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The cytotoxic and genotoxic effects of bisphenol A on neuronal cells in vitro

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Abstract

Endocrine disrupting chemicals (EDCs) have the potential to cause adverse effects to human health by disrupting normal homeostasis, reproduction, development and behaviour. They can simultaneously act as cytotoxic and genotoxic agents. Bisphenol A (BPA) is a well-documented EDC, widely used in the manufacturing of plastic and epoxy resins. Humans are regularly exposed to this chemical through ingestion of food and beverages. The adverse effects of BPA on reproduction and development have been thoroughly studied. The potential adverse effect of BPA however, on neuronal development has not been entirely explored. In this study, the potential cytotoxic and genotoxic effects of BPA were investigated on neuroblastoma cells (SH-SY5Y). While the cytotoxicity was determined using the MTT assay, genotoxicity was determined using the comet assay. Time and concentration dependent effects were observed for cellular viability in this cell line following BPA exposure. BPA significantly reduced cell viability at high concentrations (>250 µM) after 24h and (> 100 µM) 48h exposures. Nevertheless, low environmental concentrations were not found to be cytotoxic. No significant increase in DNA damage was observed after a 24h exposure, while 500 nM significantly increased DNA damage after a 48h exposure. Scanning electron microscopy (SEM) results showed no morphological changes after exposure to BPA. This study provides *in vitro* evidence of the potential adverse effects of BPA on the nervous system and possible effects on neuronal development. However, further investigation would be required to decide if environmental concentrations are likely to be potentially hazardous to human health.

Introduction

There is growing concern for the potential health implication posed by endocrine disrupting chemicals (EDC). These chemicals are suggested to interfere with the biosynthesis, metabolism and action or elimination of natural hormones within the body, which are responsible for homeostatic control, reproduction and development (Diamanti-Kandarakis et al., 2009). EDC or endocrine disrupters can occur naturally or are man-made and are released into the environment. Humans are regularly exposed to EDC via ingestion, inhalation or via direct skin contact (Gupta et al., 2010)

Bisphenol A or 2,2-bis (4-hydroxyphenol) propane (BPA) is one of the most well-known and documented endocrine disrupters, due to its ability to mimic the action of steroidal oestrogens (Ishido et al., 2007). It is considered to be a weak environmental oestrogen which can stimulate cellular response, at very low doses, by binding to the α or β form of the oestrogen receptor, disrupting the normal activity of the oestrogen nuclear hormone receptors in a range of target sites (Milić et al., 2015; Wetherill et al., 2007).
BPA is currently one of the highest production chemicals in the world with 6.5 million pounds produced per year (Vom Saal and Hughes, 2005). The xenoestrogen has been extensively used in the manufacturing of polycarbonate plastics, epoxy resins, epoxy dental sealants, flame retardants and canned foods and has been in production since the 1950s (Iwakura et al., 2010; Wetherill et al., 2007).

Under certain physical or chemical conditions (including sterilization, pH change or increased temperature), BPA can leach into food and beverage containers by the hydrolysis of polymers or via the diffusion of residual BPA, and therefore contaminate the food chain (Hoekstra and Simoneau, 2013).

This can lead to regular human exposure and widespread environmental contamination due to its high volume production (Le *et al*., 2008). The World Health Organisation estimate international dietary exposure to humans ranges from 0.4-4.2 µg/Kg/day (Milić *et al*., 2015). BPA exerts lipophilic properties with a log K_{ow} of 3.64, it's proposed that BPA has the potential to bio accumulate in storage sites such as adipose tissue and could easily accumulate into the brain (Calafat *et al*., 2008; Milić *et al*., 2015;). Because of BPAs lipophilic properties it can infiltrate the blood brain barrier and potentially cause adverse effects on the nervous system, principally brain development (Kim *et al*., 2007; Palanza *et al*., 2008). BPA has been found in human cord blood, placenta, amniotic fluid and breast milk, resulting in the exposure of foetuses and newborns, therefore the chemical might exert effects on the developing brain (Balakrishnan *et al*., 2010; Ikezuki *et al*., 2002; Mori, 2001; Schönfelder *et al*., 2002). It has also been found in urine samples as discovered by Calafat *et al*., (2008) who measured the total urinary concentration of BPA in 2517 participants of the US population, BPA was found in 92.6% of participants. Elevated gestational urinary concentrations of BPA have been linked with adverse behaviors in children including hyperactivity and aggression predominately observed in 2-year-old girls (Braun *et al*., 2010).

The adverse effect of BPA as an endocrine disrupter has been monitored in many studies showing a multitude of effects. These effects include the blocking of the estrogenic response (Richter *et al*., 2007; Rochester, 2013) effects on postnatal growth and early onset of puberty (Howdeshell *et al*., 1999), decreased testosterone synthesis and steroidogenic enzymes (Akingbemi *et al*., 2013) obesity (Milić *et al*., 2015), interference of thyroid hormone signalling (Wetherill *et al*., 2007; Zoeller *et al*., 2005) and cancer

susceptibility (Muñoz-de-Toro *et al*., 2005). However, many studies on BPA exposure are focused on its effects on reproduction and development due to its oestrogen mimicking capability (Elsworth *et al*, 2013). This study focuses solely on effects of BPA on the developing nervous system, as the central nervous system (CNS) is paramount for survival.

The developing brain is extensively sensitive to oestrogen because prenatally, and days after birth, the activity of estradiol and the enzymes responsible for estradiol syntheses, P-450 aromatase and oestrogen receptors are at their peak levels. Therefore, the brain could be potentially vulnerable to xenestrogen exposure (McCarthy, 2008). During mammalian brain development, oestrogen is vital to the organisation of neural circuits that are responsible for neuroendocrine behavioural and cognitive function (Palanza *et al*., 2008). Alterations in the estrogenic environment of the developing CNS could affect critical aspects of cellular differentiation, including myelination, neurotransmitter expression, synapse formation, neurite extension and the survival of the neuronal cells (Arnold and Gorski, 1984; Palanza *et al*., 2008).

Many studies have confirmed the adverse effects of BPA on the brain. Effects observed include degeneration of adult dopaminergic neurons and hyperactivity in juveniles (Ishido and Masuo 2014), abnormalities in ventral mesencephalon and hippocampus, and decreases in tyrosine hydroxylase expressing neurons (Elsworth *et al*., 2013) alterations in dopamine responsiveness, leading to drug dependency (Miyatake *et al*., 2006). BPA can also disrupt sexual differentiation during prenatal or neonatal exposure (Wetherill *et al*., 2007) and changes to maternal behaviour (Della Seta *et al*., 2005; Palanza *et al*., 2002). There have been suggestions of the involvement of BPA in neurodegenerative disorders such as Parkinson's disease and memory and cognitive disorders (Elsworth *et al*, 2013; Masuo and Ishido, 2011).

In vivo studies are often used to assess the toxic effect of chemicals. However, the use of animals raises many ethical implications and extremely costly procedures. Species variation also hinders our ability to use animals for predicting human responses due to the variation in metabolism and excretion of chemicals (Hartung and Daston, 2009). The human brain is more complex than a rodent making it hard to extrapolate with certainty the data collected in rodents to understand aspects of human brain development (Clowry *et al*., 2010). Therefore, *in vitro* testing is often used as it allows us to clearly see the direct effect of chemicals on the cells. The testing is often relatively quick, cheap and high volume of replicates can be produced. The use of *in vitro* studies also gives insight into the molecular and cellular mechanism of carcinogenesis caused by chemical carcinogens which is difficult *in vivo* or human systems (Tsutsui and Barrett, 1997).

The cytotoxic effects of BPA have been observed in a number of *in vitro* studies using a range of cell lines (Chen *et al*., 2013; Kafi *et al*., 2011; Kim *et al*., 2007; Kuo *et al*., 2011; Terasaka *et al*., 2005; Xu *et al*., 2002). Cytotoxicity can be determined using the MTT assay, which measures the viability of cells to an external factor. For most viable cells mitochondrial activity is persistent (Van Meerloo *et al*., 2011). The mitochondrial activity of cells is determined by the conversion of the yellow tetrazolium salt (MTT) into purple formazan crystals, which is dependent upon the action of succinate dehydrogenase enzymes to generate reducing equivalents such as NADH and NADPH. Formazan is impermeable to the cell membrane and will accumulate in healthy cells (Fotakis and Timbrell, 2006).

The chemical structure of BPA is said to be similar to that of diethylstilboestrol (DES), a potent synthetic oestrogen that has been known to cause carcinomas during prenatal exposure (Kurisawa *et al*., 2002). It has been well documented that estrogens, cause cellular proliferations and hence could act as cancer promotor or as a carcinogen (Tsutsui and Barrett, 1997). It has been suggested that hormonal metabolites may openly induce DNA damage and initiate carcinogenesis (Jha *et al*., 2000; Roy and Liehr, 1999). It is therefore not unexpected that the presence of BPA, a chemical that interferes with hormonal metabolism, causes concern to cancer development, reproduction and developmental issues (Jha *et al*., 2000).

BPA has been known to induce significant DNA damage in *in vitro* testing (Pfeifer, *et al*., 2015; Xin *et al*., 2015), providing evidence of the genotoxic effect of BPA. Alkaline comet assay was chosen to assess the genotoxic effects of BPA. This assay is a rapid and quantitative technique that is used to measure the amount of DNA damage visually. The assay is based on the quantification of denatured DNA migrating out of the cell nucleus during electrophoresis. It is commonly used as a biomarker of exposure to environmental contaminants (Liao *et al*., 2009).

To understand the mechanisms of BPA, it is important to assess the effects of environmentally relevant low doses. For *in vitro* studies low concentrations are defined as <50 ng/mL or $<2.9 \times 10^{-7}$ M (Cao *et al*., 2010; Wetherill *et al*., 2007). In this study, it was important to test both low dose concentrations within the nanomolar range and higher concentration in the micro-molar range. It has been suggested that micro-molar range of BPA could be present in the blood of patients after high dose oral supplementation or intravenous therapy (Lee *et al*., 2007).

In the backdrop of the above information, the study aims to assess the cytotoxic and genotoxic effects of BPA on SH-SY5Y neuroblastoma cells. This cell line is of neuronal origin and was originally sub cloned from the SK-N-SH cell line derived from a 4 year old female. This cell line is often used for *in vitro* models for the study of the mechanisms of action and neurotoxicity of compounds on the nervous system (Filograna *et al*., 2015) therefore it was an appropriate choice to test the toxicity of BPA. MTT assay was used to assess cytotoxicity and comet assay was used to assess genotoxicity. Scanning electron microscopy (SEM) analysis was also used to assess its effect on cellular morphology. All the assays were validated in this cell line using copper as a reference toxicant.

Materials and methods

Chemicals and reagents

BPA (CAS 80-05-7, 97%) and DMSO (CAS 67-68-5) was obtained from Sigma-Aldrich, UK and cupric sulfate pentahydrate (CAS: 7758-99-8) was purchased from BDH chemicals Ltd. MTT (thiazolyl blue tetrazolium bromide, CAS: 298-93-1, >98%) was purchased from Melford biolaboratories Ltd, UK. Dulbecco's modified eagles medium (DMEM), Dulbecco's phosphate buffer saline (DPBS) and 0.25% Trypsin were purchased from Gibco by life technologies.

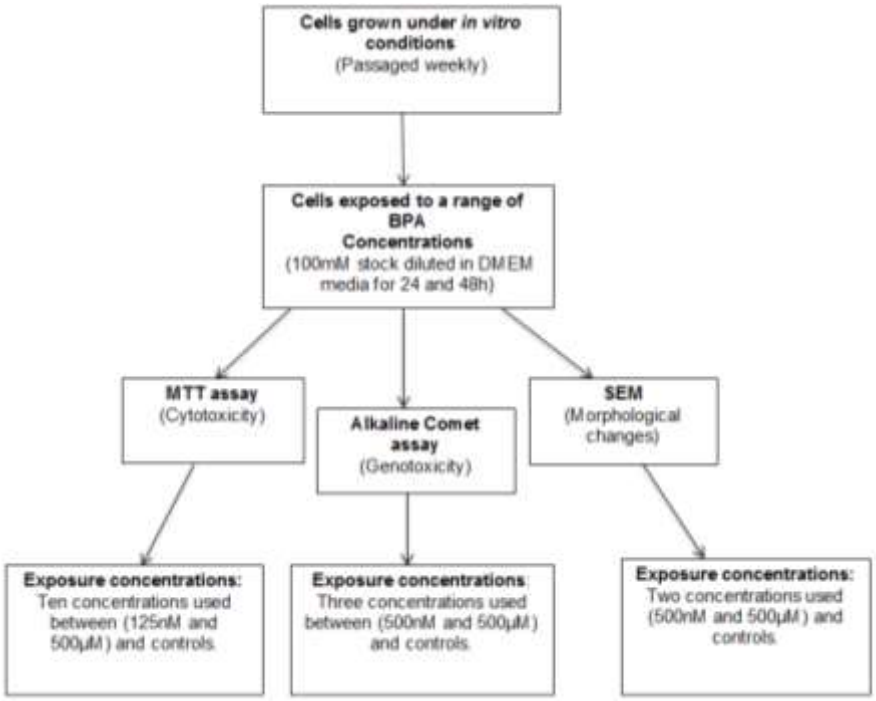

Figure 1: Schematic diagram for the methods of assessing the effects of Bisphenol A on SH-SY5Y cell line.

Cell culture and treatment to BPA

Overall experimental design has been presented in Figure 1. SH-SY5Y (neuroblastoma cells) was obtained from ATCC. Cells were grown in DMEM (Dulbecco's modified eagles medium) supplemented with 10% foetal bovine serum (50 mL) and 0.5% penicillin-streptomycin solution in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The cells were cultured in T25 flasks. Confluent cultures were sub cultured by treatment with Dulbecco's phosphate buffered solution (DPBS) and then treated with 0.5 mL of 0.25% trypsin washing solution. To ensure cells were fully detached they were viewed under an inverted microscope. The cells were re-suspended in fresh DMEM media and cell counts were carried out using a haemocytometer. For experimental purposes cells were cultured in 96 well plates, 12 well plates and 6 well plates. Cells ($1x10^4$ cells/mL) were seeded in culture plates for 24h until they became adherent. Immediately after this the required concentration of BPA was added to each well and incubated for either 24 or 48h at 37°C.

Bisphenol A was first dissolved in ethanol to a stock concentration of 100mM. Incubated at -20°C and diluted with DMEM media to the final concentrations required on the day of use. The final concentration of ethanol did not exceed 0.1%. Control cells were incubated with 0.1% ethanol. Copper was used as a positive control to validate the assays. Cupric sulfate pentahydrate was dissolved in distilled water to a concentration of 100mM, and then further diluted in distilled water to the test concentrations (125μM, 250μM and 500μM). Exposure concentrations were copper concentrations not $CuSo_4$ concentrations.

Cytotoxicity assay

Cytotoxicity of BPA was measured by the 3-[4.5-dimethylthiazol-2-yl]-2, 5 diphenyl-tetrazolium bromide (MTT) assay. SH-SY5Y cells were treated with BPA for the desired exposure time (24h, 48h). All media was removed from the 96-well plates and 85µl of media was re-added including three extra wells for media controls. MTT solution (5mg/mL) was prepared in DPBS and 15µl was added to each well and incubated for 3h at 37°C. After 3h the 96-well plates were removed from incubation and 100µl of DMSO was added to each well to dissolve the dark blue formazan crystals and incubated for a further 10 mins at 37°C. Cell cultures were then re-suspended with a 200µL pipette and absorbance was measured at 595nm using a VerseMax ELISA microplate reader. Cell viability was expressed as a percentage of the absorbance value of control cells. The effect of each concentration was analysed in triplicate and each experiment was repeated four times.

Genotoxicity assessment

Genotoxicity was measured using the alkaline comet assay. The cells were seeded in 6 well plates at ($1x10^4$ cells/mL), for 24h. Following this, the cells were exposed to the required BPA concentrations (control, 500nM, 100µM, 500µM and copper positive control 500µM) for 24 and 48h. All media was removed from each well and 200µL of trypsin was added and incubated for 10 minutes at 37°C. Following incubation 300µL of DMEM media was added to each well, cell cultures were transferred into a 1mL Eppendorf tube. Following 20µL of Sh-SY5Y cells were mixed in 75µL of 0.75% low melting point agarose and immediately spread onto a glass cover slide, pre-coated in 1.5% normal melting point agarose using cover slips. The slides were then incubated for 1.5h in a 4°C refrigerator. Cover slips were removed from the glass slides and then incubated in lysis solution (2.25M NaCl, 100mM EDTA, 10mM Tris base, 1% N-lauroyl-sarcosine, 10% DMSO and 1% Triton X-100, pH 10.0) at 4°C for 1.5h, to remove cellular proteins and cell membranes. Slides were removed from the lysis solution and placed into an electrophoresis chamber containing electrophoresis buffer (300mM NaOH, 1mM EDTA, pH 13.0). Slides were left in electrophoresis buffer for 20 mins at 4°C allowing the DNA to unwind. Electrophoresis was conducted for 25 min at 25 V, allowing the DNA to migrate to the anode. Slides were then neutralised (0.4M Tris, pH 7.5) and left to dry at room temperature. Slides were stained with 20µL ethidium bromide (20µg/ml) and analysed under a fluorescent microscope. One hundred cells were scored per each slide, as seen in Figure 2 (50 cells per each replicate on the slides), for each concentration for % tail DNA. A variety of parameters are provided from the comet assay; however, % tail DNA has been suggested to be the most reliable measurement (Kumaravel and Jha, 2006).

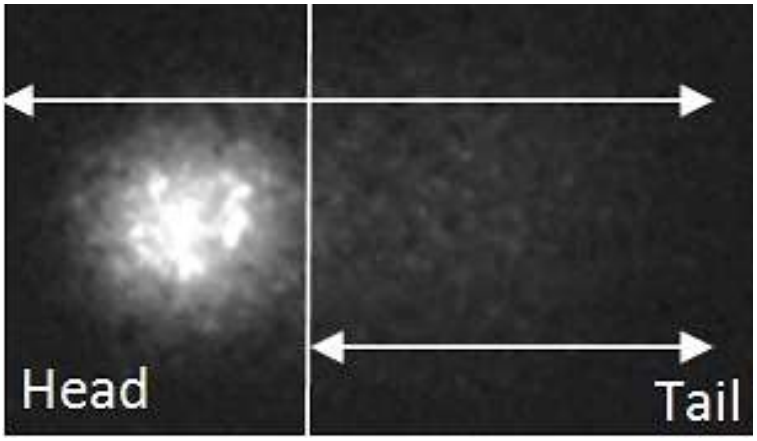

Figure 2: A typical image of comet showing analysis cell with head and tail. The % tail DNA was used to represent DNA damage in this study.

SEM analysis

Morphological changes induced by Bisphenol A were assessed using field emission scanning electron microscopy (Jeol JSM-7001). Cells ($1x10^4$ cells/mL) were grown on melinex plastic coverslips for 24h period in a 12 well cell culture plate. Following this, the required concentrations of BPA was added to each well and incubated for either 24 or 48h period at 37°C. After the desired exposure period, the cells were fixed with 2.5% glutaraldehyde and then washed twice with 0.1M sodium cacodylate buffer (pH 7.2), followed by dehydration with a series of alcohol concentration 10-100%. The coverslips were cut to size before being dried in a critical point dryer (Emitech K850), mounted on stubs and sputter coated with gold.

Statistical Analysis

MTT and comet results were tested for normality using the Shapiro-Wilks test. MTT results were normally distributed ($P>0.05$), however, the comet results were not ($P<0.05$). A Levene's test was used to verify equality of variance for both MTT and comet results, both had unequal variance ($P <0.05$). Therefore, a non-parametric Kruskal Wallis H test was used. The significant difference between control and treated cells was statistically analyzed by Mann-Whitney U tests. All statistical analysis was performed on SPSS, and the level of significance was set at $P < 0.05$.

Results

SH-SY5Y cells were exposed to BPA (0-500 µM) for 24h and 48h. Cytotoxicity was determined with the MTT assay, while genotoxicity was measured using the comet assay. Morphological abnormalities were examined using the scanning electron microscope. All results were validated with copper, however results for copper were only observed for MTT assay as seen below.

MTT assay copper validation

The validation study using copper gave a robust response with strong cytotoxicity effects observed in SH-SY5Y cells. Validation of nominal copper concentrations was tested over 24 and 48h period results can be observed in Figure 3. Copper was used based on its previous cytotoxic effects observed in SH -SY5Y cells (Arciello *et al*., 2005). 500µM of copper considerably reduced the cell viability, and therefore this concertation was used to validate all other test.

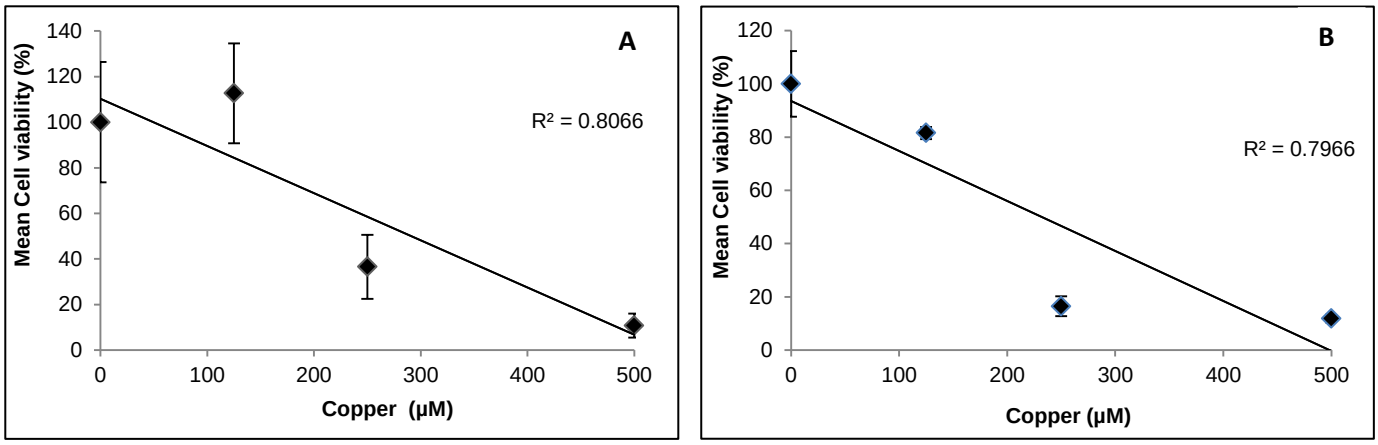

Figure 3: Effects of copper on cell viability of SHSY5Y cells. (A) MTT analysis for 24h (B) MTT analysis for 48h values are the mean ± SD N=3.

MTT assay to determine cytotoxicity

Cell viability of SH-SY5Y cells were measured whilst in solution with a range of Bisphenol A concentrations as described previously. Results for 24h exposure can be observed in Figure 4 while Figure 5 shows results for 48h. Treatments with ≥250µM BPA for 24h were cytotoxic to the SH-SY5Y cells with a significant difference ($P< 0.05$) when compared with the control. A small increase in cell viability was observed at 250nM (102 %) and 1000nM (100.4 %), but no significant difference was found from the control. All concentrations of BPA < 250µM had no significant difference when compared to the control ($P>0.05$). Cell viability dramatically decreased at concentrations above 25 µM with a cell viability of 13.36% in comparison to 125µM with a cell viability of 85.86%.

The cytotoxic effects of BPA were also assessed after 48h, as seen in Figure 5. Cells exposed to concentration ≥100µM showed a significant difference in cell viability compared to the control ($P <0.05$). No cell proliferation was observed in the lower concentrations of Bisphenol A. After a 48h exposure, no significant difference from the control was observed at concentration < 100µM ($P >0.05$). Differences in cell viability were observed between 24 and 48h exposures. Cell viability at 100µM after 24h exposure was 94.3% compared to 76.5% as seen at 48h. The percentage cell viability after exposure to 125µM almost halved after 48h from 85.86% during 24h exposure and 47.24% after 48h exposure. Again, after exposure to 250µM a large decrease in cell viability was observed after 48h exposure to 4% from 13.4% after 24h exposure. Negligible difference was observed between the two time points when exposed to 500µM.

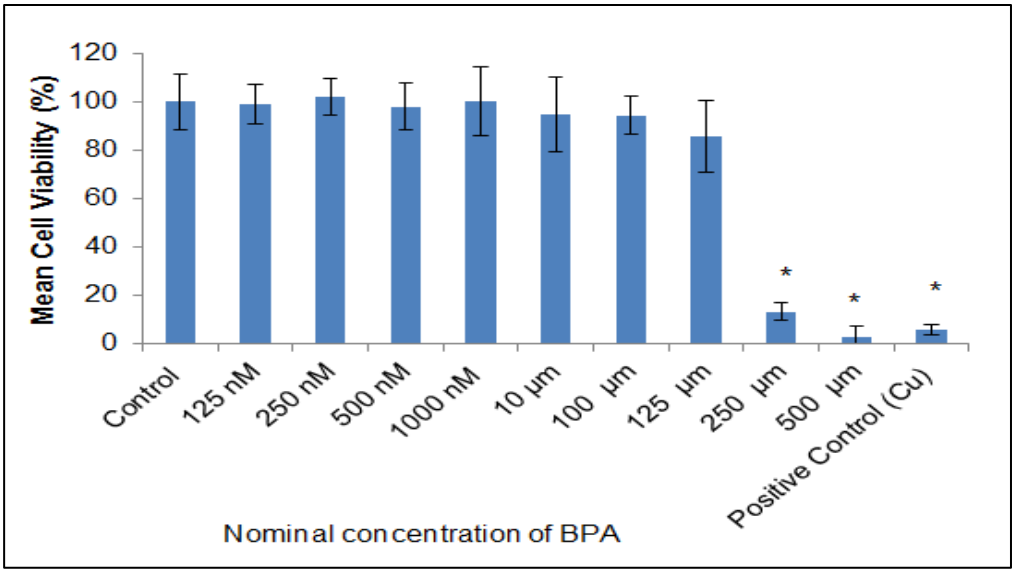

Figure 4: Bisphenol A induces cell death in SH-SY5Y cells after 24h exposure. The control used is DMEM medium with 0.1% ethanol; positive control copper (500 µM) was used to validate the assay. Data represents mean ± SD each experiment (N=4) was completed in triplicate; *asterisk indicates significant difference at P <0.05, compared with control (Kruskal Wallis with Mann-Whitney U test).

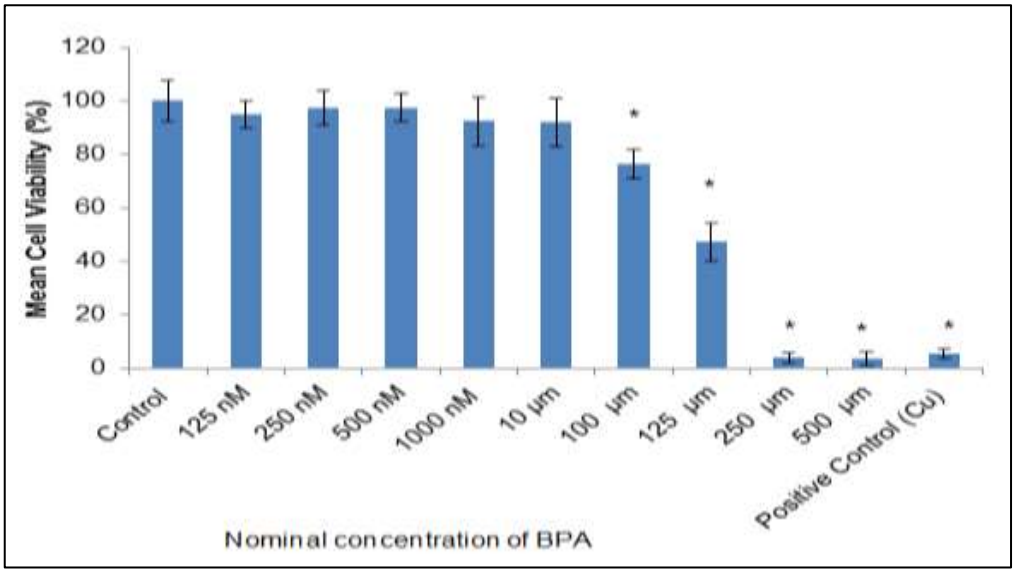

Figure 5: Bisphenol A induces cell death in SH-SY5Y cells after 48h exposure. The control used is DMEM medium with 0.1% ethanol; positive control copper (500 µM) was used to validate the assay. Data represents mean ± SD each experiment (N=4) was completed in triplicate; *asterisk indicates significant difference at P<0.05, compared with control (Kruskal Wallis with Mann-Whitney U test).

Comet assay to determine DNA strand breaks for genotoxicity

Comet assay was performed to determine whether Bisphenol A induced DNA damage. Results for both 24h and 48h can be observed in Figure 6. Figure 6 shows that after a 24h exposure no increase in % tail DNA was observed compared to the control ($P>0.05$). Both 500nM and 100µM exposure concentrations significantly lowered the % tail DNA from the control from 3.78 and 5.38 respectively from the control of 10.15 ($P<0.001$). 500µM did not significantly increase the % tail DNA from the control but the median was higher 15.67 compared to the control 10.15% tail DNA. Similar results were observed after a 48h exposure. Exposure to 500nM caused a significant increase in % tail length from the control ($P<0.001$) 2.28 to 7.30% tail DNA respectively. A 100µM exposure decreased % tail DNA to 0.129 which was significantly lower than the control ($P<0.001$) No results of 500µM exposure could be detected after 48h as cells were found floating in the medium, so could not be detected. This was also observed in the copper positive controls. As the experiment was only repeated once, more experimental replicates would be required to give more reliable evidence of DNA damage and experimental validity.

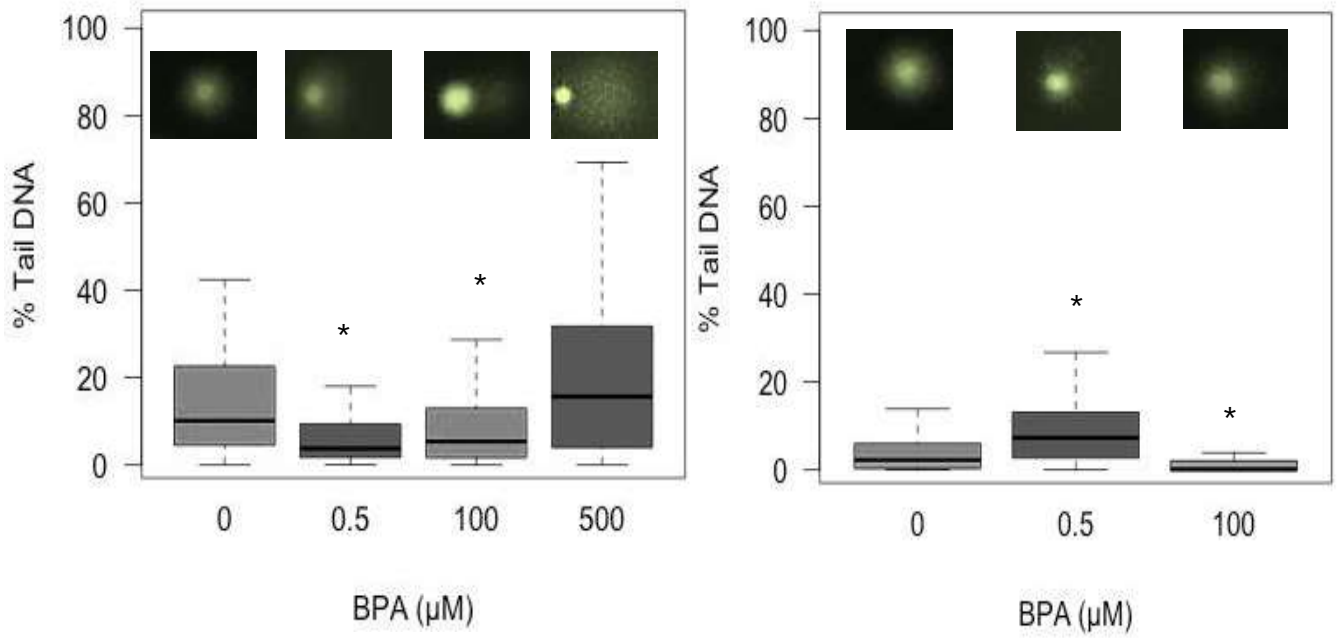

Figure 6: Induction of DNA strand breaks (represented as % tail DNA) in SH-SY5Y cells exposed to Bisphenol A. (A) 24h exposure period at (0, 500nM, 100µM, 500µM) (B) 48h exposure period at (0, 500nM and 100µM). No results for 500 µM were observed for 48h exposure. Pictures show comet assay image at different exposure conditions. The edges of the box represent the 25th and 75th percentile, a line in the box represents the median value, and the bars represent 95 % confidence intervals. *Asterisks, indicate significant difference at $P<0.001$, compared with control (Kruskal Wallis with Mann-Whitney U).

Scanning electron microscopy for morphological changes within cells

In addition to MTT and comet assay the morphological changes of SH-SY5Y cell were investigated at low concentrations of BPA. No exceptional changes in cell morphology were observed after a 24h exposure to 500nM. However, a small decrease in cell number can be observed during a 48h exposure to 500nM BPA compared to the control (Figure 7), but this was not quantitatively analysed. Some damage to the cells can be observed but this is likely due to the fixation and drying process. Higher concentrations were tested (500µM).

Conversely, no cells could be observed, this is likely due to complete cell lysis as cells had detached from the monolayer, this was also the case for the copper positive controls as seen in Figure 7 image E and F. Results from this experiment were not valid to do quantitative analysis due to experimental flaws. The results observed for 500µM BPA and copper positive control coincide with the 48h comet assay results.

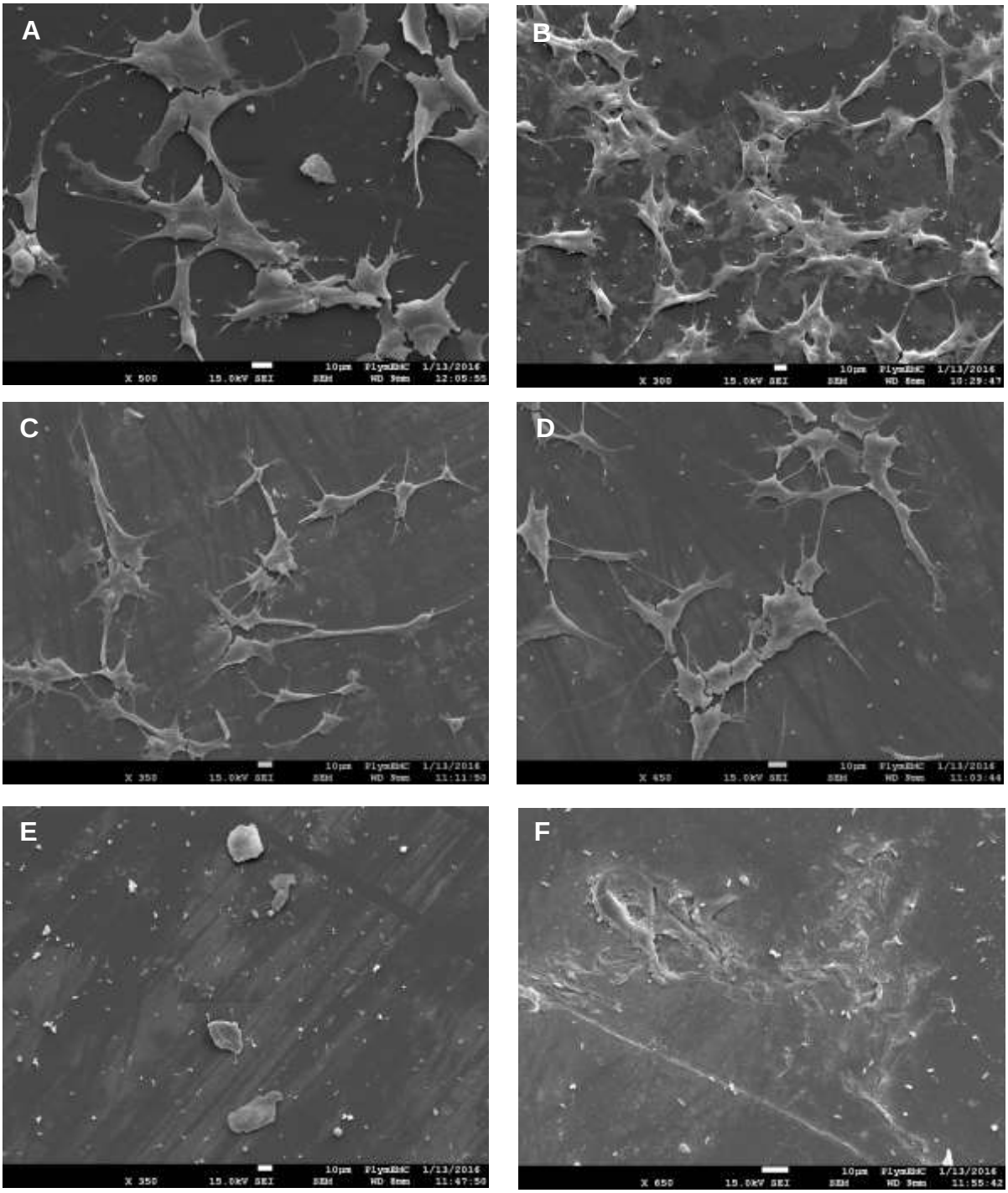

Figure 9: Scanning electron microscopy images for evaluation of morphological changes at low dose exposure to BPA: (A) 24h control, (B) 48h control, (C) 24h 500nM and (D) 48h 500nM. (E) 500µM representative of 24 and 48h exposures (F) Copper control representative of 24 and 48h exposure.

Discussion

In the present study, BPA was found to be cytotoxic in a time and concentration dependent manner. BPA caused a significant decrease in cell viability at concentrations of ≥250µM after a 24h exposure and ≥100µM after a 48h exposure, as seen in Figs. 4 and 5. Kim *et al*., (2007) found similar results where proliferation of neural progenitor cells measured by MTT was significantly decreased by 200µM of BPA after 24h. Lee *et al*., (2007) found the same effects in PC12 and cortical neurons at concentrations ≥100µM; they also found low concentrations 0-10µM increased neurite extension, leading to increased cell viability .It has been suggested that low concentrations of BPA exert neuroprotective effects against glutamate and amyloid beta toxicities (Gursoy *et al*., 2001). Low concentration of BPA caused slight increases in cell proliferation, (250 nM (102 %) and 1000 nM (100.4 %)) as seen in Fig. 4. However, there was no significant difference from the control cells. Proliferation at low concentrations has also been found in a variety of other mammalian cell lines (Bolli *et al*., 2008; Ptak *et al*., 2011; Sheng and Zhu, 2011). The cytotoxic effects of BPA found in this study was also found in a range of human cell lines (Lee *at el*, 2008; Lida *et al*., 2003; Terasaka *et al*., 2005), which was associated with apoptosis. Apoptosis is a fundamental process in shaping the neuronal system during development. However, adverse factors such as oxidative stress and neurotoxins can induce irregular apoptosis, leading to a pathological status (Sastry and Rao, 2001).

On the other hand, a previous study on SH-SY5Y cells exposed to BPA using the MTT assay found concentrations > 300nM reduced cell viability. Complete cell lysis was found at concentrations > 600nM (Kafi *et al*., 2011). The results from this study are considerably different from the results of Kafi *et al's*., (2011) study; the difference observed between concentrations to induce cytotoxicity may be attributed to different experimental designs. Un-differentiated cells were used in this study however, the use of un-differentiated cells have shown to have disadvantages. As the cells are continually dividing, the replication of the cell during the experiment makes it difficult to determine the influence of neuroprotective or neurotoxic molecules influence on proliferation or cell degeneration (Filograna *et al*., 2015). This could cause variation between laboratories. As the cells are continually dividing, the cell number throughout the experiment will also change and therefore this should be counted before the assay as seen by lida *et al*., (2003). Other limitations to cell culture include long term sub culturing which places pressure on trails of cell lines. Therefore, the use of similar or identical passage number should be used throughout to ensure reproducible results and comparisons to other studies (Hughes *et al*., 2007). To further validate the cytotoxic effects of BPA, it would be appropriate to use other cytotoxic assays. Examples of these include the LDH assay; this measures the release of lactate dehydrogenase in the culture medium after cell membrane damage, neutral red assay which measures the absorption of dye into functional lysosomes and protein assay an indirect measurement of cell viability. It has been suggested that neutral red and MTT assay are the most sensitive cytotoxic assays (Fotakis and Timbrell, 2006). Cytotoxicity of BPA on human gingival epithelial cells was found using both Neutral red and MTT assay at 0.2mM, but there was a lack of cytotoxicity found from LDH assay (Babich and Tipton, 1999).

The Comet assay was used to assess the genotoxic effects of BPA on SH-SY5Y cells. The effects can be observed in Figure 6; there was no significant increase in % tail DNA from the control after a 24hour exposure. However, exposure to 500nM and 100µM significantly lowered the % tail DNA from the control, this would most likely be due to experimental error, and therefore the test would need to be repeated to improve experimental validity. After a

48hour exposure, 500nM significantly increased the % tail DNA from the control. No results could be observed for 500µM during the 48hour exposure. This is because the cells had detached from the monolayer and were observed floating in the media which was discarded. Pfeifer *et al*., (2015) found BPA to have a genotoxic effect on mammalian cells *in vitro* at environmentally relevant doses (100nM) using the comet assay. However, cells were treated for up to 72 hours in some cell lines the damaging effect was not observed as late as 24-48h. BPA exposure ranging from 1 to 15µM significantly induced DNA damage, as measured by increased percentage of tail DNA in zebra fish (Wang *et al*., 2013). Fic *et al*., (2013) found that a 24hour exposure to BPA induced minor increases in DNA damage at low concentrations (0.1µM, 1µM and 10µM). They suggested the toxic potential of BPA was due to the reactive metabolites and oxidative stress. The metabolism of BPA by hydroxylation of one of its benzene rings leads to the formation of 3-OHBPA. Subsequent oxidation causes formation of the reactive electrophile BPA-3-4-quione, which if not adequately detoxified by GSH, will react with DNA causing depurination. This can result in an apurinic site leading to carcinogenesis (Cavalieri and Rogan, 2010; Terasaka *et al*., 2005). Conversely, Audebert *et al*., (2011), found that BPA did not produce a genotoxic effect in a range of human cell lines at non-cytotoxic concentrations using γH2AX assay, although this assay only determines double strand breaks.

Conflicting genotoxic effects of BPA have been observed within the literature. The variation in results may be due to differences in biotransformation of BPA in one cell line compared to another or variations in DNA repair mechanism and antioxidant capacities (Audebert *et al*., 2011). Variations in genotoxic effect may also be caused by false positive results due to apoptosis or cytotoxicity, in order to prevent this cell viability should be measured (Tayama *et al*., 2008). Henderson *et al*., (1998) reported the maximum concertation of test substance tested should produce > 75% cell viability. Lee *et al*., (2003), found that BPA induced tail moments at concentrations showing cell survival less than 60%, and concluded to show a negative response in comet assay. Several laboratories have reported that the onset of apoptosis can give comet images with cell aspect and tail parameter values of the same order as those of cells with moderate DNA damage. This could suggest that the comet images cannot be interpreted as a genotoxicity indicator when apoptosis risk is existent (Florent *et al*., 1999). This could indicate why no genotoxic effect was observed at high concentrations in this study, therefore cell viability counts should be completed in future studies. Varied results can occur between laboratories due to differences within protocols including agarose concentration, electrophoresis time and voltage gradients (Collins *et al*., 2014). Variation can also occur within the same laboratory due to induvial error of scoring opinion, and therefore scoring should be completed blindly to prevent bias results.

The sensitivity of the comet assay can also be improved by detecting oxidized bases by incorporating additional steps. Formamidopyrimidine DNA-glycosylase (FPG) recognises oxidized purine bases and endonuclease can detect oxidized pyrimidine bases (Collins *et al*., 2014). Micronucleus assay (MN) is used to measure the formation of micronuclei, which are extensively used as a biomarker of chromosomal damage and genome instability; therefore this assay could be used to verify the genotoxic effects of BPA (Iarmarcovai *et al*., 2008). It has been suggested that MN assay is more sensitive than the comet assay (Pfau *et al*., 1999); therefore it would be useful to compare results for both tests. The sensitivity of the comet assay can be elevated to a level higher than that of MN test by using DNA resynthesize inhibitors, such as araC and HU (Kawaguchi *et al*., 2010).

No positive control results for copper could be obtained for the comet assay. This is because 500 µM of copper caused almost complete loss of cell viability as seen in the MTT results (Figure 6 and 7) and therefore could not be detected. When repeating this experiment, it would be more appropriate to use a reference genotoxic chemical such as hydrogen peroxide to validate the assay as it causes oxidative damage in cells, causing a variety of DNA lesions, this including single and double strand breaks (Benhusein *et al*., 2010).

During neurodegenerative disease and aging, the cell shape is lost (Valdiglesias *et al*., 2012). It was therefore important to assess the effects of BPA on cell morphology using SEM. Images of the SH-SY5Y cells can be observed in Figure 7. No morphological changes could be observed between the control cells and cells treated with 500nM over a 24 and 48hour period. Kafi *et al*. (2011), found no remarkable changes in cell morphology at concentrations lower than 300nM. However, concentrations higher than 500nM of BPA decreased cell number and complete cell lysis was found at concentrations higher than 600nM of BPA. Lee *et al*., (2004) found a decrease in cell number and enlarged morphology at 200uM compared to the control. Both studies used light microscopy (LM) to study morphological changes and quantification of cell number. The results in this study could not be quantitatively measured because cells were not seeded at a high enough density and cells were not evenly dispersed, causing inaccuracy (Visser *et al*., 1996). A range of methods have been compared for the quantification of cells, including LM, SEM, trypsin and crystal violet. LM and SEM methods have been suggested to be advantages as they provide additional information for example percentage of confluence and cell morphology and considering both LM and SEM do not require the resuspension of cells they might have higher accuracy to trypsin and crystal violet methods (Visser *et al*., 1996). Visser *et al*., (1996) used SEM to quantify endothelial cell, 10 photographs were taken after random movement of the microscope stage in prearranged direction. The number of cells on each image was counted twice by blinded observes. In future, this method could be used to quantify the neuronal SH-SY5Y cells.

It was important in this study to assess the effects of environmentally relevant concentrations compared to that of micromolar concentrations. According to the World Health Organization, the estimated international dietary exposure ranges from 0.4-4.2 µg/Kg/bodyweight/day (Milić *et al*., 2015). Low dose concentrations used in the MTT assay had no effect on cell viability compare to the control. This would suggest that environmentally relevant concentrations of BPA are not cytotoxic. Although the 100-500µM (22.8-114.1µg/mL) concentrations of BPA used in this study appear to be relatively high compared to the WHO estimated values , the concentrations found within the environment can also be equally high for example BPA released from plastic dental sealants in saliva (931µg/30mL) (Olea *et al*., 1996). Although the primary BPA exposure to humans is via the diet (Sekizawa, 2008), humans are continually exposed to BPA in outdoor/indoor air and dust. If various exposure routes of BPA are taken into account such as plastic dental material, air and water 100-500µM are concentrations that humans could realistically be exposed to (Tsai, 2006). The genotoxic capability of BPA was assessed at environmental concertation (500 nM/0.114µg/mL), which induced significant DNA damage compared to the control after a 48h exposure. This provides evidence that environmental concentrations could induce adverse effects.

When testing for environmentally relevant low dose concentrations, it is important to take into account BPA leaching from laboratory equipment. It is therefore essential to eliminate

background levels of BPA, by using non polycarbonate equipment. BPA has been found in pipette tips, cell culture media and various commercial buffers (Cao *et al*., 2010). It has also been noted that certain chemical solvents can induce BPA leaching such as DMSO, a solvent used in both MTT and comet assay (Krishnan *et al*., 1993).

As it stands, there is minimal information on the effect of BPA on neurodevelopment of humans. However, two studies have been conducted showing the effects of BPA on behavioural changes in children. Hong *et al*., (2013), found association between the BPA levels in urine samples of 1008 children aged 8-11 with behavioural changes and learning development using behavioural assessments. Perera *et al.*, (2012) conducted a long term study in 198 children. BPA exposure was estimated from urine samples taken from pregnant women and in children aged 3-5 yrs. Some behavioural changes were observed, mainly in boys. Due to the uncertain effects of BPA on infants and children, the US government have banned the use of BPA in baby bottles and packaging items (U.S Food and Drug Administration (FDA) 2012). However, little regulatory actions have been made of the remaining food contact materials and so BPA is still being released into the environment (Milić *et al*., 2015). The use of *in vitro* studies contribute to the existent cytotoxic and genotoxic molecular mechanism of BPA on the nervous system which can be used to develop an understanding of the adverse effects on humans.

Conclusion

In summary, BPA is cytotoxic at high concentrations. However, low environmentally relevant concentrations do not induce a cytotoxic response. BPA was found to have a genotoxic effect at low nanomolar concentrations, but further testing is required to classify the genotoxic effect of environmentally relevant concentration of BPA. This study provides *in vitro* evidence of the potential adverse effects of BPA on the nervous system and possible effects on neuronal development. However, more research is required to assess its cytotoxic and genotoxic effects on neurodevelopment, by using further cytotoxic and genotoxic assay to validate the results observed in this study.

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