Impact of Rapeseed Pomace (RSP) extract on markers of oxidative stress and DNA damage in human SH-SY5Y cells

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Abstract

With increased longevity and subsequent rise in people with age-related neurodegenerative diseases, protection of neurons from oxidative stress damage has become an important field of study. For the first time, we highlight the neuroprotective properties of rapeseed pomace (RSP) extract in SH-SY5Y human neuroblastoma cells. We used resazurin to determine cell metabolism, 2,7'-dichlorofluorescin diacetate (H_2DCFDA) to assess the potential of RSP extracts to shield cells from reactive oxygen species (ROS) induced by H_2O_2 using flow cytometry, HPLC to analyze for malondialdehyde (MDA) as a lipid peroxidation marker and the COMET assay to assess DNA strand breakage. Protein stress arrays were used to investigate the cellular pathways affected by RSP extract. No effect on cell metabolism in SH-SY5Y cells was observed after RSP extract treatment (up to 1.5 mg/mL). Pretreatment (24 hrs) with RSP extract (1 mg/mL), before H_2O_2-induced stress, alleviated ROS production and DNA strand breakage by 68%, and 38%, respectively. At protein level, the RSP extract increased the levels of FABP-1, HIF-1α, SOD2 and Cytochrome c proteins. Under H_2O_2- induced stress, however, it helped to downregulate p38α levels, a protein kinase which is receptive to stress impulse (mitogen-activated). RSP extract shows very promising cell protective properties in relation to oxidative stress.

Practical applications

Oxidative stress has been associated with numerous diseases for example cancer, diabetes, and many neurological disorders including Parkinson's and Alzheimer's diseases. Hence there is acceptance among the scientific community of antioxidant therapy and the quest for effective, low cost and readily available sources of natural antioxidants is paramount. Rapeseed plantations are abundant around the world due to the use of rapeseed oil in cooking and as a biofuel. The resulting rapeseed pomace (by-product), specifically its extract, contains high levels of phytochemicals that protect cells against oxidative stress. Therefore, RSP extract can potentially be used/developed as functional food and nutraceuticals in the prevention of many complex neurodegenerative diseases.

Keywords

Rapeseed; plant extract; SH-SY5Y neuroblastoma cells; cytoprotection; comet assay; ROS

1 INTRODUCTION

Over the last decades, constant growth and advancement of medical services and higher standards of living, especially in the developed countries, have led to a continuous increase in human life expectancy (Dimitriadi & Hart, 2010). This positive outcome however comes as a double-edged sword, as the number of patients with age-related diseases, for example neurodegenerative diseases, has increased significantly.

Gradual and progressive degradation and loss of neurons are linked to neurodegenerative diseases with the final consequence resulting in nervous system dysfunction (Brown, Lockwood, & Sonawane, 2005). The latter can lead to either symptoms of movement deficiencies (e.g. Parkinson's disease (PD), Spinocerebellar ataxia (SCA) and Motor Neuron Disease (MND or ALS)) and/or problems with mental function (Alzheimer's disease (AD), Prion diseases). Many of these neurodegenerative diseases are well researched, however, causation and mechanisms of their etiology are not completely understood, and cures are nonexistent. Current available treatments only alleviate some of the symptoms. The main pathological landmark for many neurodegenerative diseases involves misfolded proteins that can lead to their aggregation and deposition in the central nervous system (Skovronsky, Lee, & Trojanowski, 2006). In addition, they are often associated with oxidative stress (Farooqui, 2015; Gilgun-Sherki, Melamed, & Offen, 2001; Liu, Zhou, Ziegler, Dimitrion, & Zuo, 2017; Uttara, Singh, Zamboni, & Mahajan, 2009). Whether this oxidative stress is part of the causation or a downstream effect of other factors such as protein aggregations, is not well understood. Excessive levels of cellular reactive oxygen species (ROS) can initiate protein oxidation, increased DNA damage, lipid peroxidation and mitochondrial dysfunction, all of which can be fatal to the function of neurons if not prevented (Emerit, Edeas, & Bricaire, 2004; Pohl & Kong Thoo Lin, 2018; Pollari, Goldsteins, Bart, Koistinaho, & Giniatullin, 2014).

However, several antioxidants, those obtained from natural sources, have received significant interest for their potential to treat/prevent neurodegeneration. Previous *in vitro* and *in vivo* studies had demonstrated positive outcomes in a number of different neurodegenerative disease models (Pohl & Kong Thoo Lin, 2018).

Rapeseed pomace (RSP) extracts, characterized for secondary metabolite composition as well as their *in vitro* and *in vivo* activities (Pohl et al., 2018, 2019; Yates et al., 2019), have been studied in our laboratory. After the production of edible oil from rapeseed (*Brassica napus*), RSP is obtained as the by-product. In our previous work (Pohl et al., 2018, 2019; Yates et al., 2019) we studied the potential to increase the value of RSP by determining whether it can be used to prevent and/or treat neurodegenerative disease. Studies on the RSP extract and its secondary metabolites, had identified sinapine as the main component together with several phenolic acids, flavonoids/coumarins, benzaldehydes, indoles and amines (Pohl et al., 2018; Yates et al., 2019). Furthermore, we showed that antioxidant property of the RSP extract was mainly due to sinapine and other phenolic acids (caffeic, syringic, sinapic and ferulic acids). The RSP extract also showed acetylcholinesterase inhibition activity and the capacity to protect pBR22 plasmid DNA that had been inflicted with oxidative stress initiated by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) (Pohl et al., 2018; Yates et al., 2019).

Here we report for the first time, the antioxidant protective effects of RSP extract in the human neuroblastoma cell line SH-SY5Y. This cell line, notwithstanding its limitations, has been studied regularly as an experimental model in studies that needed neuron-like cells for research into Parkinson's- and Alzheimer's diseases as well as ALS (Koriyama, Furukawa, Muramatsu, Takino, & Takeuchi, 2015; Krishna et al., 2014; Xicoy, Wieringa, & Martens, 2017; Yu et al., 2012). Previous research in the field of neurodegeneration using this cell line and natural plant extracts include, amongst others, the study of *Liriope platyphylla* (Lily turf) (Park et al., 2015), four medicinal plants (*Crinum bulbispermum, Scadoxus puniceus Zanthoxylum capense*, and *Lannea schweinfurthii*) (Seoposengwe, van Tonder, & Steenkamp, 2013), tea extracts (green and black tea) (Levites, Youdim, Maor, & Mandel, 2002) as well as Ginkgo biloba extract (EGb761) (Shi et al., 2009). After oxidative stress was induced, the potential of RSP extracts to prevent the induced cellular changes were determined. This included ROS production, cellular nuclear DNA strand breakage, lipid peroxidation and the expression of cell stress-related proteins.

2 MATERIALS AND METHODS

2.1 Rapeseed pomace extract

RSP (harvest 2014) was supplied by Mackintosh of Glendaveny (Mains of Buthlaw, Glendaveny, Peterhead, Scotland, (www. macintoshofglendaveny.co.uk) and the extract prepared as previously described (Pohl et al., 2018; Yates et al., 2019). Briefly, 6 g ground RSP (125-710 μm) was weighed into a filtration extraction thimble (Fioroni S.A X25 cellulose thimble 33 x 80 mm) in a Soxhlet extractor (Gerhardt; Soxtherm SE 416) and lipids were extracted using petroleum ether (140 mL) for 45 min at 150°C. That oil fraction was discarded. After overnight drying, the de-fatted pomace was subjected to another Soxhlet extraction with ethanol/water mixture (95:5, v:v, 140 mL, 45 min at 240°C). The resulting extract was evaporated to dryness (Büchi Rotavapor R-114) and freeze dried (Edwards, Freeze Dryer Modulyo)(Pohl et al., 2018). After several extractions, all the extracts were combined, homogenized and vacuum packed prior to storage at -80°C until further use (Pohl et al., 2019).

2.2. Cell culture

SH-SY5Y neuroblastoma cells (from ECACC, cat number 94030304, PHE, UK) were cultured as described before (Smith et al., 2005) with slight modifications. Supplemented media was prepared with DMEM medium (DMEM, high glucose, pyruvate), FBS (10%), non-essential amino acids (1%, NEAA) and Penicillin/Streptomycin (1%, Pen/Strep); all from Fisher Scientific, UK. All cell culture flasks and plates/flasks were kept at 37°C in a humidified incubator under 5% CO_2 atmosphere. Cells were passaged when the levels of confluency reached 80% to 90%. In all experiments, only adherent cells were used. The latter were washed with PBS and/or lysed prior to proceeding with the experiments described in sections 2.3 to 2.7.

2.3. Cell viability assay using Resazurin

In 96-well plates, SH-SY5Y cells (10^4/100 μL/well) were seeded and left to incubate for 24 hours. The cells were then exposed to RSP extract (0-5 mg/mL) for 24 hours. After the addition of resazurin (20 μL, R&D systems, UK) to each well, the plate was incubated (37°C) for 4 hours before measuring fluorescence intensity at 530/25 nm (excitation) and 590/35 nm (emission). The experiment was carried out 3 times independently with 3 replicates for each treatment per plate. The fluorescence intensity (fl) means of the three experiments (exp) were added and divided by 3 ($mean = (fl\ exp\ 1 + fl\ exp\ 2 + fl\ exp\ 3)/3$). The mean of untreated cells (0 mg/ml RSP extract) was set to 100% and the other wells normalized to the untreated control using GraphPad Prism software.

2.4. ROS detection

2,7'-dichlorofluorescindiacetate (H_2DCFDA, Invitrogen, Life Technologies, Fisher Scientific, UK) was used to determine ROS production in SH-SY5Y cells. Cells (7 x 10^5) were cultured in T25 culture flasks and treated with RSP extract (1 mg/ml) for 24 hours. The RSP extract was then removed and cellular oxidative stress was triggered with H_2O_2 (1 mM) for 30 minutes. The H_2O_2 was removed followed by the addition of H_2DCFDA (1 μM in medium) and exposed for 45 min. The cells were collected after trypsinization. After resuspension of cell pellet (PBS), the latter was subjected by flowcytometric analysis on a Coulter Epics XL-MCL flow cytometer (EXPO32 ADC XL 4 color, Beckman Coulter, UK) at 525 nm fluorescence signal. To record and analyze the 10,000 events, the EXPO32 ADC software (Beckman Coulter, UK) was applied. The % of ROS production (% of events/cells after set threshold of 9th decade of 1st log) was determined from the FL-1 plots (**Supplementary Information**, Figure 1). The experiment was carried out 4 times independently (n=4) with each treatment in duplicate and means ($mean = (mean\ \%\ exp\ 1 + mean\ \%\ exp\ 2 + mean\ \%\ exp\ 3 + mean\ \%\ exp4)/4$) and standard deviations calculated using GraphPad Prism software.

2.5. Lipid peroxidation

To determine malondialdehyde (MDA), cells were cultured, treated (1 mg/mL RSP extract) and harvested as described above for ROS detection. Following cell lysis (~200 µL, 150 mM NaCl, 50 mM Tris pH 8.0, 1.0% Triton-X100) for 30 minutes on ice and sonication for a further 15 minutes (4°C), the MDA assay was performed following the method of Domijan *et al.* (Domijan, Ralić, Radić Brkanac, Rumora, & Žanić-Grubišić, 2015). Chromatographic analysis was carried out on a Shimadzu Prominence liquid chromatography system (LC-20AD) with degasser, fitted with a Phenomenex Hyperclone ODS (C18) 120 column (150 x 4.6 mm; 3 µm) and maintained at 40°C. The HPLC was coupled to an SPD-M20A diode array detector (DAD) and a Shimadzu RF-10A XL fluorescence detector. The DAD was set at 532 nm whilst the fluorescence detector was operated at wavelengths 527 nm (excitation) and 551 nm (emission). Quantification was carried out by external standard calibration.

The mobile phase conditions included buffers A (20 mM ammonium acetate in water) and B (20 mM ammonium acetate in methanol) (Fisher Scientific, UK). The chromatographic conditions were flow rate of 1 mL/min over 20 min and an injection volume of 10 µL. The gradient programme was: 90% A – 10% B initially, changed to 5% A – 95% B over 10 min, held for 5min then changed to 90% A – 10% B over 5 min. There was an equilibrium step after each run.

2.6. Comet assay (alkaline)

SH-SY5Y cells were incubated with RSP extract (1 mg/mL) or vehicle control for 24 hours, followed by removal of the RSP extract and exposure to H_2O_2 (300 and 1000 µM) in medium for 30 minutes; 37°C. Cells were harvested after trypsinization and DNA strand breakage was analyzed using single-cell gel electrophoresis (SCGE, comet assay) as reported by Barron *et al.* (Barron et al., 2015) and Duthie *et al.* (Duthie, Narayanan, Blum, Pirie, & Brand, 2009). To determine the concentration of H_2O_2 that induced DNA strand breakage, initially a concentration gradient (100-1000 µM H_2O_2) was tested. Concentrations of 300 and 1000 µM H_2O_2 were chosen for subsequent study of the RSP extract. All the comets were manually scored following the method of Heuser *et al.* (Heuser, Erdtmann, Kvitko, Rohr, & da Silva, 2007). Briefly, a fluorescence microscope (Leica DMRB (Germany), 200x magnification) with wavelengths 340-380 nm (excitation) and 425 nm (emission) was used to score a total number of 100 comets with arbitrary numbers based on 5 recognizable classes of comet, from class 0 (undamaged, no discernible tail) to class 4 (almost all DNA in tail, insignificant head) as described by Duthie *et al.*(Duthie, Ma, Ross, & Collins, 1996) and is shown in **Supplementary Information,** Figure 2. 100 comets are recorded from each gel giving a score between 0-400 for each scored gel. Three gels were scored for each experimental condition per experiment and the mean value determined ($mean = (score\ gel1 + score\ gel2 + score\ gel3)/3$). Statistical analysis was applied on three independent experiments (n=3) using GraphPad Prism software.

2.7. Protein array (cell stress)

The relative levels of selected human cell stress proteins was studied with the proteome profiler (Cat# ARY018, R&D Systems, UK) and carried out following the instructions from the manufacturer (R&D Systems Inc., 2014). Briefly the cells were seeded (1 x 10^6) in T25 flasks followed by RSP extract treatment (1 mg/mL) for 24 hours, then H_2O_2 (1 mM in medium) for 30 minutes after the RSP extract had been removed. The cells were trypsinized, collected and lysed according to the proteome profiler kit instructions (R&D Systems Inc., 2014). Each treatment was performed in duplicate and the protein contents were deduced with the DC protein assay (BIO-RAD, UK) according to the manufacturer's instructions [22]. The membranes were exposed to X ray films (CL Xposure, Thermoscientific, UK) for 10 to 15 min. After the development of the films (Developer and Fixer solution from Sigma, UK, **Supplementary Information**, Figure 3), spot analysis was performed as described in (Jaul & Barron, 2017) using a FUSION FX7™ imaging instrument with Fusion 1 and BIO-1D™ imaging software (PeqLab; VWR International Ltd., UK). The data for the same treatment were pooled together prior to calculating the ratio of Treatment/Control.

2.8. Statistical analysis

All experiments, except the protein array, were carried out at least 3 times independently. Statistical analysis was undertaken using GraphPad Prism 7. Data was checked for normal distribution and outliers, and comparisons were made using one-way ANOVA followed by Bonferroni post-hoc test (multiple comparison).

3 RESULTS AND DISCUSSION

3.1. RSP extract toxicity towards SH-SY5Y cells

Several plant extracts have previously been tested for their toxicity and *in vitro* properties, and have therefore been suggested for potential use as treatments for neurodegenerative diseases (Mohd Sairazi & Sirajudeen, 2020; Pohl & Kong Thoo Lin, 2018). Here in this study, for the first time, we studied the effect of RSP extract on human SH-SY5Y neuroblastoma cells for its toxicity and cytoprotective properties.

Undifferentiated human SH-SY5Y cells were chosen as the cellular system to study the protective properties of RSP on markers of oxidative stress and DNA damage due to their many advantages over other models as reviewed by Xicoy *et al.* (Xicoy et al., 2017), Kumar Dubey *et al.* (Dubey et al., 2019) and Kovalevich and Langford (Kovalevich & Langford, 2013). Nevertheless, they have a number of limitations, such as their cancer properties, as they are derived from SH-N-SH, uncloned neuroblastoma cells (Biedler, Helson, & Spengler, 1973). However, they are easy to culture and hence have been used in many publications as a first study model for testing protective properties of natural products in neuron like cells (Koriyama et al., 2015; Krishna et al., 2014; Morán-Santibañez et al., 2019; Xicoy et al., 2017; Yu et al., 2012).

SH-SY5Y cells showed high tolerability towards the RSP extract with concentrations up to 1.5 mg/mL exhibiting no significant impact on cellular metabolism (toxicity). Only concentrations above 2 mg/mL of RSP extract had a significant effect on cell viability (Figure 1).

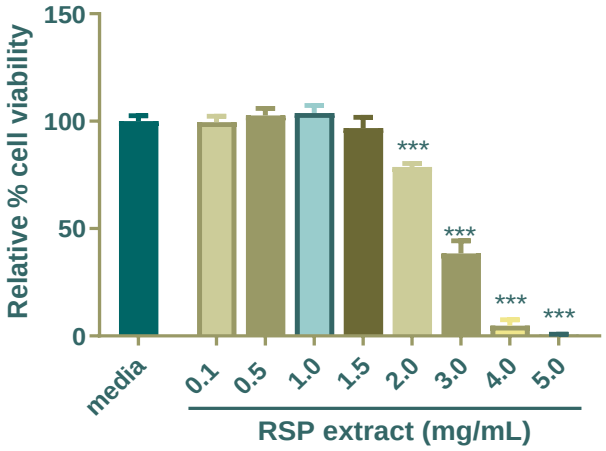


Fig. 1 Cell viability assay of RSP extract (0.1-5.0 mg/mL, 24 h treatment) using Resazurin. One-way ANOVA and Bonferroni's multiple comparison analysis was used to assess statistical significance relative to media control: ***p≤0.001, n=3

The SH-SY5Y cells exhibited higher tolerance to the RSP extract in comparison to other natural product extracts published previously. For example, H.E. Moore (corozo fruit) reported lower SH-SY5Y cell viability with an ethanol extract of *Bactris guineensis* (L.) at a concentration of 0.15 mg/mL (López et al., 2017), meaning that the latter plant extract is 10 times more toxic than our the RSP extract. Similarly, even lower concentrations (0.05 and 0.10 mg/mL) were found to cause significant decrease in cell viability with *Agaricus blazei* (almond mushroom) extract (Venkatesh Gobi et al., 2018). An elevated tolerance towards the RSP extract is advantageous, since higher extract concentrations can be used to study cells protection from oxidative stress in the current study.

3.2. Protection of SH-SY5Y cells with RSP extract from induced H_2O_2 ROS production

Previously, plant extracts have demonstrated *in vitro* protection from ROS. In the case of RSP extract, treatment of SH-SY5Y cells with extracts at 0.25-1.0 mg/mL did not induce ROS production; however, significant increase ($p \leq 0.001$) in ROS levels was observed with H_2O_2 (1 mM, 30 min, Figure 2a, **Supplementary Information**, Figure 1). However, the pre-treatment of cells with RSP extract (0.25-1.0 mg/mL) for 24 hours followed by H_2O_2 (1 mM) incubation for 30 minutes, resulted in significant suppression of ROS production by 60% ($p \leq 0.05$) and 68% ($p \leq 0.01$) for 0.75 and 1.0 mg/mL RSP extract, respectively (Figure 2b, **Supplementary Information,** Figure 1).

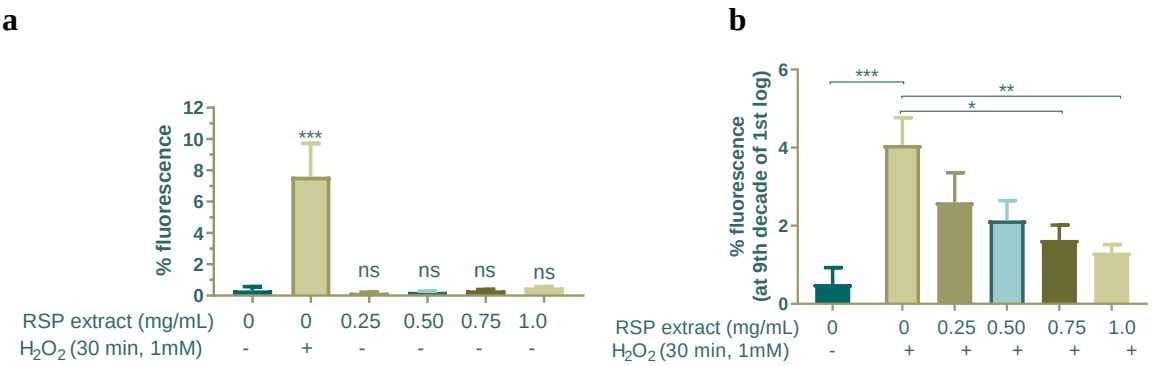


Fig. 2 (a) Generation of ROS in SH-SY5Y cells treated with H_2O_2 (1 mM, 30 min) at varying RSP extract concentrations (0.25-1.0 mg/mL); (b) ROS generation after 24 h RSP extract pre-treatment followed by exposure to H_2O_2 (1 mM, 30 minutes); One-way ANOVA and Bonferroni's multiple comparison analysis was used to assess statistical significance relative to media control: ns: not significant; * $p \leq 0.05$, ** $p \leq 0.01$,*** $p \leq 0.001$, $n \geq 3$

Of note, Park *et al.* (Park et al., 2015) reported that SH-SY5Y cells stressed with H_2O_2 (100 µM) after pretreatment with *Liriope platyphylla* extract (0.5-50 µg/mL) for 6 hours, resulted in a decrease of ROS concentrations. In another study, phenolic extracts of *Inula helenium* (0.5, 5 µg/mL and 1 hour cellular pretreatment) significantly reduced ROS production after 200 µM H_2O_2 treatment (Wang, Zhao, Zhang, & Guo, 2015). In contrast to the latter two papers in which extracts' pre-treatment was carried out for one and six hours respectively, in our study pre-treatment with RSP extract was for 24 hours. Direct comparison among previous studies to determine which extract showed better protection are difficult due to the different concentrations of H_2O_2 and extract employed. ROS are well accepted to be the root of oxidative stress within a cellular system. This is because oxidative stress can lead to DNA strand breaks (nuclear) and lipid peroxidation. Therefore, here we studied the ability of RSP extract to prevent or reduce these consequences as a result of increased ROS levels.

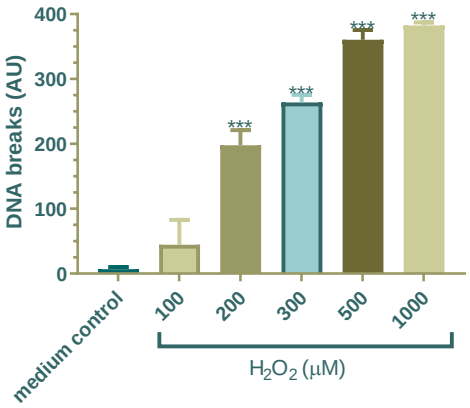
3.3. H_2O_2 (1 mM) does not induce measurable levels of MDA as result of lipid peroxidation in SH-SY5Y cells

The degree of lipid peroxidation induced by treating SH-SY5Y cells with H_2O_2 (1 mM) for 30 minutes was very minimal and below the limit of detection (data not shown). Interestingly similar observation was reported when H_2O_2 was utilised as the stressor against Jurkat T cells in a previous work by Erba *et al* .(Erba, Riso, Criscuoli, & Testolin, 2003).

3.4. Prevention of DNA strand breaks induced by H_2O_2 (300 mM) with RSP extract pretreatment

In previous studies we demonstrated that RSP extract prevented plasmid DNA damage (cuts from supercoiled to circular and linear forms) *in vitro* induced by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) (Pohl et al., 2018; Yates et al., 2019). To determine whether DNA damage can be reduced/prevented in a cellular environment, the comet assay (single cell gel electrophoresis) was carried out on SH-SY5Y cell line. Increasing H_2O_2 concentrations (100-1000 µM) showed the expected increase in DNA breaks (Figure 3a). However, when cells were pre-treated with only RSP extract, no increase in DNA strand breaks was observed (Figure 3a), thus confirming the safe RSP extract concentration (1 mg/mL) towards SH-SY5Y cells used in this study.

a



b

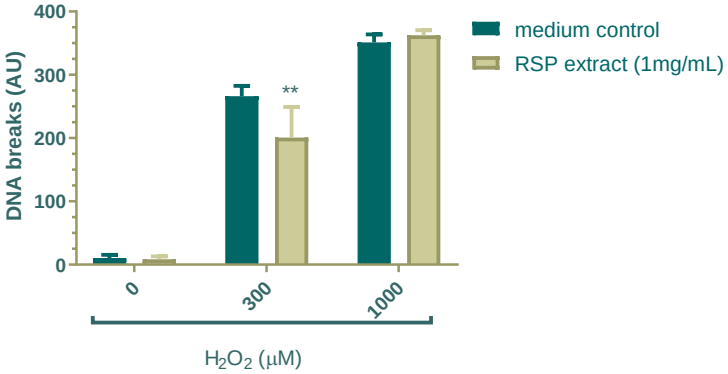


Fig. 3 (a) DNA strand breaks induced by H_2O_2 (30 minutes) in a concentration-dependent manner; (b) Cellular DNA protection from H_2O_2 (300 but not 1000 µM) induced DNA strand breaks by the RSP extract (1 mg/mL, 24 h preincubation); One-way ANOVA and Bonferroni's multiple comparison analysis was used to assess statistical significance relative to media control: **p≤0.01***p≤0.001, n≥3; AU-arbitrary units

In addition, RSP extract (1 mg/mL) pretreated cells were able to partially prevent H_2O_2-induced DNA strand breaks (Figure 3b). This effect further supports the RSP extracts protective properties at molecular level *in vitro*. We would like to highlight that this is the first reported study where RSP extract has been used to protect cellular DNA from H_2O_2-induced DNA strand breaks. There are only a handful of similar studies in the literature where natural compounds/extracts had previously been used to prevent DNA strand breakage in SH-SY5Y cells. For example, in a report by Kim *et al.* (H. S. Kim, Lee, Kang, Lee, & Hyun, 2012), they showed similar positive results with the comet assay. They applied a pre-treatment with phloroglucinol (1,3,5-trihydroxybenzene), a phenolic (phlorotannin) commonly found in *Ecklonia cava* (brown algae). The latter prevented high ROS levels, induced by H_2O_2 (0.8 mM) and showed DNA protective properties (10 µg/mL). Furthermore, boysenberry and blackcurrant extracts (rich in

phenolic or/and anthocyanin) exhibited DNA protective properties in HL-60 cells with the comet assay (Ghosh, McGhie, Zhang, Adaim, & Skinner, 2006).

3.5 Cell stress protein levels show changes upon RSP extract and H_2O_2 treatment

Protein, DNA and lipid damage to neuron cells is the main contributary factor to the development of many neurodegenerative diseases. Those compounds with the ability to significantly reduce ROS levels and protect DNA strand breakage when under oxidative conditions, could lead to candidates that can further be developed to prevent, manage, and treat these neurological disorders. Hence, further future studies to decipher the mechanism of how RSP extract protects DNA from damage is required. However, a possible explanation for the protection properties of the RSP extract on SH-SY5Y cells, may arise directly from its inherent antioxidant properties. Furthermore, the RSP extract could also impart indirect cellular protection *via* the modulation of cellular signaling pathways. From the literature there has been several reports showing that many extracts and isolated compounds activate the Nrf2 pathway both *in vitro* and *in vivo* models. For example, curcumin, sulforaphane and resveratrol are a selection of compounds that are known to trigger antioxidant and detoxification enzymes after the upregulation Nrf2 expressions (Hodges & Minich, 2015; Stefanson & Bakovic, 2014). Here further *in vitro* investigation to the effect of the RSP extract on the cell stress protein expression was carried out with protein arrays analysis.

Protein arrays were used to study the potential stress related pathways affected by pre-treating SH-SY5Y cells with RSP extract followed by H_2O_2- induced stress (**Supplementary Information**, Figure 3). For the array analysis, a cut-off of 2-fold increase and/or 0.5-fold decrease was set. Sixteen out of 26 cell stress related proteins studied showed no change after either RSP extract (24 h, 1 mg/mL) and/or hydrogen peroxide treatment (30 min, 1 mM) (Table 1) and were not studied further.

Table 1 List of stress related proteins not affected by treatment with 1 mM H_2O_2 for 30 min (H_2O_2/C), 1 mg/mL RSP for 24 hr (RSP/C), or both treatments (RSP+H_2O_2/C), and their relative expression (treatment to control ratio between 0.5 and 2, the set cut-off points) in SH-SY5Y cells

Cell stress protein	H_2O_2/C	RSP/C	RSP+H_2O_2/C
ADAMTS1	1.3	1.3	1.3
Carb. Anhydrase	1.2	1.0	1.1
Cited-2	1.3	1.8	1.5
COX-2	1.4	1.4	1.4
Dkk-4	1.5	1.8	1.6
HIF-2a	1.5	0.8	1.0
HSP60	1.7	1.5	1.6
HSP70	1.5	1.7	1.8
IDO	1.0	1.0	1.1
NFkB1	1.5	1.6	1.5
p21	1.6	1.8	1.4
p27	1.4	1.9	1.2
Phospho-p53	1.7	0.8	1.7
PON3	1.1	0.6	1.1
Thioredoxin-1	1.5	1.8	1.8
SIRT2	1.5	1.7	1.7

On the other hand, protein array analysis showed upregulation of eight cell stress proteins (FABP-1, HIF-1α, phospho-HSP27, JNK Pan, p38α, PON2, SOD2 and Cytochrome c). Of those, phospho-HSP27, p38α and PON2 cell stress proteins were upregulated to ≥ 2.0 upon RSP extract and/or H_2O_2 treatment (Figure 4) while only two proteins

(Bcl-2 and PON1) were down regulated to ≤ 0.5 (Figure 5). p38α was upregulated 5-fold by H_2O_2 treatment but upon RSP extract pretreatment, its upregulation was decreased 4-fold (Figure 4). p38α is a mitogen-activated protein kinase (MAPK) known to be triggered by the presence of ROS and other physical and chemical stressors (Matsuzawa & Ichijo, 2008). In our analysis, p38α levels were highly increased (5-fold) by H_2O_2 but only changed slightly (1.6-fold) by RSP extract treatment compared to the control. Similarly to our observations in H_2O_2 treated SH-SY5Y cells, Xia *et al.* (Xia, Dickens, Raingeaud, Davis, & Greenberg, 1995) demonstrated in PC12 (neuron-like pheochromocytoma cells) that withdrawal of nerve growth factors can lead to similar upregulation of p38α and that this was required for induction of apoptosis. This implies that a reduction in p38α would reduce neuronal cell death, which is an important factor in neuro-degenerative diseases.

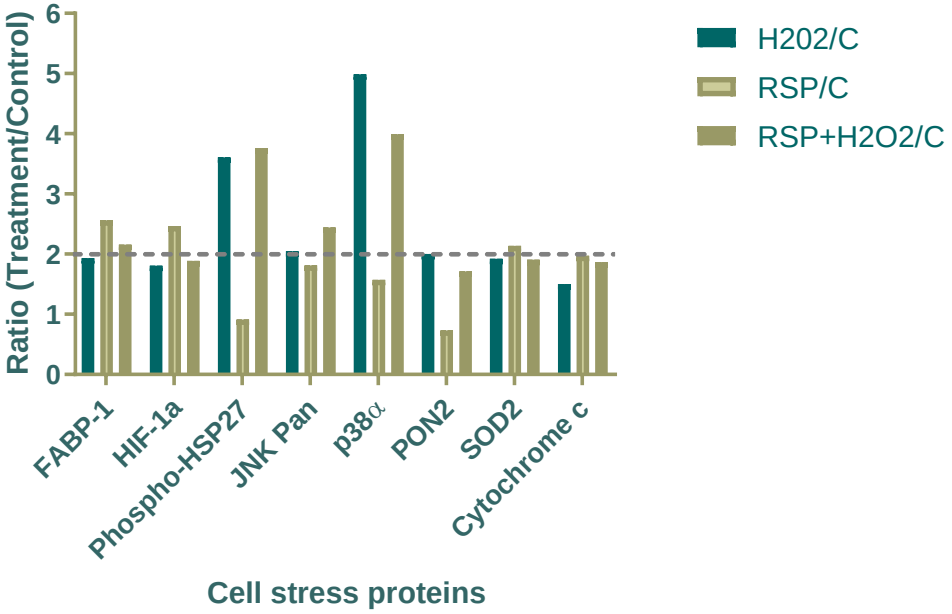


Fig. 4 Relative proteins' amount in SH-SY5Y cells treated with 1 mg/ml RSP for 24 hr and/or 1 mM H_2O_2 for 30 min with ratio treatment to control ≥ 2.0; experiment performed in duplicate

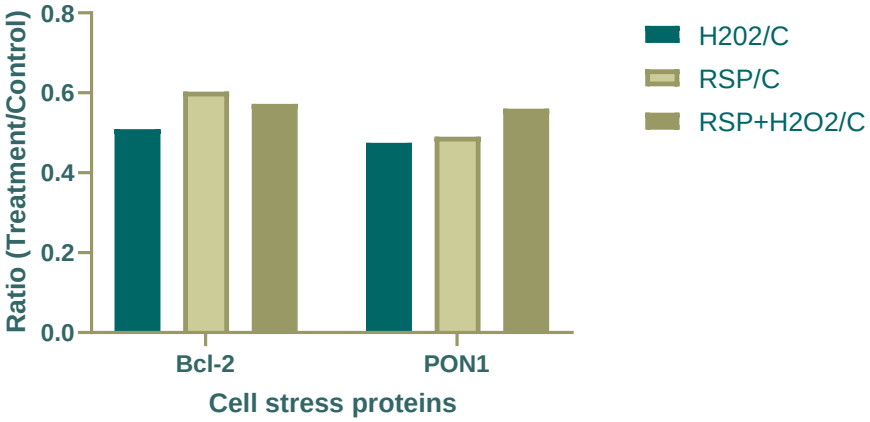


Fig. 5 Relative proteins' amount in SH-SY5Y cells treated with 1 mg/ml RSP for 24 hr and/or 1 mM H_2O_2 for 30 min with ratio treatment to control ≤ 0.5, experiment performed in duplicate

Interestingly two other cell stress related proteins i.e. Bcl-2 and PON1 were down regulated after H_2O_2 treatment (1 mM for 30 minutes) to 0.5 whereas only PON1 was down regulated by RSP extract (1 mg/mL) treatment to 0.5 (Figure 5) and there were no further changes with RSP pre-treatment. It is known that the regulation of Bcl-2 is associated with apoptosis and cell death (Chi, Chang, & Sang, 2018) while PON-1 has previously shown to have protective properties (Borowczyk, Shih, & Jakubowski, 2012; M. J. Kim et al., 2015; Levy, Reichert, & Bydlowski, 2019).

Importantly, most proteins in this study, were either not affected by the pretreatment with the RSP extract compared to untreated cells (10 proteins) nor there was a change in levels compared to the H_2O_2 treatment alone (six proteins). It is noteworthy to mention that interpretation of the protein array is however limited due to the number of proteins up ($\geq$ 2-fold) and/or down ($\leq$ 0.5-fold) regulated (10 in total) and the complexity of their interactions in this cell system.

4.0 CONCLUSION

Damage to biomolecules such as DNA, protein and lipid in neurons are well known to be responsible for the development of many neurodegenerative diseases. The ability of compounds to protect DNA strand breakage and reduce ROS levels when under oxidative attack, could provide potential therapeutic candidates in the prevention, management and treatment of these neurological disorders. Here we have demonstrated at cellular level that RSP extract at a non-toxic concentration reduces the levels of ROS and provide cellular protection by reducing oxidative DNA damage in the presence of an oxidizing agent (H_2O_2). Furthermore, the potential indirect antioxidant activity of the RSP extract through the modulation of antioxidant pathways within the cell system was exploited. We found in pretreated cells, the downregulation of p38α, a mitogen-activated protein kinase amenable to stress stimuli. Taken together the protective properties of the RSP extract presented in this study while considering the promising effects showed *in vivo* studies in *C. elegans* (Pohl et al., 2019), warrant further studies to understand the full potential of the RSP extract on oxidative stress and in particular, in the context of neuro-degenerative diseases.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information

Impact of Rapeseed Pomace (RSP) extract on markers of oxidative stress and DNA damage in human SH-SY5Y cells

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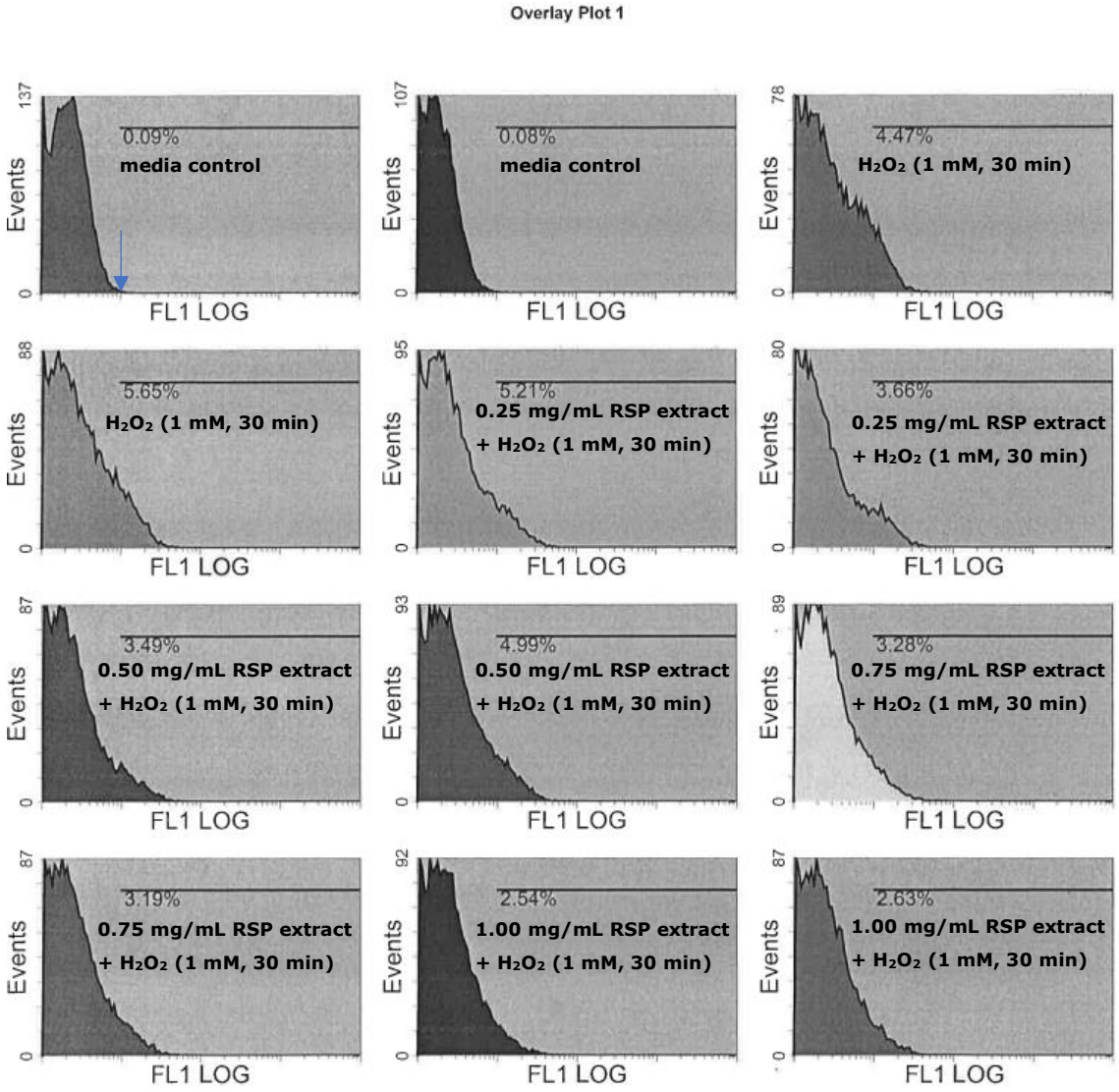
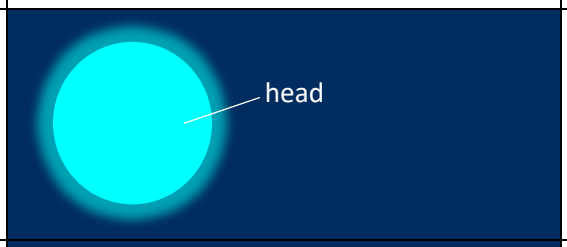
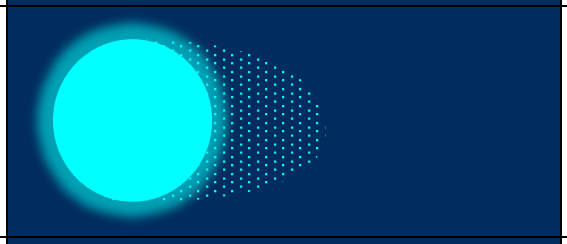
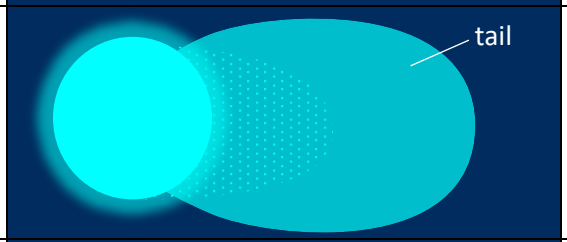
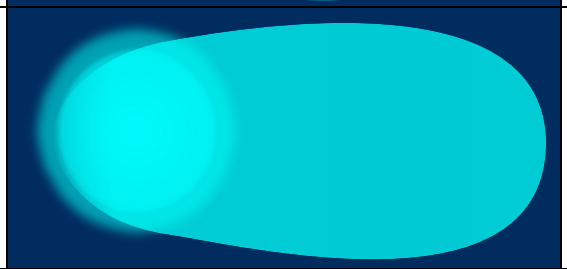
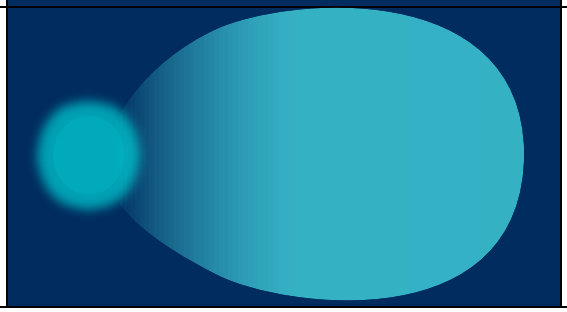
Figure 1



Overlay Plot 1 Data Source	Statistic	Value
media a.LMD Ungated FL1 LOG	LM1	0.09
media b.LMD Ungated FL1 LOG	LM1	0.08
H2O2 a.LMD Ungated FL1 LOG	LM1	4.47
H2O2 b.LMD Ungated FL1 LOG	LM1	5.65
0.25 a.LMD Ungated FL1 LOG	LM1	5.21
0.25 b.LMD Ungated FL1 LOG	LM1	3.66
0.5 a.LMD Ungated FL1 LOG	LM1	3.49
0.5 b.LMD Ungated FL1 LOG	LM1	4.99
0.75 a.LMD Ungated FL1 LOG	LM1	3.28
0.75 b.LMD Ungated FL1 LOG	LM1	3.19
1 a.LMD Ungated FL1 LOG	LM1	2.54
1 b.LMD Ungated FL1 LOG	LM1	2.63

Figure 1. Raw data obtained from flowcytometry, showing Events (number of cells) related to their fluorescence intensity on a logarithmic scale (FL1 LOG) and percentage of events (cells) counted after threshold of 9th decade of 1st log (blue arrow)

Figure 2

Comet score	Schematic	Criteria
0		• Bright big circular comet head • No indication of tail
1		• Big and bright circular comet head (same as 0) • Sparkle like tail visible; at least the length of the head
2		• Bright circular comet head, same size as 0 and 1 • Comets show significant tail • Tail is less bright than head • Head still has a sharp outer contour
3		• Big head, but fainter in brightness, similar to tail brightness • Tail and head begin to merge in shape • Head loses sharp circular contour
4		• Small head with decreased brightness • Head is smaller in diameter than tail and looks separated from tail

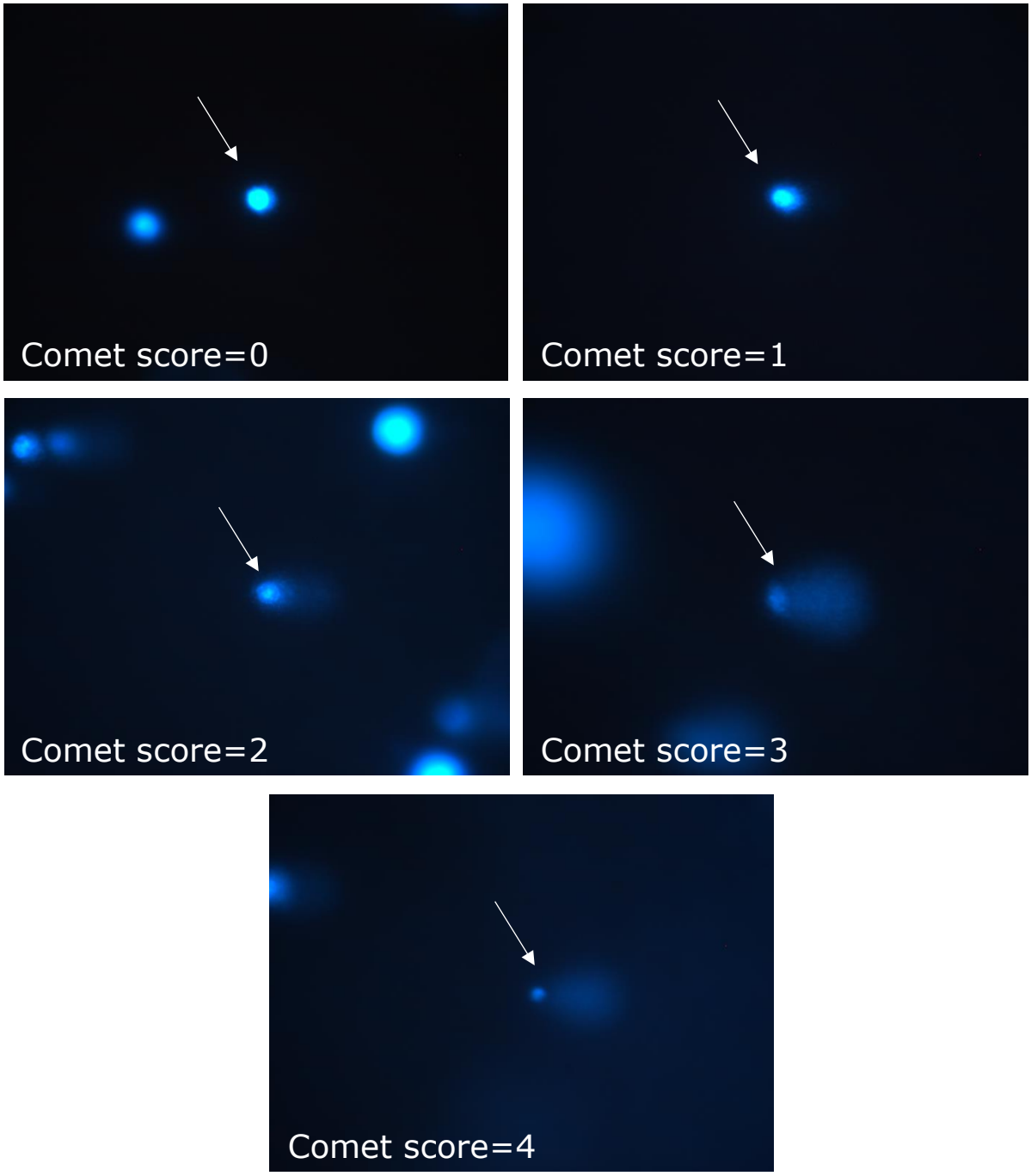
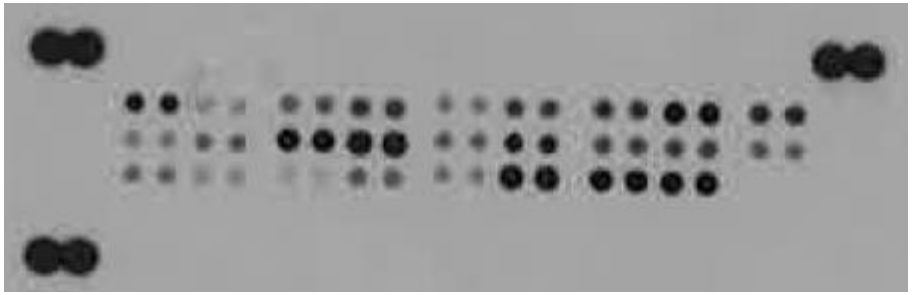


Figure 2: Comet scores and their criteria and SH-SY5Y examples

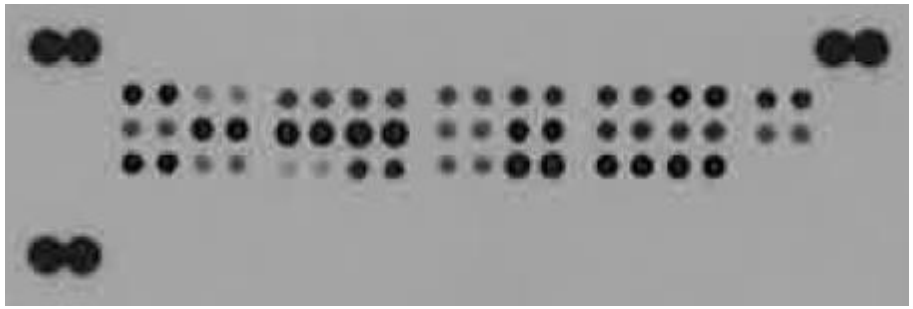
Figure 3

Control

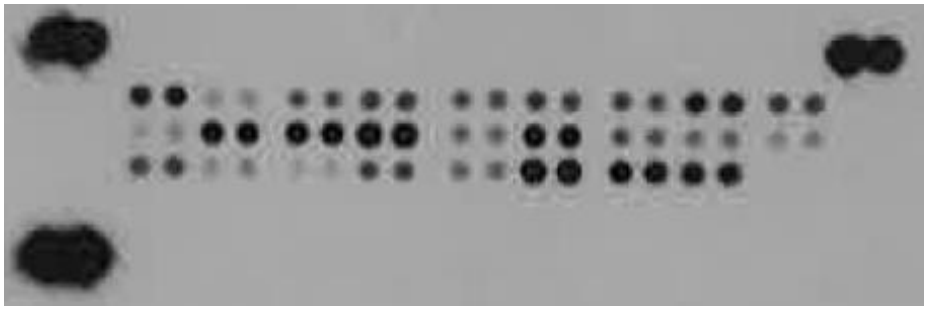
RP

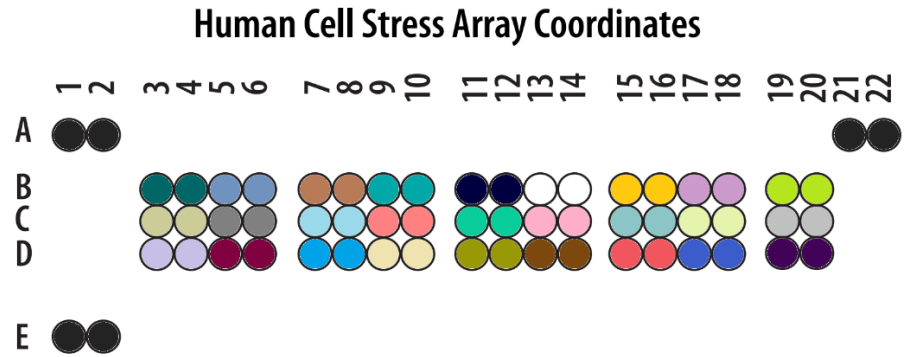


H_2O_2



RP + H_2O_2





Coordinate	Colour	Analyte/Control	Alternative name
A1, A2, A21, A22, E1, E2		Reference Spot	/
B3, B4		ADAMTS1	/
C3, C4		HIF-2α	EPAS1
D3, D4		Phospho-p38 (T180/Y182)	
B5, B6		Bcl-2	
C5, C6		Phospho-HSP (S78/S82)	
D5, D6		Phospho-p53 (S46)	
B7, B8		Carbonic Anhydrase IX	CA9
C7, C8		HSP60	
D7, D8		PON1	
B9, B10		Cited-2	
C9, C10		HSP70	
D9, D10		PON2	
B11, B12		COX-2	
C11, C12		IDO	Indoleamine 2,3-dioxygenase
D11, D12		PON3	
B13, B14		Cytochrome c	
C13, C14		Phospho-JNK Pan (T183/Y182)	
D13, D14		Thioredoxin-1	
B15, B16		Dkk-4	
C15, C16		NFκB1	
D15, D16		SIRT2	Sirtuin 2
B17, B18		FABP-1	L-FABP

C17, C18		p21/CIP1	CDNK1A
D17, D18		SOD2	MnSOD
B19, B20		HIF-1α	
C19, C20		p27	Kip1
D19, C20		Negative control	Control (-)

Figure 3 Human cell stress proteins included in the protein array adapted from (R&D Systems Inc., 2014)

R&D Systems Inc. (2014). Proteome Profiler™ Array-Human Cell Stress Array Kit. 1–11.