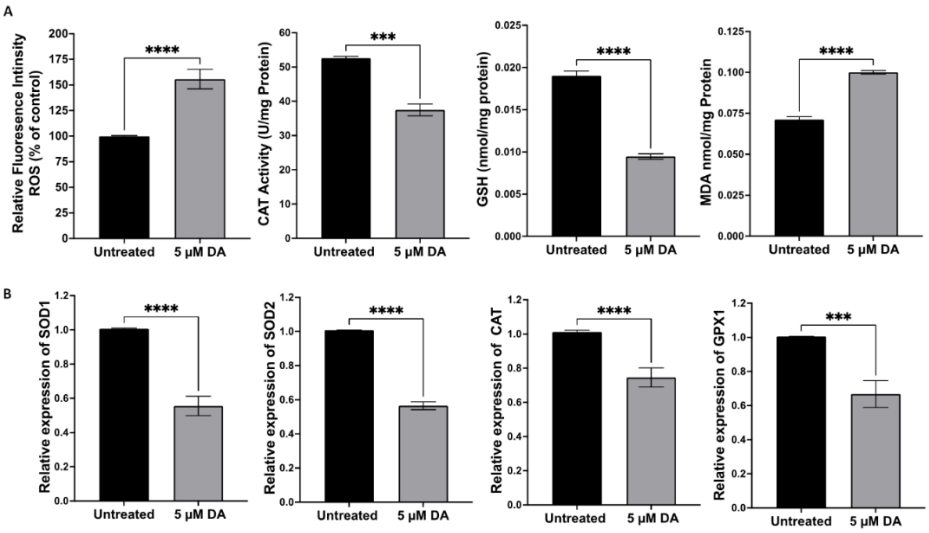

Figure 1. Effect of domoic acid (DA) on anti-oxidative capacity in human retinal pigment epithelial cells. (A) Reactive oxygen species (ROS) production, catalase (CAT) activity, GSH and MDA levels. (B) Expression of antioxidant genes. Data were analysed by a nonparametric *t* test and are displayed as mean ± SEM (n=3). ***$P < 0.001$; ****$P < 0.0001$.

Since DA induced ROS overproduction, we examined whether the cellular imbalance of the antioxidant system is linked with the change of related gene expression. We quantified the expression of the targeted genes – *SOD1* (superoxide dismutase 1), SOD2 (superoxide dismutase 2), *CAT* and *GPX1* (glutathione peroxidase 1) – using qRT-PCR and found that *SOD1* exhibited a significant decrease in mRNA level by 43.5% ($P < 0.0001$) in the cells treated with DA, while *SOD2* expression was significantly decreased by 44.1% ($P < 0.0001$) compared to the untreated cells. In addition, our results also showed a marked reduction in the expression of these genes by 26% ($P < 0.0001$) for *CAT* and 32% ($P < 0.001$) for *GPX1* in the ARPE-19 cells treated with DA for 24h compared to the untreated control group (Figure 1B).

Given that NRF2 regulates expression of antioxidant response element–dependent genes to mediate the physiological and pathophysiological processes associated with oxidant exposure, we investigated whether DA exposure alters the relative expression of NRF2. Our data showed that ARPE-19 cells exposed to DA for 24h had a significant decrease by 24% ($P < 0.001$) in mRNA level measured by qRT-PCR (data not shown). We also observed a significant decrease in its protein level in both cytosolic and nuclear fractions by,

respectively, 17.7% ($P < 0.001$) and 16.9% ($P < 0.01$), when compared to the untreated cells (Figures 2 and S2).

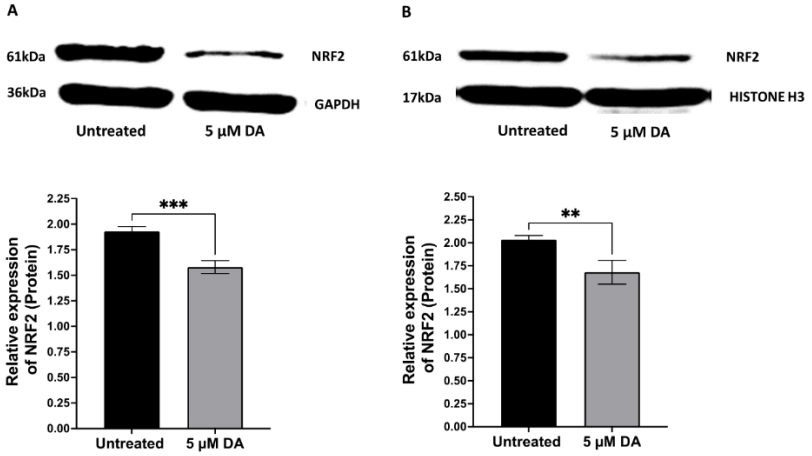

Figure 2. NRF2 in cytosolic (A) and nuclear (B) fractions of untreated and DA-treated ARPE-19 cells, detected by Western blot and quantified by normalizing GAPDH and HISTONE H3, respectively. Data were analysed by a nonparametric *t* test and are displayed as mean ± SEM (n=3). ** $P < 0.01$; ***$P < 0.001$.

3.3 DA induced inflammation in RPE cells

DA treatment resulted in oxidative stress, which stimulates inflammation. Accordingly, we used ELISA to quantify the secreted proinflammatory mediators IL-1β, IL-6, IL-8 and TNFα in the media of cells treated with/without 5 μmol/L DA for 24hours. The data showed a significant increase in these cytokines by, respectively, 67.1% ($P < 0.001$), 158.2% ($P < 0.0001$), 57.8% ($P < 0.001$) and 53.1% ($P < 0.001$) when compared to levels in untreated cells (Figure 3A). We also examined the expression of the relevant mediator genes by qRT-PCR and found that the mRNA level of *IL-1β* was increased by 63.3% ($P < 0.0001$), *IL-6* was increased by 77.8% ($P < 0.0001$), *IL-8* was increased by 83.3% ($P < 0.0001$) and *TNFα* expression was increased by 83.8% ($P < 0.0001$), compared to untreated cells (Figure 3B).

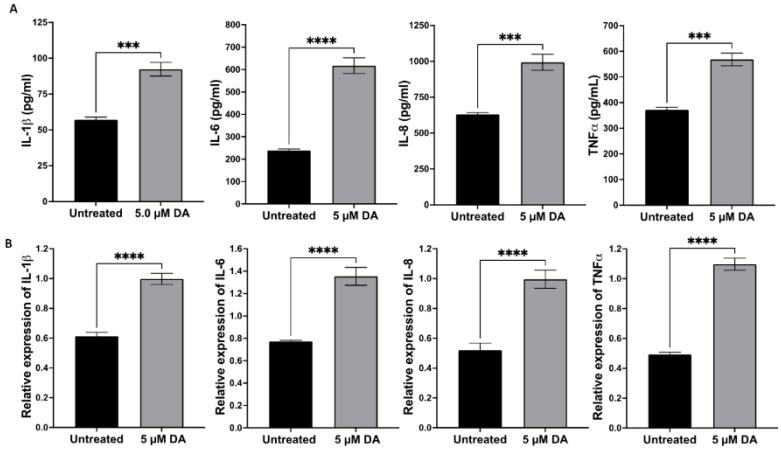

Figure 3. DA exposure increased expression of proinflammatory mediators. (A) Secreted IL-1β, IL-6, IL-8 and TNFα in untreated and DA-treated ARPE-19 cells detected by ELISA. (B) Quantification of *IL1-β, IL-6, IL-8 and TNFα* mRNA levels in untreated and DA-treated ARPE-19 cells, measured by qRT-PCR and normalized to the housekeeping gene, *GAPDH*. Data were analysed by a nonparametric *t* test and are displayed as mean ± SEM (n=3). *** $P < 0.01$; ****$P < 0.0001$.

NF-κB activation induces transcription of proinflammatory genes and has been reported as a central proinflammatory mediator that plays a simultaneous role in innate and adaptive immune cells. The pathway can be activated upon a specific cellular stimulation usually related to pathogens or stress signals (Tak &

Firestein, 2001). We quantified the level of p65, a component of NF-κB complex, in both cytosolic and nuclear fractions of ARPE-19 cells treated with or without 5 μmol/L DA. P65 level was significantly higher in the cytosolic fraction by 102% ($P < 0.0001$) and increased in nuclear fraction by 59.2% ($P < 0.01$), when compared to the untreated cells (Figures 4 and S3), implicating activation of NF-κB signalling pathway.

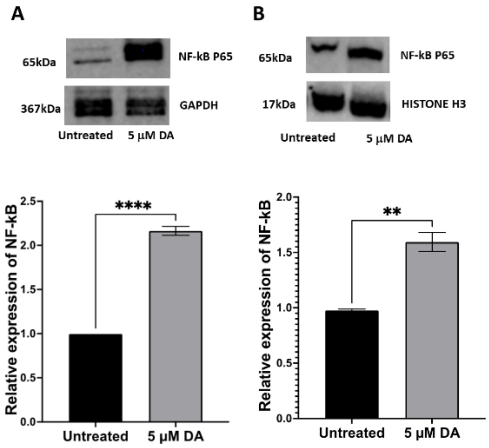

Figure 4. NF-kB p65 protein levels in cytosolic (A) and nuclear fractions (B) of untreated and DA-treated ARPE-19 cells, detected by Western blot and quantified by normalizing GAPDH and HISTONE H3, respectively. Data were analysed by a nonparametric *t* test and are displayed as mean ± SEM (n=3). ** $P < 0.01$; **** $P < 0.0001$.

3.4 DA caused RPE cell apoptosis

Given that DA exposure decreased RPE cell viability and caused oxidative stress and inflammation, we used a TUNEL assay to detect whether DA caused cell death. ARPE-19 cells exposed to 5 μmol/L DA for 24 hours had an increase by 1690% ($P < 0.001$) in cell death, compared to the untreated control group (Figure 5).

We also examined whether or not this DA-induced cell death is caspase-dependent, initially by quantifying the expression of caspase-3 gene. We found that DA exposure significantly increased the mRNA level of caspase-3 in RPE cells by 92.9% ($P < 0.0001$) compared to the untreated control group (Figure S4A). We also measured caspase 3/7 activities and found a marked increase by 249.3% ($P < 0.0001$) in cells exposed to 5 μmol/L DA compared to untreated cells (Figure S4B).

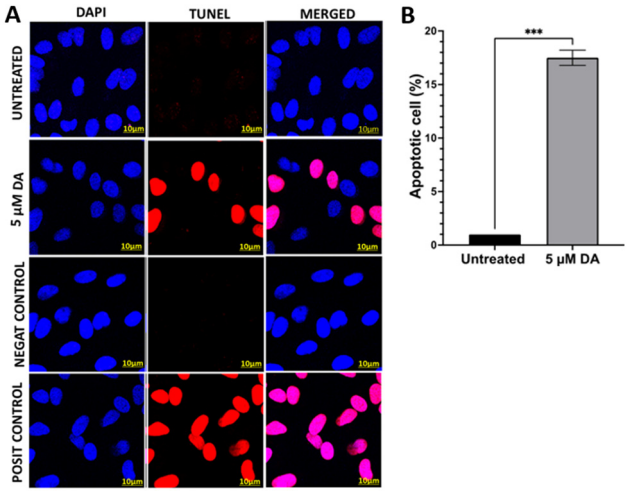

Figure 5. DA exposure induced apoptosis in ARPE-19 cells. Treated cells significantly underwent increased apoptosis as detected by a TUNEL assay. (A) Images of dead cells in untreated and DA-treated conditions. The positive control was treated with DNase. Samples were stained with TUNEL reagents (red) and co-stained with DAPI (blue) to detect apoptotic cells. Scale bar: 10μm. (B) Quantification of apoptotic cells using 300 cells as a base and presented as a percentage [(number of

apoptotic cells/300) × 100]. Data were analysed by a nonparametric *t* test and are displayed as mean ± SEM (n = 3). *** $P < 0.001$.

Early studies reported that disruption of the cytoskeleton induces apoptotic cell death (Kulms et al., 2002; Hinoue et al., 2005). A recent study showed that DA treatment downregulates genes involved in the maintenance of axonal cytoskeleton (Panlilio et al., 2020). To determine whether the structure of the cytoskeleton was disrupted by DA exposure, we stained the ARPE-19 cells with phalloidin and found that DA-exposed cells had significantly disrupted filaments. The untreated cells exibited long and well-structured filaments; by contrast, DA-treated cells had shortened filaments (Figure 6), suggesting that disruption of the cytoskeleton may contributedto DA-associated apoptosis.

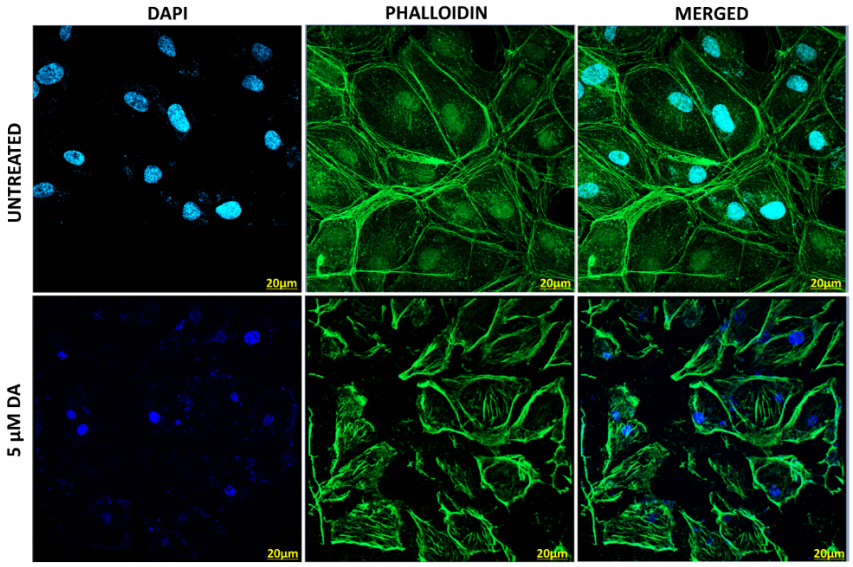

Figure 6. DA exposure caused cytoskeleton damage in ARPE-19 cells. Treated cells exhibited a significant disruption in cytoskeleton compared to the untreated control cells. Cells were stained with phalloidin and visualized under a confocal microscope to detect the filaments, and nuclei were labelled with DAPI (blue). Scale bar: 20µm.

3.5 Toxic effects of DA in zebrafish embryos

To assess the toxicity of DA in the zebrafish embryos, we exposed the embryos to DA at different concentrations (0.5, 1.0, 2.5, 5.0, 7.5 and 10 µmol/L) from 1dpf to 5dpf and monitored mortality, hatching, heartbeat and malformations. Zebrafish embryos treated with 0.5, 1.0 or 2.5 µmol/L DA did not cause a significant difference in mortality after two days of administration, whereas there was an increased mortality rate in embryos exposed to 5.0 µmol/L by 22% ($P < 0.05$), 7.5 µmol/L by 38% ($P < 0.01$) and 10 µmol/L by 72% ($P < 0.0001$) following treatment of two days (Figure S5). The mortality rate was proportional to the period of exposure and drug concentration; all embryos exposed to 10 µmol/L were dead within three days of treatment. The hatching rate was notably decreased by 40 % ($P < 0.001$), 54% ($P < 0.001$) and 65% ($P < 0.001$) in embryos exposed to, respectively, 5 µmol/L, 7.5 µmol/L or 10 µmol/L at 2dpf (24 hours of exposure) compared to the untreated embryos (Figure S5). Heartbeat was also slightly decreased by 10.4% ($P < 0.01$) in embryos exposed to 5.0 or 10 µmol/L DA compared to the control group. Morphological abnormalities including curved body axis and pericardial oedema were also monitored. Embryos exposed to 0.5, 1.0, 2.5 or 5.0 µmol/L DA displayed normal morphology until 5dpf, whereas samples exposed to 7.5 or 10 µmol/L DA

demonstrated abnormalities until 3dpf. Based on these observations, 5 μmol/L DA was chosen for subsequent experiments.

3.6 DA exposure caused oxidative stress and inflammation in zebrafish embryos

Our *in vitro* data from human retinal cells showed that DA exposure caused oxidative stress and inflammation; consequently, we also investigated oxidative stress and inflammation in DA-exposed embryos. We found that ROS was markedly increased by 52% ($P < 0.0001$) in DA-treated embryos compared to the untreated control group. Zebrafish embryos exposed to DA also had a significant decrease in Catalase activity by 23.4% ($P < 0.001$), GSH level was notably reduced by 54.4% ($P < 0.001$), while MDA level was significantly increased by 32.2% ($P < 0.001$) in DA exposed embryos compared to the control group (Figure 7A). We further examined the effect of DA on expression of some antioxidant genes and found that DA-exposed embryos had a significant decrease in *sod1, sod2, cat* and *gpx1* genes by, respectively, 23.4% ($P < 0.001$), 26.2% ($P < 0.001$), 27.1% ($P < 0.0001$), 48.3% ($P < 0.0001$) compared to the controls (Figure 7B).

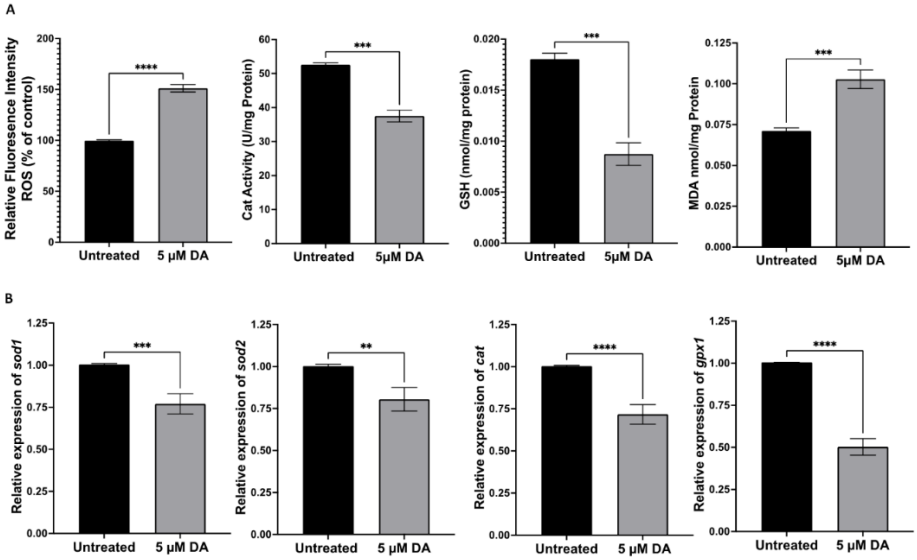

Figure 7. Effect of DA exposure on oxidative stress in zebrafish embryos. (A) Increased ROS production, decreased catalase (Cat) activity and GSH levels, and increased MDA level in DA-treated embryos. (B) Lowered expression of sod1, sod2, catalase (cat) and gpx1 in DA-treated embryos, compared to untreated embryos. Data were analysed by a nonparametric *t* test and are displayed as mean ± SEM (n=3). ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

As oxidative stress induces inflammation, we also investigated whether DA exposure resulted in inflammation in the zebrafish embryos. We measured expression of *il-1β, il-6, il-8* and *tnfα* by qRT-PCR. Our result showed a significant increase in expression of these cytokine genes by, respectively, 63.3% ($P < 0.0001$), 34.8% ($P < 0.001$), 67.6% ($P < 0.0001$), and 58.2% ($P < 0.001$) in DA-exposed embryos compared to the controls (Figure S6).

3.7 DA exposure caused photoreceptor loss in zebrafish embryos

After four days of DA-exposure, zebrafish embryos were sectioned and subjected to H&E staining to examine the retinal morphology. The retina of embryos exposed to 5.0 μmol/L DA showed a significant decrease in the thickness of the photoreceptor layer by 28% ($P < 0.01$) compared to the control group, suggesting a loss of photoreceptor cells (Figure 8). We further investigated which type of photoreceptors was

lost, by detecting rod cells with anti-rhodopsin antibody and cone cells with ZPR1 antibody, respectively. We found that DA treatment significantly reduced the fluorescent signals of rod cells by 48.6% ($P < 0.01$) compared to the controls (Figure 9A); similarly, the fluorescent signals of cone cells were significantly reduced by 26.6% ($P < 0.01$) compared to the controls (Figure 9B).

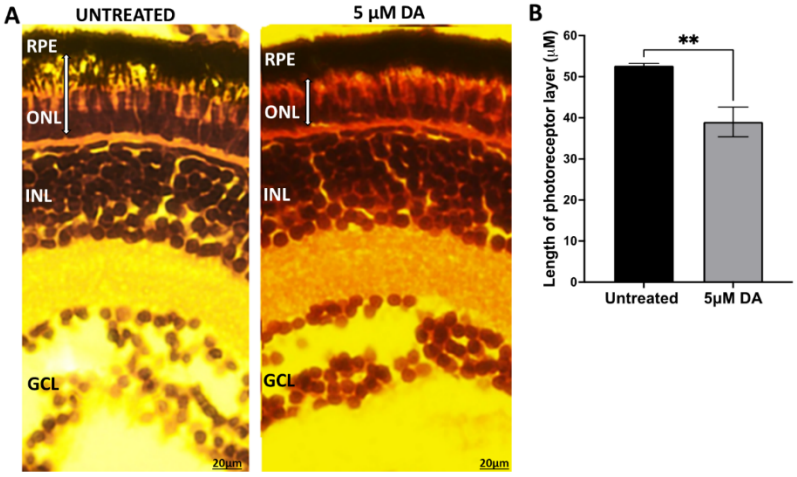

Figure 8. Effect of DA exposure on the photoreceptor layer in zebrafish embryos. (A) Retinal sections of control and DA-treated embryos, stained with haemotoxylin and eosin. The photoreceptor layer is labelled with a white bar. Scale bar: 20µm. (B) The thickness of the photoreceptor layer was measured using Image J software. Data were analysed by a nonparametric *t* test and are displayed as mean ± SEM (n=5). $^{**}P < 0.01$. Scale bar: 20µm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

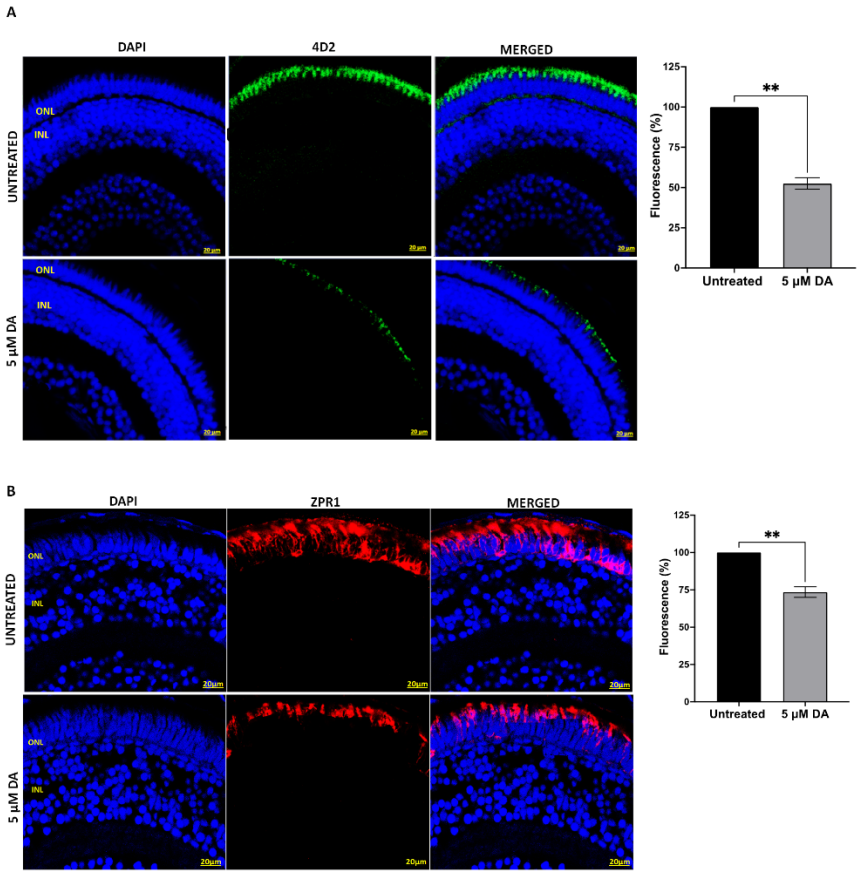

Figure 9. Effect of DA exposure on photoreceptors in zebrafish embryos. (A) Rod cells were detected in the retinal sections of untreated and DA-treated embryos using anti-rhodopsin antibody (green signals). Fluorescent signals were quantified and compared between both groups. (B) Cone cells were detected in the retinal sections of untreated and DA-treated embryos using anti-arrestin antibody (red signals). For quantification of fluorescent signal, ten retinal sections from five eyes (2

sections from each eye) of untreated and DA-treated zebrafish were chosen. Fluorescent signals in a 10μm×10μm (under 400× magnification) area were quantified using Image J software. Data were analysed by a nonparametric *t* test and are displayed as mean ± SEM (n=5). $^{**}P < 0.01$. Scale bar: 20μm.

4. Discussion

This study aimed to assess the toxic effects of DA on human RPE cells and zebrafish embryos. In keeping with earlier studies demonstrating toxic effects of DA in different models, the results of our current study indicated a toxic effect of DA on human RPE cells and zebrafish embryos. We demonstrated that DA significantly decreased the cell viability in a dose-dependent format, induced ROS overproduction, altered MDA and GSH levels as well as CAT activity in RPE cells, lowered antioxidant capacity, promoted inflammation, and caused photoreceptor loss in zebrafish embryos.

Cells produce free radicals as a result of metabolic processes, together with antioxidants that neutralize these free radicals. Cells generally strike a balance between antioxidants and free radicals, maintaining a reducing environment in which specific enzymes preserve the reduced state through the continuous input of metabolic energy. Imbalance of the anti-oxidative system caused by overproduction of ROS leads to inability of the cell to detoxify the toxic conditions, resulting in cell dysfunction and even cell death (Schafer & Buettner, 2001; de Diego-Otero et al., 2009). Previous *in vitro* and *in vivo* studies have demonstrated that DA increased ROS production in rodents and nervous-associated cell lines. A study demonstrated that 20 μg/ml of DA induced ROS overproduction in Caco-2 cell exposed for 24h (Radad et al., 2018). DA exposure has also been shown to induce mitochondrial Ca^{2+} overload and ROS generation in spinal motor neurons *in vitro (*Carriedo et al., 2000*).* Our results are in keeping with several previous experimental studies, indicating an overproduction of ROS in both ARPE-19 cells and zebrafish embryos, suggesting cellular macromolecules and supramolecular damage or activation of specific signalling pathways.

Several enzymes such as CAT, SOD1, SOD2 and GPX1 are involved in ROS detoxification. Since catalase efficiently converts H_2O_2 to water and oxygen (DeJong et al., 2007), we investigated whether CAT activity was changed following DA exposure and found that exposure significantly decreased CAT activity both in RPE cells and in zebrafish embryos. These results are consistent with the previous studies in rodents and nervous-related cell lines (Hiolski et al., 2014; Hiolski *et al.,* 2016; Ventoso *et al.,* 2021). We also quantified the GSH level in both ARPE-19 cells and zebrafish embryos treated with DA. GSH is widely known to play a crucial role in cell protection from oxidative damage and xenobiotic electrophile toxicity, and in maintaining redox homeostasis (Egea et al., 2017; Garza-Lombó et al., 2018). Several studies in different models have demonstrated that DA exposure reduces the intracellular level of GSH. For instance, Giordano and collaborators (2008, 2009 and 2013) argued that DA exposure reduced the GSH level in mouse cerebellar granule neurons. Our results have corroborated previous studies, demonstrating that DA exposure significantly decreased the GSH level in RPE cells and in zebrafish embryos. This suggests interference with its protective role as the essential antioxidant guardian in cells, leading to the exacerbated inflammation that may trigger some cellular dysfunction and organ failure in zebrafish embryos. In contrast to GSH, DA exposure causes an imbalance in MDA by raising its intracellular level (Hiolski et al., 2014). It is widely

accepted that MDA is one of final products of polyunsaturated fatty acid peroxidation; an increase in free radicals induces overproduction of MDA, which is a biomarker of oxidative stress and antioxidant status in several diseases, including retinal degenerative conditions (Kubota et al*.,* 2020). Our results both in RPE cells and zebrafish embryos recorded a significant increase in the MDA level of DA-exposed samples, in agreement with previous studies.

Several studies in different models have demonstrated that DA exposure induces low expression of antioxidant genes (Giordano et al., 2008; Song, Choi, & Park, 2020). Studies *in vivo* in liver and gill tissues of Nile tilapia demonstrated that DA exposure induced low expression of the SOD1 gene (Mazmanci, & Cavaş, 2010). Bose *et al.* (1992) had previously demonstrated that DA treatment decreased the expression of SOD1 in mouse brains. Exposure to DA in rats induced low expression of SOD2 (Motaghinejad et al., 2017), corroborating our results in both models. Studies *in vitro* in different models demonstrated that exposure to DA exposure and its derivates reduced GPX1 gene expression (Mazmanci & Cavaş, 2010; Motaghinejad & Motevalian, 2016). Our studies show that DA treatment significantly reduced expression of *SOD1*, *SOD2*, *CAT* and *GPX1* genes both in RPE cells and zebrafish embryos, suggesting that disruption to the scavenging of free radicals caused an imbalance in intracellular redox homeostasis and possibly resulted in cellular damage.

Nrf2 is an enzyme that enables the ubiquitination of its target substrates and recruits the substrates to the proteasome (Yan et al., 2021); it is one of the most widely studied agents targeting antioxidant response. Nrf2 has been described as a prominent regulator of cellular resistance to oxidants, controlling the cellular redox homeostasis and inducing expression of several antioxidant response-element–dependent genes to modulate the physiological and pathophysiological effects of oxidant exposure (Ma, 2013). Studies in mice revealed that DA exposure inhibited Nrf2 gene expression in the hippocampus (Wang et al., 2018); our results also demonstrated a significantly decreased level of NRF2 in both cytosolic and nuclear fractions, suggesting inactivation of the NRF2 signalling pathway and downregulation of antioxidant gene expression.

It has been widely demonstrated that many marine biotoxins, including domoic acid, can cause inflammation in different types of cells and tissues. Mayer et al (2001) reported increased expression of TNFα and MMP9 in cultured DA-treated rat neonatal microglia. DA treatment has been reported to upregulate expression of IL-1β, IL-6 and TNFα in cultured rat primary astrocytes (Gill et al., 2008); similarly, DA-treated mice had higher levels of proinflammatory mediators, including IL-1β, TNFα, GFAP and COX2, in the hippocampus (Lu et al., 2013). In the current study, our data also showed markedly increased expression of IL-1β, IL-6, IL-8, and TNF-α in human RPE cells and in zebrafish embryos. The NF-κB pathway plays a predominant role in regulating expression of inflammatory mediators (Liu et al., 2021). DA has been shown to activate the NF-κB pathway in mouse hippocampus and to upregulate expression of NF-κB pathway-associated inflammatory genes (Lu et al., 2013). We also observed that DA treatment activated the NF-κB pathway and upregulated associated inflammatory mediators in human retinal cells, suggesting it may contribute to DA-induced retinal toxicity.

It is well documented that oxidative stress and inflammation promote neural cell death in neurodegenerative disorders (Fischer & Maier, 2015). DA has been widely used to induce experimental neurodegeneration models due to its induction of oxidative stress, inflammation, mitochondrial dysfunction, neuronal cell death and cognitive deficits (Jakaria et al., 2018). Vieira et al (2015) reported substantial neuronal cell death in rat hippocampus, a structure critical for learning and memory. We detected a high level of apoptosis in DA-treated human RPE cells and loss of both rod and cone photoreceptors in DA-treated zebrafish embryos. The death of RPE and photoreceptors is possibly caspase-dependent, since we noticed increased expression and activity of caspases in RPE cells; it may also be caused by disruption of the cytoskeleton, as we demonstrated in our study. Other types of cell death, such as necrosis and ferroptosis, may also contribute to DA-induced death of RPE cells and photoreceptors, a possibility that requires further investigation.

In conclusion, DA treatment resulted in significantly decreased viability, reduced anti-oxidative capacity, increased inflammation and apoptosis in human RPE cells; DA exposure caused delayed hatching, reduced heartbeat, morphological abnormality and mortality in zebrafish embryos; in the latter, DA also induced death of both rod and cone cells. The data suggests that DA-contaminated seafood may cause retinal toxicity when consumed.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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