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Mechanistic basis for protection of differentiated SH-SY5Y cells by oryzanol-rich fraction against hydrogen peroxide-induced neurotoxicity

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Abstract

Background: Apoptosis is often the end result of oxidative damage to neurons. Due to shared pathways between oxidative stress, apoptosis and antioxidant defence systems, an oxidative insult could end up causing cellular apoptosis or survival depending on the severity of the insult and cellular responses. Plant bioresources have received close attention in recent years for their potential role in regulating the pathways involved in apoptosis and oxidative stress in favour of cell survival. Rice bran is a bioactive-rich by-product of rice milling process. It possesses antioxidant properties, making it a promising source of antioxidants that could potentially prevent oxidative stress-induced neurodegenerative diseases.

Methods: Thus, the present study investigated the neuroprotective properties of oryzanol-rich fraction (ORF) against hydrogen peroxide (H₂O₂)-induced neurotoxicity in differentiated human neuroblastoma SH-SY5Y cells. ORF was extracted from rice bran using a green technology platform, supercritical fluid extraction system. Furthermore, its effects on cell viability, morphological changes, cell cycle, and apoptosis were evaluated. The underlying transcriptomic changes involved in regulation of oxidative stress, apoptosis and antioxidant defence systems were equally studied.

Results: ORF protected differentiated SH-SY5Y cells against H₂O₂-induced neurotoxicity through preserving the mitochondrial metabolic enzyme activities, thus reducing apoptosis. The mechanistic basis for the neuroprotective effects of ORF included upregulation of antioxidant genes (catalase, SOD 1 and SOD 2), downregulation of pro-apoptotic genes (JNK, TNF, ING3, BAK1, BAX, p21 and caspase-9), and upregulation of anti-apoptotic genes (ERK1/2, AKT1 and NF-κB).

Conclusion: These findings suggest ORF may be an effective antioxidant that could prevent oxidative stress-induced neurodegenerative disorders.

Keywords: Neuroprotective, Rice bran, Oryzanol-rich fraction, Hydrogen peroxide, Supercritical fluid extraction system, Multiplex GeXP, SH-SY5Y cells

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Background

Antioxidant defense systems scavenge reactive oxygen species (ROS) in biological systems as a way to prevent build-up of their levels beyond physiologically acceptable limits. At a certain threshold, however, cells become unable to remove excess ROS, leading to oxidative stress, which is linked to the degenerative processes of aging as well as pathogenesis of many diseases [1]. Cellular response to oxidative stress is dependent on the type of cell. Responses to neuronal oxidative stress are particularly interesting because of their contrasting nature in comparison to other cells. Furthermore, with increasing longevity due to better health care systems, and the delicate nature of the nervous system, considerable interest has grown in factors linked to neurodegenerative diseases in a bid to improve quality of life of the elderly.

Superoxide anions, hydrogen peroxide (H₂O₂) and the hydroxyl radical have been indicated as potent mediators of neuronal oxidative stress [2]. They elicit a complex cascade of events that may eventually remove the initiating stimulus or result in apoptotic death of the cells, depending on the severity of the damage [1]. Mitochondria have been suggested as early targets of oxidative damage, in which cause damage leads to cytochrome c release through a process closely regulated by the Bcl-2 family proteins (Bcl-2, Bax and Bid). Cytochrome c in conjunction with Apaf-1 then activates the caspases, which cleave DNA repair enzymes including PARP, eventually leading to cellular damage and apoptosis [3]. Interestingly, cell survival signaling pathways are closely linked to those that end up in apoptosis. Notably, the major signaling pathways in response to oxidative stress insults include mitogen-activated protein kinases (MAPKs), Akt pathway and nuclear factor- κ B (NF- κ B) signaling. MAPKs encompass a large number of serine/threonine kinases involved in regulating cellular processes including proliferation, differentiation, stress adaptation, and apoptosis. These include the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK), and the p38 kinases. The ERK pathway is linked to the regulation of cell proliferation, while the JNK and p38 pathways are more strongly tied to stress [1].

Furthermore, Akt activation mediated through phosphatidylinositol-3 kinase (PI3K) pathway has been reported to inhibit apoptosis by inhibiting caspase-9 and Bad. ERK1/2 activation through active Ras and PKA has also been shown to block cytochrome c release through Bad-mediated Bcl-xL inhibition [3]. On the other hand, NF- κ B also regulates inflammation, immune responses, control of cell division and apoptosis, and its manipulation is reported to be valuable in treating ischemic stroke, physical trauma to the brain or spinal cord, and neurodegenerative disorders including Alzheimer's disease and

Parkinson's disease [4]. Overall, the modulation of these complex pathways constitutes an important avenue for therapeutic interventions aimed at limiting oxidative damage or attenuating its consequent effects [1].

In recent years, growing concerns of side effects associated with pharmacological agents have generated interest in the therapeutic potentials of plant bioresources. Rice bran is a by-product of the rice milling process, and was previously considered a waste. Now, it is known to contain fat, proteins and bioactives including γ -oryzanol (a mixture of ferulic acid esters of triterpene alcohols and sterols), tocopherols (tocopherols and tocotrienols) and unsaturated fatty acids [5-9], phytosterols, stanols and policosanols [10]. Its functional effects include antioxidant, anti-inflammatory, cholesterol-lowering and anti-diabetic, anti-cancer, anti-hypertensive and glucose metabolism [5,11-15]. Simultaneous extraction and use of multiple bioactive compounds from plants have been reported to potentiate the effects of any one of the bioactives through synergy. Hence, our choice of oryzanol-rich fraction (ORF) in the current study was to maximize the benefits from the rice bran bioactives, while using a green technology, supercritical fluid extraction (SFE) system [16-23].

In the present study, extracted ORF was studied for its ability to regulate processes leading up to oxidative stress and apoptosis in differentiated SH-SY5Y cells.

Methods

Reagents

Rice bran samples were obtained from local milling company, Padiberas National Berhad (BERNAS) at Kuala Selangor, Malaysia. The human neuroblastoma SH-SY5Y cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Minimum essential Eagle's medium, Ham's nutrient mixture F-12, fetal bovine serum and gentamicin were obtained from Sigma (St. Louis, MO, USA). Total RNA Isolation kit was obtained from RBC Bioscience Corp. (Taipei, Taiwan), GenomeLab™ GeXP Start Kit was purchased from Beckman Coulter Inc. (Miami, FL, USA), Magnesium chloride and DNA Taq polymerase were from Thermo Fisher Scientific (Pittsburgh, PA, USA).

Extraction of ORF by SFE system

ORF was prepared using SFE system (Thar 1000 F, Thar Technologies, Inc., Pittsburgh, PA, USA). Briefly, 100 g of stabilized rice bran was placed into the SFE extraction vessel and extraction parameters were set at 600 bars pressure, temperature of 40°C and carbon dioxide flow rate of 30 g/min. ORF was collected from collection vessel when the ranges of pressure and temperature reached 100 – 300 bar and 40°C – 60°C, respectively.

Cell culture

The human neuroblastoma SH-SY5Y cells were maintained in complete culture medium containing 1:1 mixture of Minimum essential Eagle's medium and Ham's nutrient mixture F-12, supplemented with 10% fetal bovine serum, 1% MEM non-essential amino acids and 50 µg/mL gentamicin. Cells were maintained at 37°C under 5% CO₂/95% air.

MTT assay

SH-SY5Y cells were seeded into 96-well culture plates at a density of 2×10^5 cells/mL and allowed to attach. Then, 24 h after seeding, the cells were differentiated with retinoic acid (10 µM) for 6 days prior to treatment. The differentiated cells were then pretreated for 24 h with ORF prepared in serum-free medium at concentrations of 1, 10 and 100 µg/mL. The treated cells were then challenged with 250 µM H₂O₂ for 24 h, as reported in our previous publication [24]. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma, St. Louis, MO, USA] was added to the wells and allowed to incubate in the dark at 37°C for 4 h. The amount of MTT formazan product was determined by measuring absorbance using a Microplate reader (Opsys MR, Thermo LabSystems, Franklin, MA) at 570 nm.

Acridine orange (AO)–propidium iodide (PI) double staining cell morphological assessment

Approximately 2×10^5 cells/mL of SH-SY5Y were seeded into 6-well plate and allowed to attach. Then, 24 h after seeding, the cells were differentiated with retinoic acid (10 µM) for 6 days prior to treatment. The differentiated cells were then pretreated for 24 h with 100 µg/mL ORF prepared in serum-free medium, and subsequently exposed to 250 µM H₂O₂ for 24 h. The cells were then trypsinized and 10 µL of the cell suspension was mixed with 10 µL of AO (50 µg/mL) and PI (50 µg/mL) and placed on a glass slide. The cells were viewed under a fluorescence microscope (Leica, Germany).

Cell cycle analysis

SH-SY5Y cells were seeded into 6-well plates at a density of 2×10^5 cells/mL. The cells were differentiated with 10 µM retinoic acid for 6 days prior to treatment. The cells were pretreated with 100 µg/mL ORF for 24 h with subsequent exposure to 250 µM H₂O₂ for 24 h. The cells were harvested using 0.1% trypsin-EDTA, fixed in 70% ethanol and kept at -20°C overnight. After fixation, the pellets were washed with PBS to remove ethanol and further resuspended in 25 µL of RNase, 50 µL of propidium iodide and 425 µL of PBS to make up the volume to 500 µL. After 30 min of incubation in the dark at 4°C, the DNA contents of the cells were analyzed using flow

cytometer with Summit v4.3 software (Cyan ADP, Beckman Coulter, Brea, CA, USA).

Annexin V-FITC and propidium iodide staining assay

SH-SY5Y cells were seeded in 6-well plates at a density of 2×10^5 cells/mL. The cells were differentiated with 10 µM retinoic acid for 6 days prior to treatment. The cells were pretreated with 100 µg/mL ORF for 24 h followed by exposure to 250 µM H₂O₂ for another 24 h. The subsequent procedures were carried out according to the instructions provided by the manufacturer of APOTEST-FITC kit (Beckman Coulter, Brea, CA, USA). Briefly, cells were harvested using 0.1% trypsin-EDTA and cell pellets were resuspended in ice-cold 1X binding buffer. One microliter of Annexin V-FITC solution and 5 µL of propidium iodide were added to 100 µL of the cell suspension. The tube was incubated on ice for 15 min in the dark followed by addition of 400 µL ice-cold 1X binding buffer and mixing gently. The samples were analyzed using flow cytometer with Summit software v4.3 (CyAN ADP, Beckman Coulter, Brea, CA, USA).

GeXP multiplex gene expression analysis

RNA extraction

SH-SY5Y cells were seeded into 6-well plates at a density of 2×10^5 cells/mL. The cells were differentiated with 10 µM retinoic acid for 6 days prior to treatment. The cells were pretreated with 100 µg/mL ORF for 24 h with subsequent exposure to 250 µM H₂O₂ for 24 h. Total RNA was extracted using Total RNA Isolation kit (RBC Bioscience Corp., Taiwan) according to the manufacturer's protocol. RNA concentration was quantified using NanoDrop spectrophotometer (Thermo Scientific Nanodrop, NanoDrop Technologies, Wilmington, DE, USA), and ratios of A260/230 and A260/280 between 1.8 and 2.0 were used to indicate RNA of high purity.

Primer design

Nucleotide sequences of the genes of interest and house-keeping genes (Table 1) were obtained from National Center for Biotechnology Information GenBank Database, while the internal control (KanR) was supplied by Beckman Coulter Inc. (Miami, FL, USA). The specificity validation of the nucleotide sequences was performed using NCBI-nucleotide-BLAST. Additional 37 base pair of universal tag sequences were attached to each forward and reverse primers. Synthesis of primers was done by First Base Ltd. (Selangor, Malaysia) and diluted according to instructions from Beckman Coulter Inc (Miami, FL, USA).

cDNA synthesis

The complementary DNA (cDNA) was synthesized using 50 ng/µL RNA of each sample. The reverse transcription

Table 1 Gene name, accession number, reverse and forward primer sequences used in GeXP multiplex gene expression analysis

Gene name	Accession number	Primer sequences* with universal tags	
		Forward	Reverse
<i>Antioxidant genes</i>			
Catalase	NM_001752	AGGTGACACTATAGAATAGAAGTGCGGAGATTCAACACT	GTACGACTCACTATAGGGAACACGGATGAACGCTAAGCT
SOD 1	NM_000454	AGGTGACACTATAGAATATCATCAATTCGAGCAGAAGG	GTACGACTCACTATAGGGATGCTTTTTCATGGACCACC
SOD 2	NM_000636	AGGTGACACTATAGAATACATCAAACGTGACTTTGGTTC	GTACGACTCACTATAGGGACTCAGCATAACGATCGTGTT
<i>Downstream apoptotic genes</i>			
BAD	NM_004322	AGGTGACACTATAGAATACGGAGGATGAGTGACGAGTT	GTACGACTCACTATAGGGAGGAGTTTCGGGATGTGGAG
TNF	NM_000594	AGGTGACACTATAGAATACTACTCTGGGAGGGTCTTCC	GTACGACTCACTATAGGGAATGTCCTCCTCACAGG
ING3	NM_198267	AGGTGACACTATAGAATAAGGTTCAAGTTGGCAAACCCAG	GTACGACTCACTATAGGGAAGCCAAAAGTGAGCATGTGTT
BAK1	BC004431	AGGTGACACTATAGAATAAGCCTGTTGAGAGTGGCAT	GTACGACTCACTATAGGGAAGTGATGCAGCATGAAGTCG
BAX	BC014175	AGGTGACACTATAGAATACCCTTTTGCTTCAGGGTTTC	GTACGACTCACTATAGGGACAAAGTAGAAAAGGGCGACAA
p21	NM_000389	AGGTGACACTATAGAATATTAGCAGCGGAACAAGGAGT	GTACGACTCACTATAGGGAAGCCGAGAGAAAACAGTCCA
CAS-9	NM_001229	AGGTGACACTATAGAATAGGGCTCACTCTGAAGACCTG	GTACGACTCACTATAGGGATCTGGAAGCTGCTAAGAGCC
BCL-2	M14745	AGGTGACACTATAGAATAACCACTAATTGCCAAGCACC	GTACGACTCACTATAGGGATTTTCCATCCGTCTGCTCTT
<i>Upstream apoptotic genes</i>			
ERK1/2	NM_002745	AGGTGACACTATAGAATAGGAGCAGTATTACGACCCGA	GTACGACTCACTATAGGGAGATGTCTGAGCACGTCCAGT
p53	NM_001126117	AGGTGACACTATAGAATAGGGGAGCAGGGCTCA	GTACGACTCACTATAGGGAAAATGGCAGGGGAGGG
JNK	NM_139046	AGGTGACACTATAGAATACAGAAGCTCCACCACCAAAGAT	GTACGACTCACTATAGGGAGCCATTGATCACTGCTGCAC
PARP1	NM_001618	AGGTGACACTATAGAATATATCGAGTCGAGTACGCCAA	GTACGACTCACTATAGGGAGTGTGGGACTTTTCCATCAA
AKT1	NM_001014431	AGGTGACACTATAGAATAGAGGAGATGGACTTCCGGTC	GTACGACTCACTATAGGGAAGGATCTTCATGGCGTAGTAGC
NF-kB	NM_001077493	AGGTGACACTATAGAATAGCGGGCTCTAAAATTCTG	GTACGACTCACTATAGGGATTCCACGATCACCAGGTAGG
p38	NM_001315	AGGTGACACTATAGAATATTCAGTCTTTGACTCAGATGCC	GTACGACTCACTATAGGGAGTCAGGCTTTTCCACTCATCT
<i>Housekeeping genes</i>			
GAPDH^a	NM_002046	AGGTGACACTATAGAATAAAGGTGAAGGTCGGAGTCAA	GTACGACTCACTATAGGGAGATCTCGCTCCTGGAAGATG
Hyaluronidase^a	AJ000099	AGGTGACACTATAGAATACAGCAGTTCATGCTGGAGAC	GTACGACTCACTATAGGGACCAGGTAGACAGACGGGAAG
18 sRNA^a	M10098	AGGTGACACTATAGAATAGGAGTGGAGCCTCGCGCTTAA	GTACGACTCACTATAGGGATAGCATGCCAGAGTCTCGTT
Actb^{a,#}	NM_001101	AGGTGACACTATAGAATAGATCATTGCTCCTCCTGAGC	GTACGACTCACTATAGGGAAAAGCCATGCCAATCTCATC
Kan(r)^b	-	AGGTGACACTATAGAATAATCATCAGCATTGCATTGATTCTCTGTTG	GTACGACTCACTATAGGGAATCCGACTCGTCCAACATC

*Based on the *Homo sapien* gene sequences adopted from the National Center for Biotechnology Information GenBank Database.

^aHousekeeping genes; ^bInternal control; [#]Normalization gene.

(RT) reaction was performed according to GenomeLab™ GeXP Start Kit instructions (Beckman Coulter Inc., Miami, FL, USA): 1 µL of RNA sample, 4 µL of 5X RT buffer, 2 µL of RT multiplex reverse primers, 1 µL of KanR, 1 µL of reverse transcriptase and 11 µL of DNase/RNase free water. cDNA was synthesized according to the reaction protocol: 48°C for 1 min, 42°C for 60 min, 95°C for 5 min and 4°C hold in XP Thermal Cycler (BIOER Technology, Hangzhou, China).

PCR amplification

PCR reactions were carried out using GeXP Start Kit (Beckman Coulter, Miami, FL, USA) consisting of cDNA sample taken from the RT reaction (9.3 µL each), 5X PCR buffer, 25 mM magnesium chloride, PCR multiplex forward primer and Thermo-Start DNA polymerase. Amplification was done in an XP Thermal Cycler (BIOER Technology, Hangzhou, China) using 95°C for 10 min, followed by 34 cycles of 94°C for 30 sec, 55°C for 30 sec, 70°C for 1 min and 4°C hold.

GeXP multiplex data analysis

The PCR product (1 µL each) was mixed with 38.5 µL of sample loading solution and 0.5 µL of DNA Size Standard-400 (Beckman Coulter Inc., Miami, FL, USA). The PCR products were then separated in the GenomeLab GeXP Genetic Analysis System (Beckman Coulter, Brea, CA, USA) by capillary gel electrophoresis according to their nucleotide sizes. The dye signal strength was measured in arbitrary units (A.U.) of optical fluorescence. The data were analyzed using the Fragment Analysis module of the GeXP system software and then transferred to the analysis module of eXpress Profiler software. Normalization was performed with β -actin as reference gene, according to manufacturer's instructions.

Statistical analysis

Statistical analysis ($n = 3$) was conducted by one-way analysis of variance with Tukey's multiple comparison test using Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois, USA) version 21.0 and $p < 0.05$ was considered as significantly different.

Results

ORF-protected SH-SY5Y cells against H₂O₂-induced neurotoxicity

Treatment of SH-SY5Y cells with ORF (1–100 µg/mL) did not show much toxicity (Figure 1A). However, treatment of the cells with 250 µM H₂O₂ for 24 h resulted in significant cell death [24]. When cells were pretreated with ORF for 24 h and subsequently exposed to 250 µM H₂O₂ for another 24 h, the resulting toxicities observed were not as significant as with H₂O₂ treatment alone (Figure 1B).

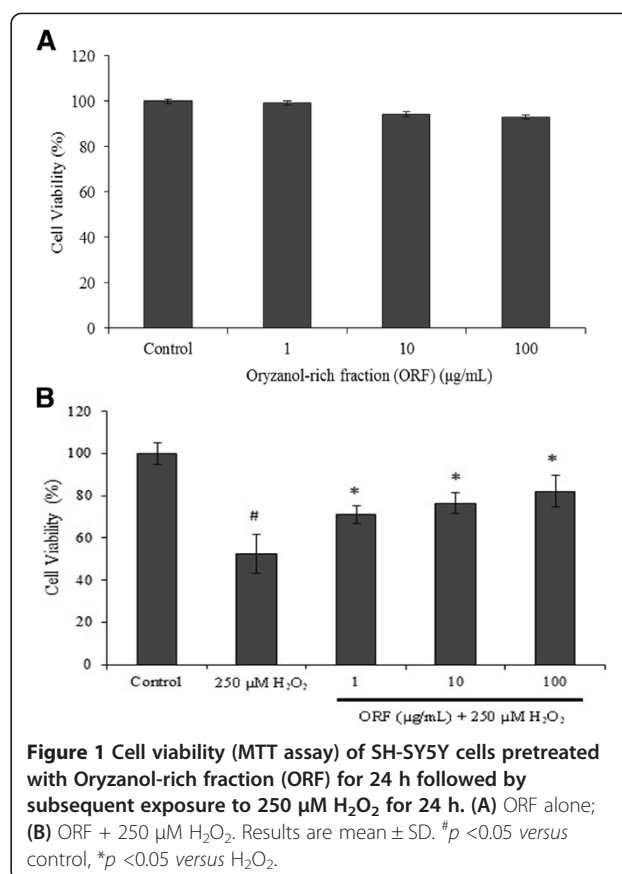


Figure 1 Cell viability (MTT assay) of SH-SY5Y cells pretreated with Oryzanol-rich fraction (ORF) for 24 h followed by subsequent exposure to 250 µM H₂O₂ for 24 h. (A) ORF alone; (B) ORF + 250 µM H₂O₂. Results are mean ± SD. [#] $p < 0.05$ versus control, ^{*} $p < 0.05$ versus H₂O₂.

ORF prevented H₂O₂-induced morphological changes in SH-SY5Y cells

AO/PI double staining distinguishes between viable, apoptotic and necrotic cells. Viable cells will normally show round and green nuclei, similar to those of the untreated cells in this study (Figure 2A). Late apoptotic and necrotic cells will stain orange and red as displayed by the H₂O₂-treated cells (Figure 2B). In this study, the nuclei of the cells pretreated with ORF stained orange and red but with less intensity that those of H₂O₂-treated cells (Figure 2C).

ORF protected SH-SY5Y cells against H₂O₂-induced cell death

Figure 3 showed significant cell death (at Sub G1) (40% ± 5.88%) upon exposure to 250 µM H₂O₂ in comparison to untreated cells (8% ± 2.34%), $p < 0.05$. In contrast, pretreatment with 100 µg/mL ORF did not produce as much dead cells (14% ± 5.0%) as with H₂O₂ treatment alone, $p < 0.05$. In addition, there was no significant difference in cell populations at S and G2/M phases among control, H₂O₂ alone and ORF treatment.

Figure 4 showed that exposure of SH-SY5Y cells to H₂O₂ demonstrated significant differences in viable, late

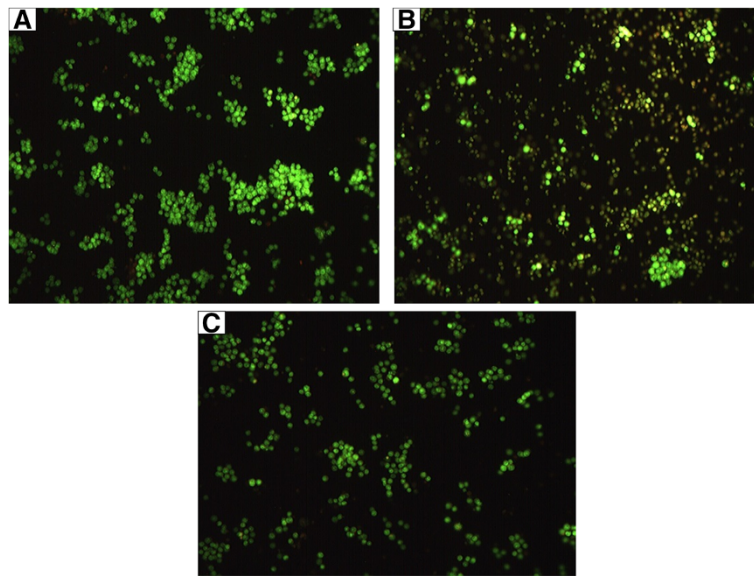


Figure 2 Acridine orange (AO)-propidium iodide (PI) double staining cell morphological assessment. Morphological changes in SH-SY5Y cells pretreated with Oryzanol-rich fraction (ORF) for 24 h followed by subsequent exposure to 250 μM H_2O_2 for 24 h. **(A)** untreated cells (control); **(B)** 250 μM H_2O_2 alone; **(C)** 100 $\mu\text{g}/\text{mL}$ ORF +250 μM H_2O_2 . Viable cells are stained green by acridine orange; Late apoptotic and necrotic cells are stained orange and red by propidium iodide.

apoptosis/early necrosis and late necrosis states in comparison to untreated cells. The untreated cells showed $94\% \pm 3.67\%$ viability, while incubation with 250 μM H_2O_2 reduced the cell viability, to only $16\% \pm 3.97\%$ represented by early apoptosis ($2\% \pm 0.05\%$), late apoptosis ($39\% \pm 2.2\%$) and necrosis ($43\% \pm 3.85\%$). The results indicated that H_2O_2 at 250 μM was highly toxic to SH-SY5Y cells. Pretreatment with ORF (100 $\mu\text{g}/\text{mL}$), however,

protected the cells against H_2O_2 -induced apoptosis, as demonstrated by $92.41\% \pm 3.3\%$ viability, which was similar to the untreated cells (Figure 4).

Effects of ORF on transcriptomic regulation of apoptotic and antioxidant genes in SH-SY5Y cells exposed to H_2O_2
 H_2O_2 -treatment induced upregulation of antioxidant genes in SH-SY5Y cells in comparison to untreated cells

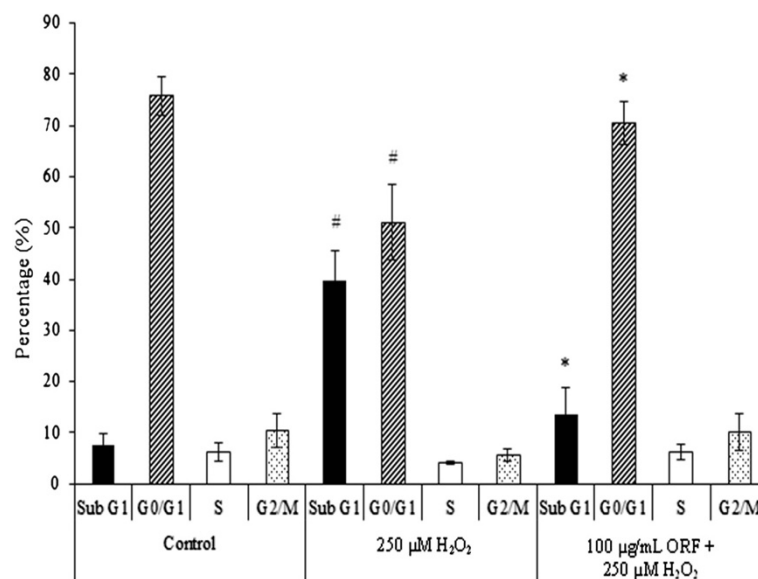


Figure 3 Cell cycle analysis. Flow cytometric measurement of cell death and cell cycle on SH-SY5Y cells pretreated with Oryzanol-rich fraction (ORF) (100 $\mu\text{g}/\text{mL}$) before exposure to 250 μM H_2O_2 over 24 h. Results are mean \pm SD. [#] $p < 0.05$ versus control, ^{*} $p < 0.05$ versus H_2O_2 .

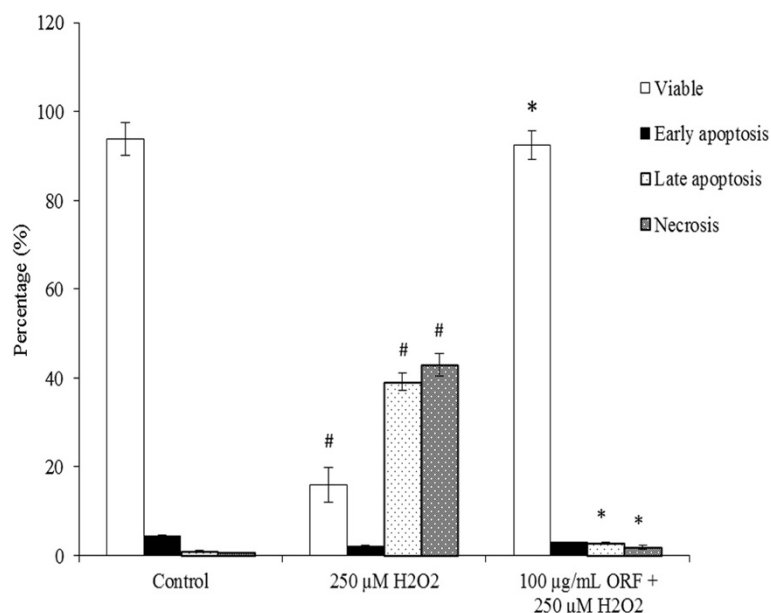


Figure 4 Annexin V-FITC and propidium iodide staining assay of SH-SY5Y cells following exposure to 250 µM H₂O₂ over 24 h, in the presence or absence of 24 h Oryzanol-rich fraction (ORF) (100 µg/mL) pretreatment. Results are mean ± SD. [#]*p* < 0.05 versus control, ^{*}*p* < 0.05 versus H₂O₂.

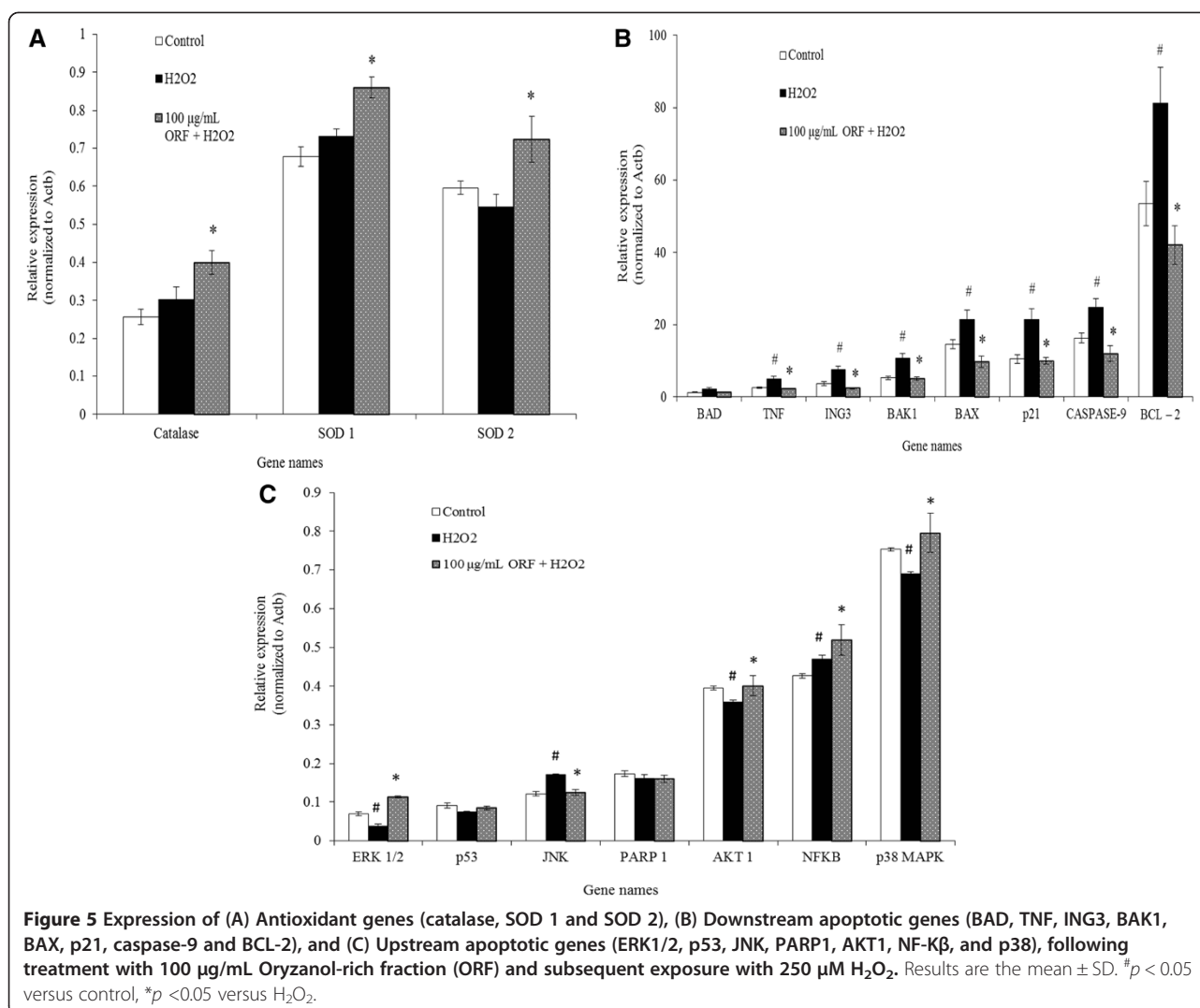
(Figure 5A). Furthermore, pretreatment of SH-SY5Y cells with ORF (100 µg/mL) prior to H₂O₂ exposure upregulated catalase, SOD 1 and SOD 2 genes more than with H₂O₂ exposure alone or in untreated cells (*p* < 0.05). Additionally, H₂O₂ significantly upregulated (*p* < 0.05) the expression levels of TNF, ING3, BAK1, BAX, p21, caspase-9 and Bcl-2 genes, but not BAD gene, in comparison to untreated cells (Figure 5B). Pretreatment with ORF (100 µg/mL) resulted in downregulation of the expression levels of TNF, ING3, BAK1, BAX, p21 and caspase-9 genes, (*p* < 0.05). In the presence of H₂O₂, SH-SY5Y cells showed significantly upregulated (*p* < 0.05) JNK and NF-κβ gene expression levels, and downregulated ERK1/2, AKT1 and p38 levels, in comparison to untreated cells. No changes were observed for p53 and PARP1 expression levels (Figure 5C). Pretreatment with ORF (100 µg/mL) upregulated the expression of ERK1/2, PARP1, AKT1 and NF-κβ genes, (*p* < 0.05), but downregulated that of JNK.

Discussion

In the present study, the neuroprotective effects of ORF were evaluated against toxicity caused by H₂O₂ on differentiated SH-SY5Y cells. Also, to minimize hazards associated with extraction of plant bioresources, a green technology (SFE) was used to extract ORF. In neuronal cells, H₂O₂ activates intracellular defence mechanisms including the upregulation of endogenous antioxidants, which are meant to protect cells from damage by H₂O₂. However, in excess, apoptosis and cell death ensue when the defence mechanisms fail to counter the effects of the

H₂O₂ [25]. The increasing interest in plant bioresources that can potentiate antioxidant systems is based on the notion that beyond the normal cellular threshold for apoptosis and cell death, such exogenous antioxidants could complement the cellular defence systems in countering H₂O₂-induced damage [1,26,27]. In this study, cell viability and morphology of SH-SY5Y cells were preserved by ORF in the presence of H₂O₂, suggesting that ORF likely was able to alter some pathways leading upto oxidative damage and/or apoptosis. The protective effects of ORF against H₂O₂-induced cell death were further corroborated by flow cytometric analyses, which indicated that ORF reduced the apoptotic and necrotic cells' population in SH-SY5Y cells.

Changes in the expression of antioxidant genes in the present study due to H₂O₂ are similar to what we have reported previously [28]. These changes suggested activation of antioxidant defence systems in SH-SY5Y cells to counter H₂O₂. The presence of ORF potentiated the expression of the antioxidant genes, suggesting further protection for the cells. As can be recalled, a very close link exists between pathways leading up to cell survival and those that mediate apoptosis and cell death due to oxidative damage. MAPKs and Akt are believed to be involved in these processes including cell growth, survival, differentiation and apoptosis responses [29]. Oxidative stress is reported to induce apoptotic cell death through activation of transcriptional factors such as MAPKs [25], and especially JNK [30,31], which is known to be involved in pro-apoptotic signaling [32]. JNK activation facilitates



the decrease of mitochondrial membrane potential followed by release of cytochrome c, which then activates caspase-9 and caspase-3, eventually leading to cell death [33]. Our results showed that H₂O₂ induced JNK and caspase-9 activation, which were both attenuated by ORF treatment, suggesting that the protective effects of ORF against H₂O₂-induced injury in SH-SY5Y cells were partly mediated through its protection of the mitochondria.

Activation of ERK due to oxidative stress is reportedly mediated by growth factor receptors [34-36]. When growth factor receptors undergo phosphorylation in response to oxidative insults such as H₂O₂, the resulting changes attenuate ERK activation. Similarly, expression of inactive mutant forms of various growth factor receptors reduces activation of ERK by oxidative stress [34], while over expression of certain normal growth factor receptors in rat PC12 cells exposed to H₂O₂, results in enhanced activation of ERK [37]. Observations like these have given rise to suggestions of ERK activation as a survival factor

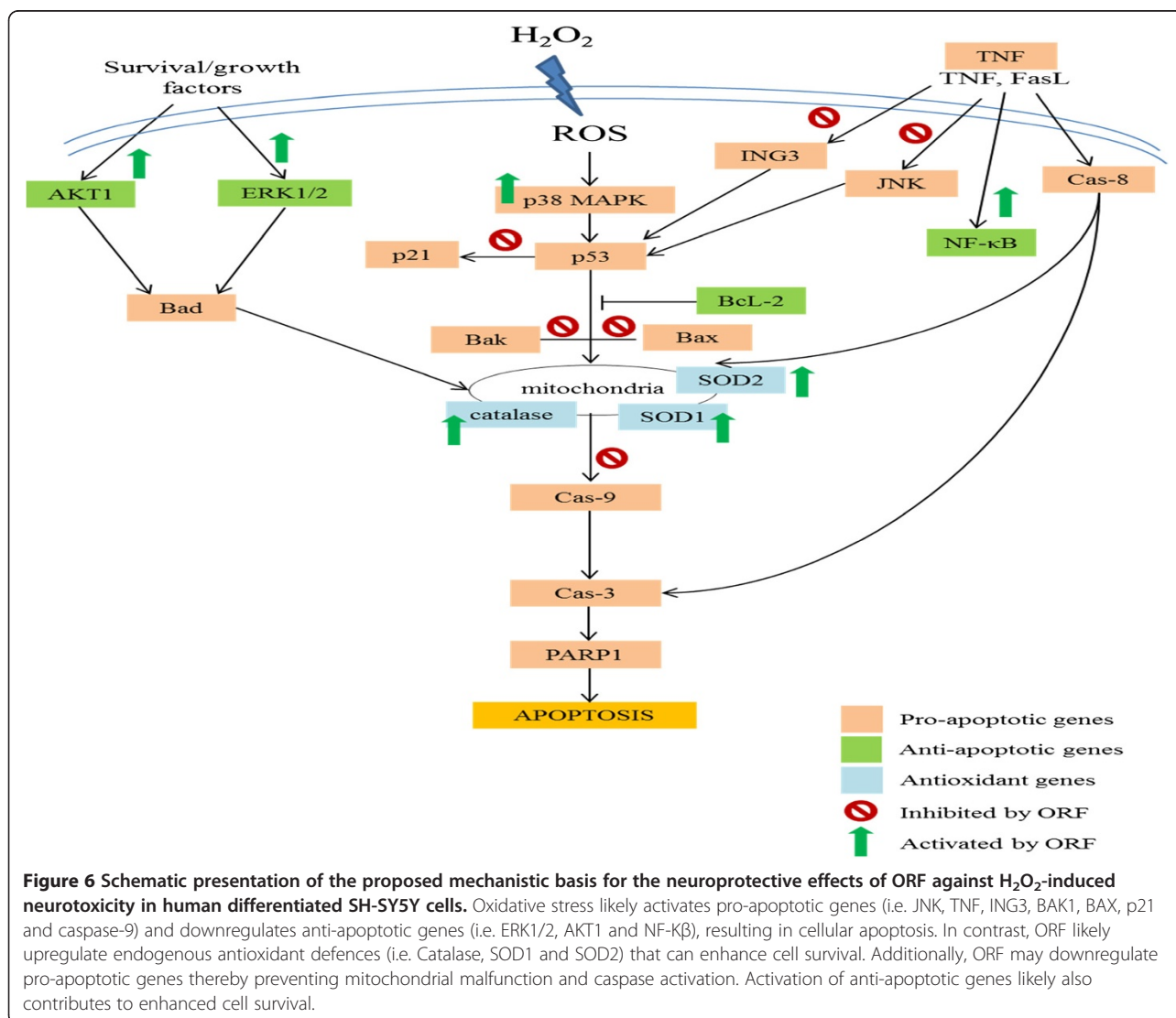
following oxidative injury [38,39]. In the present study, H₂O₂ downregulated ERK1/2 gene expression, while ORF pretreatment resulted in upregulation of the gene. This suggested that in response to H₂O₂-induced oxidative stress, ORF pretreatment may trigger the expression of growth factor receptors in SH-SY5Y cells leading to activation of ERK1/2 gene as a protective mechanism. In addition, p38 MAPK is known to be a stress kinase, and its activation may lead to cell death. However, while this assumption is correct in most cases, cause-effect studies have also found that activation of p38 MAPK by stress stimuli may not necessarily promote death, but sometimes could enhance cell survival and DNA repair [40]. Similarly, activation of Akt in response to oxidant exposure appears to be mediated through growth factor receptors also [41]. In SH-SY5Y cells, Akt activation is linked to inhibition of apoptosis especially in the presence of oxidative stress [42,43]. In the present study, upregulation of Akt by ORF pretreatment suggested that ORF is anti-apoptotic.

Dysregulation of TNF production has been implicated in a variety of human diseases. Binding of TNF to its receptor may result in activation of NF-κB, activation of MAPK pathways or induction of death signaling. NF-κB is a heterodimeric transcription factor and translocates to the nucleus to mediate the transcription of a vast array of proteins involved in cell survival and proliferation, inflammatory response and anti-apoptotic factors. ORF pretreatment enhanced the gene expression of NF-κB, indicating that NF-κB signaling pathway was likely involved in promoting survival and anti-apoptosis in SH-SY5Y in the presence of oxidative damage.

Oxidative stress often leads to cell death through apoptosis, and in the present study, activation of the genes encoding the Bcl-2 family proteins (i.e. Bcl-2, Bak1 and Bax) and caspase-9 suggested the activation of apoptosis in the cells. Caspase-9 is thought to activate caspase-3, which is known to cleave many nuclear DNA repair enzymes, such

as PARP, resulting in nuclear DNA damage and apoptosis [3]. Moreover, ING3 overexpression was also reported to have induced apoptosis in RKO cells, through activation of p21 and Bax [44]. Upregulation of ING3 in the present study due to H₂O₂, therefore, also suggested induction of apoptosis in the cells. Interestingly, ORF pretreatment resulted in downregulation of Bcl-2, Bak1, Bax, p21, ING3 and caspase-9, indicating a tendency for anti-apoptosis.

Taken together, ORF was found to protect SH-SY5Y cells against H₂O₂-induced neurotoxicity possibly through multi-signaling pathways (Figure 6). The protective effects of ORF on SH-SY5Y cells were likely mediated through upregulation of antioxidant genes (catalase, SOD 1 and SOD 2), downregulation of pro-apoptotic genes (JNK, TNF, ING3, BAK1, BAX, p21 and caspase-9), and upregulation of anti-apoptotic genes (ERK1/2, AKT1 and NF-κB). The findings from this study suggest that rice bran could potentially be a source of antioxidants that



may have huge implications on the study and management of neurodegenerative diseases.

Conclusions

In this study, ORF protected SH-SY5Y cells against H₂O₂-induced neurotoxicity as evidenced by the reduced cytotoxicity, inhibition of apoptosis, and gene expression changes (upregulation of antioxidant genes, downregulation of pro-apoptotic genes, and upregulation of anti-apoptotic genes) that tended towards cell survival. The results suggested that ORF could protect SH-SY5Y cells against oxidative stress-mediated apoptosis. These findings could have huge implications on future studies on the potential use of ORF in managing neurodegenerative diseases caused by oxidative injury.

Abbreviations

AKT- v: Akt murine thymoma viral oncogene; AO-PI: Acridine orange-propidium iodide; BAD: Bcl-2-associated death promoter; BAK1: BCL2-antagonist/killer 1; BAX: Bcl-2-associated X; ERK: Extracellular regulated kinase; H₂O₂: Hydrogen peroxide; ING3: Inhibitor of growth 3; JNK- c: Jun N-terminal kinase; MAPK: Mitogen-activated protein kinases; MTT: (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide); NF-κB: Nuclear factor kappa B; ORF: Oryzanol-rich fraction; P13K: Phosphoinositide 3-kinase; p21: Tumor suppressor gene 21; ROS: Reactive oxygen species; SFE: Supercritical carbon dioxide fluid extraction; SOD: Superoxide dismutase; TNF: Tumor necrosis factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NI and MI designed the study. NI, MUI, NHA, SFF and JBF performed the experiments. NI and MUI drafted the manuscript, while MI reviewed the manuscript before final submission. MFAB prepared stabilised rice bran and ORF for the study. All authors read and approved the final manuscript.

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