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The cytotoxic and genotoxic potential of titanium dioxide (TiO_2) nanoparticles on human SH-SY5Y neuronal cells *in vitro*

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Abstract

Titanium dioxide (TiO_2) nanoparticles are one of the most commonly used nanomaterials. They are used in plastics, sunscreens, personal care products, pharmaceuticals and the food industry due to their photocatalytic properties, high refractive index and generally unreactive nature. Their vast range of applications, and hence potential exposure to humans, raises concern over the safety of the nanomaterial. TiO_2 nanoparticles have been extensively studied; however, their toxic effect on humans is still poorly understood, particularly the adverse effects they may have on the nervous system. *In vivo* studies have shown that TiO_2 nanoparticles are able to enter the brain via circulation and through the olfactory pathway. Because of their potential genotoxicity and carcinogenic effect, the aim of this study was to assess the cytotoxic and genotoxic potential of TiO_2 nanoparticles on human SH-SY5Y neuronal cells. Anatase TiO_2 nanoparticles were used in this experiment due to their extensive use in industry. Results obtained from this study show that TiO_2 nanoparticles are able to induce DNA damage in neuronal cells at concentrations of 3000µM (239.61µg/ml) after 24 and 48 hours of exposure, but do not significantly alter cell viability even at concentrations as high as 10,000 µM (798.66 µg/ml). Cell morphology, as determined by scanning electron microscopy, was also unaltered in this study. Further study is required to determine cell internalisation and the mechanisms behind the genotoxicity induced by TiO_2 nanoparticles.

Introduction

Nanotechnology is the manipulation and engineering of materials with at least one dimension <100nm in length (Oberdörster *et al.,* 2007). In recent years the demand for engineered and manufactured nanomaterials has increased rapidly (Borm and Berube, 2008) due to their vast range of applications in medicine, the food industry and energy conversion (Seigrist *et al*., 2008). Current applications of nanomaterials include; anti-microbial food processing and packaging, antibacterial milk bottles for babies (Siegrist *et al.,* 2008), and water purification (Liga et al., 2011). Due to their small size and large surface area to volume ratio, nanoparticles are readily transported around the body, able to enter cells and capable of releasing drugs over time enabling the production of nano scale implants and drug delivery systems (Langer and Weissleder, 2015; Roco, 2003).

Titanium Dioxide (TiO_2) is one of the most commonly used nanoparticles due to its photocatalytic properties, high refractive index (Borm and Berube, 2008), thermal stability and its generally insoluble, unreactive nature (Lee *et al.,* 1985). It is used as a pigment in antifouling paints (Borm and Berube, 2008), plastics, enamels, foods, sunscreens, pharmaceuticals, personal care products such as toothpaste, shampoos and deodorants (Weir *et al.,* 2012) and in the treatment of drinking water (Liga *et al.,* 2011). The vast use of TiO_2 nanoparticles in everyday products and foods has sparked a great interest in the safety of the nanomaterial, particularly after the International Agency for the Research of Cancer (IARC) have classified pigment grade TiO_2 as a potential carcinogen to humans (Wang *et al.,* 2008a). Nanomaterials have different physiochemical properties to their bulk forms, such as size, surface area:volume, surface charge, redox activity and surface hydrophobicity, and therefore have different mechanisms of toxicity. As a result, not only do nanomaterials provide a benefit to industry but also a risk (Oberdörster *et al.,* 2007; D'agata *et al.,* 2013).

The toxicity of TiO_2 has been studied in a variety of conditions to assess its safety in humans and in the environment. Previous studies have reported TiO_2 induced cytotoxic effects such as altered cell cycle, shrinkage of nuclear membranes and apoptosis (Acar *et al.,* 2015; Coccini *et al.,* 2015; Gosch *et al.,* 2010; Hu *et al.,* 2011; Valdiglesias *et al.,* 2013; Wang *et al.,* 2007), inhalation risks and correlation with Crohn's disease in humans (Weir *et al.,* 2012), as well as gill pathologies and biochemical brain disturbances in fish (Boyle *et al.,* 2013; Federici *et al.,* 2007; Ramsden *et al.,* 2009). Studies have also found that TiO_2 nanoparticles are capable of causing DNA damage (Jugan *et al.,* 2012; Petkovic *et al.,* 2011; Shukla *et al.,* 2011; Trouiller *et al.,* 2009; Valdiglesias *et al.,* 2012; Vevers and Jha, 2008), which is increased with the interaction of UV light (Vevers and Jha, 2008) due to its photocatalytic properties (Borm and Berube 2008). TiO_2 is considered a polymorphic material as it is found in three different forms; Rutile, Anatase and less commonly, Brookite. The property of TiO_2 and hence its toxicity, is closely related to its crystal structure. Anatase and Rutile are the two forms of TiO_2 used most. Anatase has more applications in industry than the other two crystal forms due to its high specific surface area and increased activity in photocatalysis (Bourikas *et al.,* 2014; Li *et al.,* 2004), it is however considered to be far more toxic than rutile (Weir *et al.,* 2012).

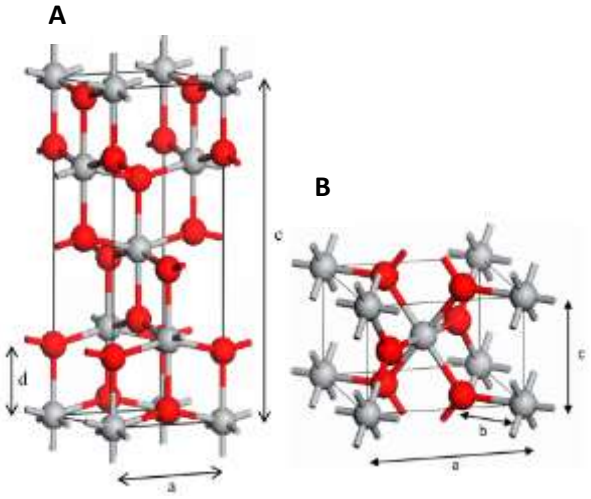


Figure 1. Comparative tetragonal structures of (A) anatase and (B) rutile crystal forms of TiO_2. Grey balls represent titanium and red balls represent oxygen (adapted from Bourikas *et al.,* 2014).

Previous studies have shown that the crystalline structure and size of TiO_2 determines the toxicity, aggregation and agglomeration of the nanoparticle. Smaller particles of TiO_2 have been shown to induce higher levels of inflammation in the lung when inhaled (Oberdörster *et al.,* 2007) and are migrated around the body more readily than bulk TiO_2 (Ma *et al.,* 2010). In comparison to this D'agata *et al.,* (2013) found that although nano-sized TiO_2 showed higher levels of accumulation, bulk TiO_2 actually caused a higher genotoxic effect in marine mussels. Similarly Magdelenova *et al.,* (2011) found that dispersions with larger agglomerates of TiO_2 NPs induced greater cell damage than dispersions with smaller agglomerates. Published studies on the toxicity of TiO_2 are conflicting, reflecting the great effect aggregation, agglomeration, size of nanoparticle and crystal structure can have on the property of TiO_2 (Li *et al.,* 2004; Ma *et al.,* 2010; Oberdörster *et al.,* 2007).

Humans are exposed to TiO_2 nanoparticles via multiple exposure routes on a daily basis. One route of exposure is through the consumption of food and water, either directly, as a result of packaging or through environmental pollution and bioaccumulation within the food chain. Previous research has assessed the content of TiO_2 in food and water. Gottschalk *et al.,* (2009) found there to be 0.021µg/L TiO_2 present in surface water and 4.28µg/L present in waste water treatment plants. Foods with particularly high values of TiO2 NPs include coffee, sweets and chewing gum. It is estimated that the average 3-4 year old male in the western world will consume 2mg TiO_2/kg BW per day, (Weir *et al.,* 2012).

Once in the body, nanoparticles can enter the bloodstream and be taken up by cells (Rothen-Rutishauser *et al.,* 2006), effecting vital organs such as the liver, brain and placenta (Borm and Berube, 2008). TiO_2 NPs can affect the brains of offspring, inducing brain damage when present in the abdominal cavity (Ma *et al.,* 2010) and effecting behaviour and reproduction rates in future generations (Jacobasch *et al.,* 2014). Previous work has shown that nanoparticles form nanomaterial-protein

coronas when in the blood stream as plasma proteins are adsorbed to the surface of the nanoparticle, conferring their biological identity and masking the nanoparticle, enhancing cell uptake (Cedervall *et al.,* 2007; Doak *et al.,* 2009; Lynch and Dawson, 2006). Apolipoprotein E, a lipoprotein involved in brain trafficking (Lynch and Dawson, 2006), has been found to associate with some nanoparticles, facilitating transport to the brain and across the blood brain barrier (BBB) (Bramini *et al.,* 2014; Kim *et al.,* 2007; Michaelis *et al.,* 2006). Although a lot of nanoparticles are more likely to accumulate in lysosomes, some nanoparticles are small enough to cross the blood brain barrier (BBB) and accumulate in regions of the brain such as the olfactory bulb and the hippocampus (Borm and Berube, 2008; Bramini *et al.,* 2014; Hu *et al.,* 2011). The mechanisms for the uptake of the NPs across the BBB appears to be receptor – mediated endocytosis in endothelial cells in the brain (Brun *et al., 2012;* Wohlfart *et al.,* 2012). Czajka et al. (2015) illustrates the potential routes of exposure and mechanisms of TiO2 nanoparticle induced toxicity on the nervous system.

Humans are also exposed to nanoparticles through the air. Manufactured and engineered nanoparticles could potentially erode to form nano scale particulate matter, exposing humans to NPs in the air (Handy and Shaw, 2007). The vast majority of research on inhaled TiO_2 nanoparticles has been carried out on lung cells however; inhaled TiO_2 can also reach the brain via the olfactory pathway (Elder *et al.,* 2006; Wang *et al.,* 2008a; Wang *et al.,* 2008b). Once inhaled TiO_2 can accumulate in areas such as the olfactory nerve layer, olfactory ventricle, cerebral cortex, thalamus, and hippocampus regions CA1 and CA3 (Wang *et al.,* 2008a).

Once in the brain TiO_2 nanoparticles mainly accumulate in the hippocampus causing lipid peroxidation, protein oxidation, oxidative damage (Wang *et al.,* 2008a; Wang *et al.,* 2008b), a decreased antioxidant capacity and an increase in the production of reactive oxygen species (ROS) (Hu *et al.,* 2011). Other effects in the brain include inhibited proliferation, induced morphological changes, apoptosis (Márquez – Ramirez *et al.,* 2012), shrinkage of nuclear membranes (Hu *et al.,* 2011), changes in copper (Cu), potassium (K) and Zinc (Zn) levels (Federici *et al.,* 2007) and dysregulation of the BBB (Brun *et al.,* 2012).

Given the potential carcinogenic effect of TiO_2 nanoparticles, the aim of this study was to assess the cytotoxic and genotoxic potential of TiO_2 nanoparticles on the developing nervous system using undifferentiated SH-SY5Y cells. SH-SY5Y cells are undifferentiated noradrenergic cells capable of exhibiting dopamine β hydroxylase, cloned from a four year old female with neuroblastoma. The neuroblastoma cells are sub cloned from cell line SK-N-SH, and are used largely in neuroscience (Filograna *et al.,* 2015). Cell viability was determined using the methyl thiazole tetrazolium (MTT) assay and DNA damage was assessed using the alkaline comet assay. Scanning electron microscopy was used to assess morphology of the cells and x-ray microanalysis was used to analyse the presence, aggregation and location of the nanoparticles. In order to validate these assays copper was used as a reference toxic agent.

Materials and Methods

Chemicals

Anatase TiO_2 (nanopowder, <25 nm particle size, 99.7% trace metals basis, CAS: 1317-70-0, Batch # 07324KD) and low melting point agarose gel (LMPA) (CAS: 39346-81-1) were purchased from Sigma Aldrich. Cupric Sulphate Pentahydrate ($CuSO_4H_2O$) ACS grade (CAS: 7758-99-8) was purchased from BDH chemicals LTD. MTT (Thiazolyl blue tetrazolium bromide, $C_{18}H_{16}N_5^{SBr}$, CAS: 298-93-1) was purchased from Melford Chemical and Biochemical Manufacturing. Dulbecco's modified Eagle's medium (DMEM, Gibco) was purchased through Thermo Fisher Scientific. Sterile A Dulbecco's Phosphate Buffered Saline (DPBS) and Sterile A 0.25% trypsin were purchased from Gibco by Life Technologies.

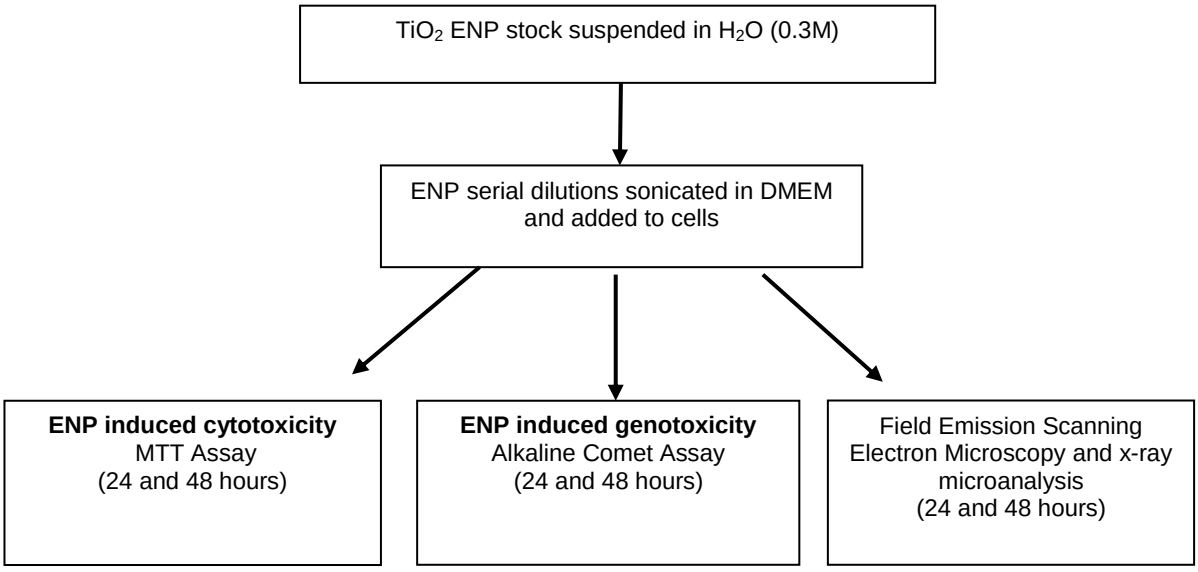


Figure 2. Schematic diagram for TiO_2 ENP toxicity assessment methods.

Cell Culture

Human neuroblastoma SH-SY5Y cells were purchased from ATCC. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, CA, USA) supplemented with 10% foetal bovine serum (FBS) and 0.5% Penicillin Streptomycin. Cells were maintained in a humidified incubator with 5% CO^2 and 95% air at 37°C in filtered cap flasks (CELLSTAR). To passage cells, all media was removed and cells were washed with 4 ml Dulbecco's Phosphate buffered saline before adding 500µl 0.25% trypsin, 0.53mM EDTA to detach cells with 3.5ml fresh DMEM. To ensure the cells had detached from the flask, cells were viewed under an inverted light microscope. Cell counts were carried out using a haemocytometer.

TiO_2 Nanoparticle Preparation

Anatase TiO_2 NPs were suspended in sterile distilled water at a stock concentration of 0.3M, and sonicated using a Fisher Scientific sonicator. Serial dilutions were made to obtain concentrations ranging from 10µM to 10,000µM (0.79866 µg / ml – 798.66 µg / ml). TiO_2 0.3M stock was sonicated for 1 hour 30 minutes prior to making diluted concentrations to ensure nanoparticles were suspended and to prevent aggregation

and agglomeration. Individual concentrations were mixed with a pipette and sonicated for at least 5 minutes prior to cell exposure. All concentrations of TiO_2 had equal amounts of PBS to water and TiO_2 to ensure ratio of water to DMEM media was not influencing cell health. Controls were used with equal amounts of media, PBS and water/TiO_2 suspension, made up of 8:1:1 parts respectively.

Validation of Assays with Copper

Prior to experimentation, MTT assays were carried out in SH-SY5Y cells exposed to Cupric Sulphate Pentahydrate ($CuSO_45H_2O$) for 24 and 48 hours to obtain a concentration of copper suitable for use as a positive control throughout experiments in order to validate each assay. The concentration of copper within the Cupric Sulphate Pentahydrate was calculated. $CuSO_45H_2O$ was then used as a positive control throughout all 24 and 48 hour MTT experiments, SEM analysis and Comet assays at a copper concentration of 300µM.

MTT assay

Cell viability was assessed using the methyl thiazole tetrazolium (MTT) assay. The assay assesses the capability of viable cells to transform the methyl thiazole tetrazolium into a formazan dye. Cells were seeded at a density 1 x 10^4 /ml onto a 96 well plate, 100µl/well. MTT was prepared in DPBS 5mg/ml. After exposure periods of 24 and 48 hours, all media was removed from wells and replaced with 85µl DMEM and 15µl MTT and incubated for 3 hours at 37°C. Blue formazan crystals inside cells were dissolved by adding 100µl Dimethyl sulfoxide (DMSO) to each well and incubating for a further 10 minutes at 37°C. Contents of wells were mixed thoroughly before the absorbance was read at 595nm using a VERSA max microplate reader. Media blanks were used to eliminate the interference of the DMEM media and MTT with the results. TiO_2 blanks, comprising of media, TiO_2 NP suspension and MTT were used to prevent the interference of the nanoparticles with the absorbance obtained. Blank values were subtracted from concentration values and represented as a percentage of the control. All experiments were carried out in triplicate (three wells per plate) and repeated four times.

Assessment of DNA damage by alkaline comet assay (Single-cell gel electrophoresis)

Cells were seeded in a 6 well plate at a density of 1 x 10^4 / ml exposed to titanium dioxide nanoparticles for periods of 24 and 48 hours. Cells were exposed to concentrations of 30 µM, 300 µM and 3000 µM TiO_2 NPs. Cells were trypsinized and centrifuged. Cell suspension was mixed with low melting point agarose gel and pipetted onto normal melting point agarose (NMPA) coated slides, and covered with cover slips. Slides were refrigerated at 4°C for an hour. Cover slips were removed and slides were placed back to back in lysis buffer and refrigerated at 4°C for a further hour. Slides were then placed in electrophoresis chamber and left to unwind for 20 minutes. Electrophoresis chamber ran for 20 minutes at 25V. Slides were then placed in a coplin jar with neutralisation buffer for 10 minutes. Slides were left to air dry for scoring. Slides were stained with 20µl of ethidium bromide (20 µg /ml) and scored using a fluorescent microscope and Comet IV software. 50 cells per gel were scored, with duplicate slides per treatment condition and exposure period. Copper was used as a positive control during this experiment at both 24 and 48 hour exposure periods. %Tail DNA was used to measure genotoxicity caused by TiO_2 as it is a recommended parameter to analyse from the alkaline comet assay for

regulatory purposes as it has the best correlation with dose. % tail DNA consists of DNA released as fragments and relaxed loops (Kumaravel and Jha, 2006).

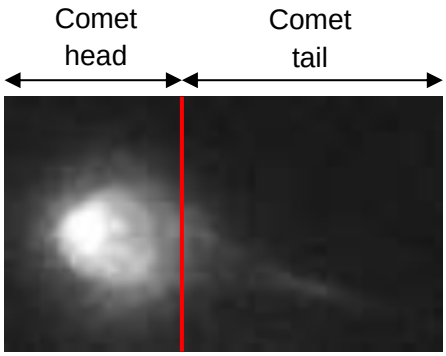


Figure 3. Image of comet head and tail taken from SHSY5Y cell exposed to 300µM TiO_2 nanoparticles for 48 hours following fluorescent staining (as seen in figure 11).

Figure 3 shows a typical 'comet', a cell with a visible 'tail' of DNA. Images of comets are portrayed in each figure for their respective concentrations. A longer tail and corresponding increased % tail DNA indicates an increase in DNA strand breaks. Tails of comets are hard to identify in all images obtained from these results but are slightly more apparent at higher concentrations.

SEM Analysis

Field emission scanning electron microscopy (Field Emission SEM; JEOL JSM-7001F) was used to observe any morphological differences. Cells were grown on plastic cover slips (Melinex) in a twelve well plate, seeded at a density of 1 x 10^4/ml. Cells were exposed to concentrations of 300µM and 1000µM TiO_2 nanoparticles for 24 and 48 hours before fixation. All media and TiO_2 concentrations were removed and cells were fixed in their plate using 2.5% glutaraldehyde (pH 7.2) and 0.1M Sodium cacodylate buffer for one hour. Glutaraldehyde was removed and cover slips containing cells were placed into individual containers with fresh 0.1M Sodium cacodylate buffer. Cells were dehydrated with a series of ethanol concentrations ranging from 10% to 100%, with 15 minute intervals between each concentration. Ethanol was replaced with liquid carbon dioxide and dehydrated using a critical point dryer (Emitech K850 Critical Point Dryer). Cells were then mounted on stubs and coated in carbon using a Quorum Q150 T-ES Carbon Coating Unit. Copper was used as a positive control to compare cell morphology to.

X-ray microanalysis

X-ray microanalysis was carried out on control cells, and cells exposed to 300µM and 1000µM TiO_2 nanoparticles for 24 and 48 hours. Cells were coated in carbon due to its low atomic number, allowing easier identification of other elements with a high atomic number. Copper was used as a positive control for x-ray microanalysis. Element maps and spectrums were produced when elements with a high atomic number were identified using back scattered electron microscopy.

Statistical analysis

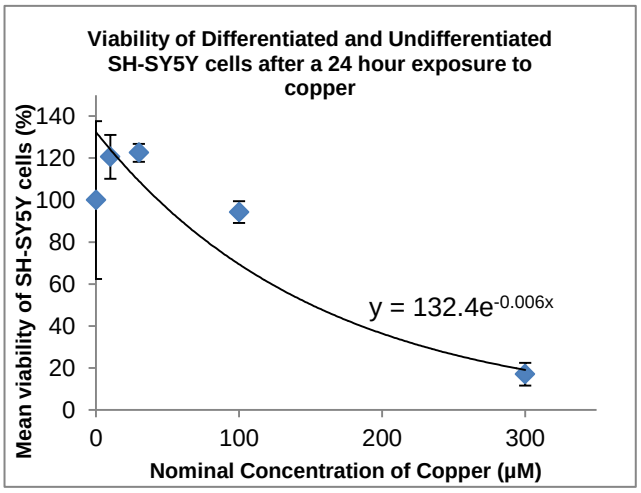
In order to determine if all data was parametric, normal distribution was tested using the Shapiro-Wilk test and equality of variance was tested using Levene's test. Data from MTT was normally distributed and had an equality of variance ($P>0.05$) so a one way analysis of variance (ANOVA) with an LSD post hoc test was used at a significance level of $P<0.05$. Data for comet assay was not normally distributed and variances are unequal ($P<0.05$) so a Kruskal-Wallis H test was used to determine if there was a significant difference between results. Mann-Whitney U tests were used between all concentrations and controls used in the comet assay ($P<0.05$). All statistics were carried out in SPSS.

Results

Validation of MTT Assay

Cells were exposed to Copper Sulphate Pentahydrate ($CuSO_45H_2O$) for exposure periods of 24 and 48 hours in order to obtain a concentration of copper suitable for use as a positive control for experiments. At copper concentrations of 300µM a significant decrease in cell viability was observed. 300µM copper was used as a positive control in all other assays throughout the project.

A



B

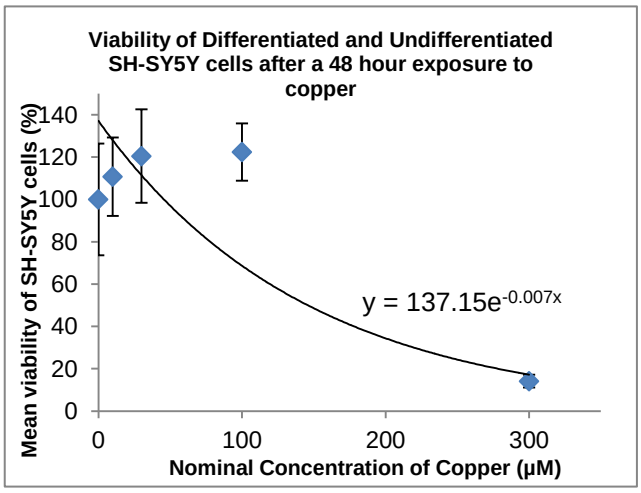


Figure 4. Viability of differentiated and undifferentiated SH-SY5Y cells after a 24 hour (A) and 48 hour (B) exposure to Copper Sulphate pentahydrate ($CuSO_45H_2O$) represented by means ± SD and line of best fit.

Cell Viability

As seen in figures 5 and 6, no significant decrease ($P>0.05$) in cell viability was observed in response to any of the concentrations of TiO_2 nanoparticles compared to the control following exposure periods of 24 (figure 6) and 48 hours (figure 6). Copper was used as a positive control at 300 µM in all repeats of these experiments.

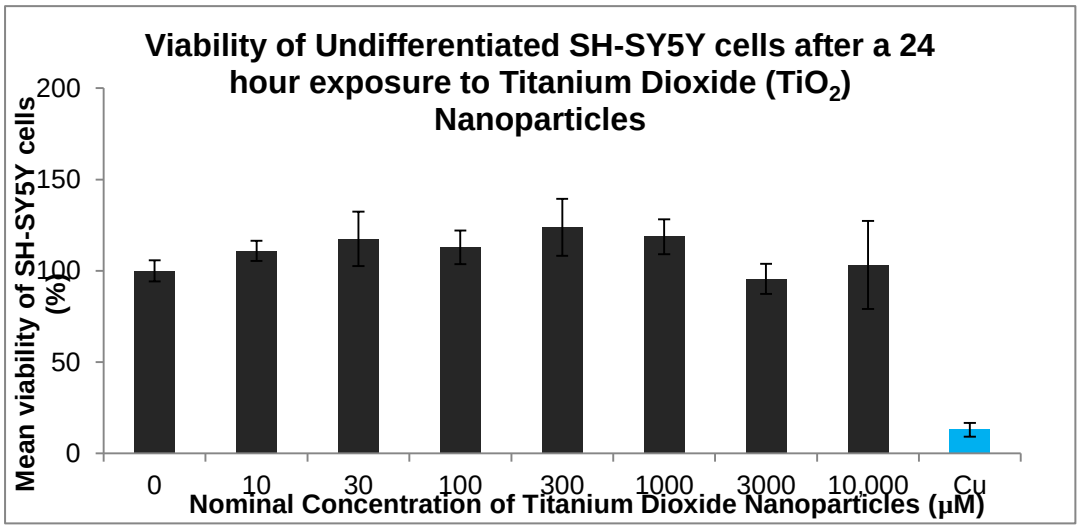


Figure 5. Cell Viability of differentiated and undifferentiated SH-SY5Y cells following a 24 hour exposure to TiO_2 nanoparticles presented by mean ± SD (N=3) with positive control (300µM Cu).

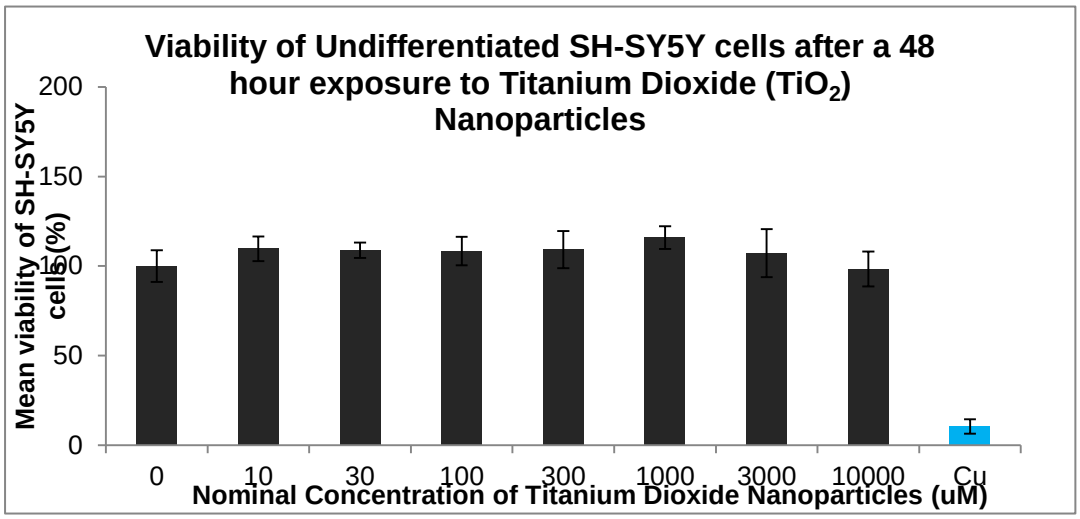


Figure 6. Cell viability of differentiated and undifferentiated SH-SY5Y cells following a 48 hour exposure to TiO_2 nanoparticles presented by means ± SD (N=3) with positive control (300µM Cu).

Assessment of DNA damage using the Alkaline Comet Assay (Single gel electrophoresis)

An alkaline comet assay (single gel electrophoresis) was carried out to assess the genotoxic potential of TiO_2 nanoparticles at concentrations of 30 µM, 300 µM and 3000 µM. Kruskal Wallis H tests revealed significant differences in % tail DNA following exposure periods of 24 and 48 hours, ($P<0.01$). Mann Whitney U tests were then performed between concentrations. A significant difference was observed

between the control and 3000 µM following both 24 and 48 hours of exposure with increased % tail DNA (P<0.01) as seen in figure 8. TiO_2 nanoparticles at the highest concentration used (3000 µM) were able to induce DNA damage following exposure periods of 24 and 48 hours. A significantly lower % tail DNA was observed following 24 hours of exposure to 30 µM TiO_2 nanoparticles, assessed by the Mann- Whitney U test (P<0.01). Although statistically significant, further tests need to be conducted to improve experimental validity. Copper was used as a positive control in this study, however due to loss of cell viability; cells were not present for scoring purposes.

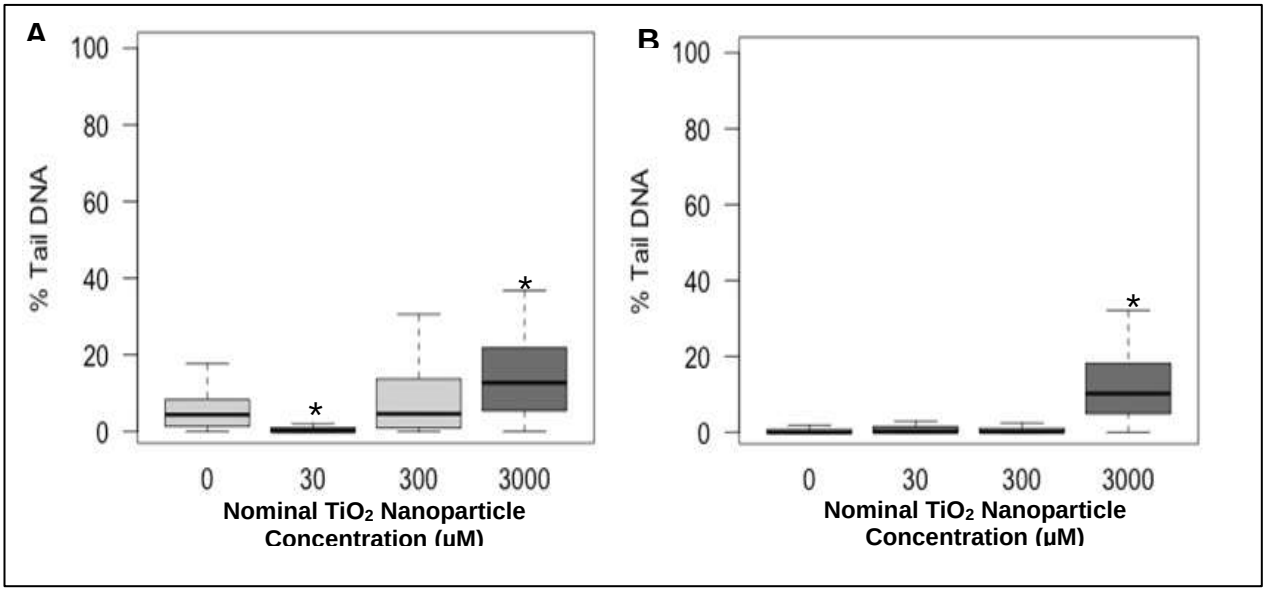


Figure 7. Box and whisker plots for comet assay values (% Tail DNA) following a 24 hour (A) and 48 hour (B) exposure to TiO_2 nanoparticles. Boxes plots show 25th and 75th percentile values, with median value and 90th percentile values (whiskers). The asterisk (*) represents a significant (P<0.01) difference in % tail DNA compared to the control.

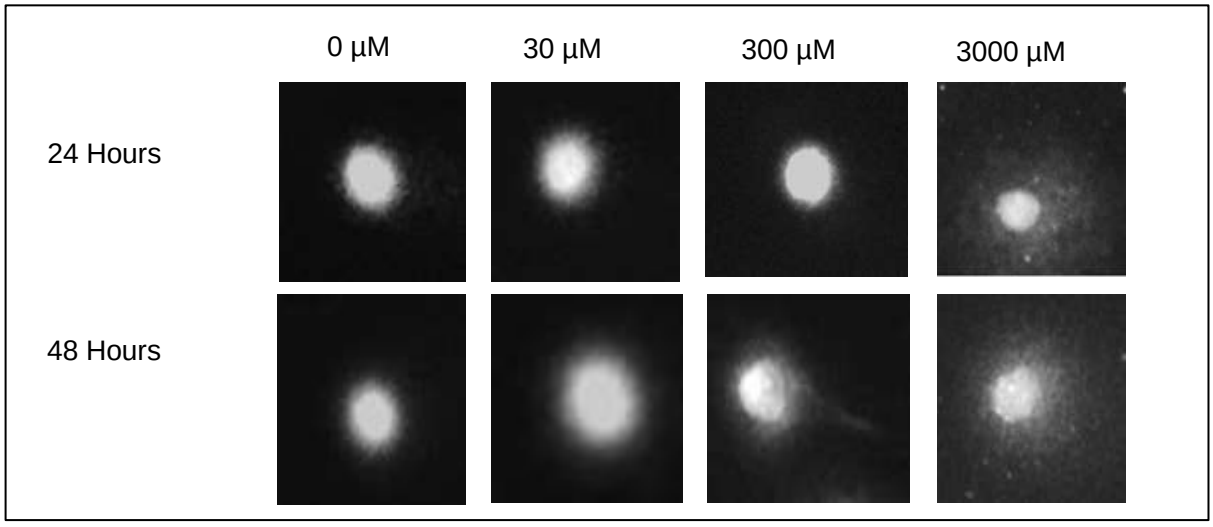


Figure 8. DNA comets following single gel electrophoresis after 24 and 48 hour exposure periods to varied concentrations of TiO_2 nanoparticles (30 µM, 300 µM and 3000 µM).

Figure 8 shows DNA comets from each treatment condition. A clear difference can be seen between the cells in the control group and those exposed to 3000 µM TiO_2 nanoparticles, with an increased portion of migrated DNA outside of the nucleus (tail DNA) in the 3000 µM treated cells. No DNA comets were present for copper treated cells due to loss of cell viability.

Morphology of cells

No clear differences in cell morphology was observed between control cells and cells exposed to TiO_2 nanoparticle concentrations of 300µM and 1000µM. Cells were also exposed to copper to provide a positive control for the experiment however, cell morphology could not be assessed due to excessive cell lysis and detachment from the melinex cover slip. Damage can be seen in all images potentially caused from dehydration and fixation.

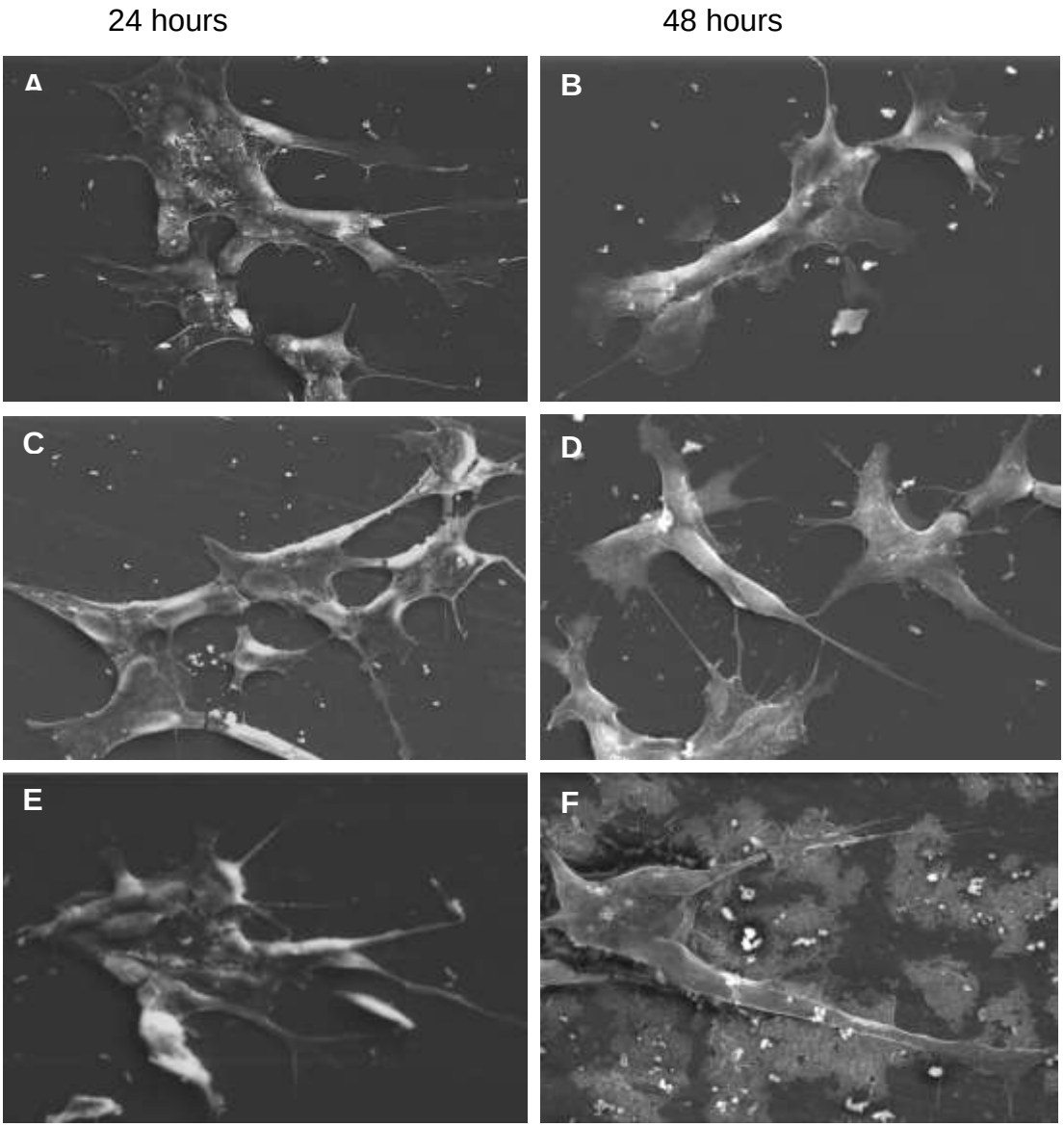


Figure 9. Morphology of control cells (A and B), cells treated with 300µM TiO_2 nanoparticles (C and D) and cells treated with 1000µM TiO_2 nanoparticles (E and F) for exposure periods of either 24 hours (A, C and E) or 48 hours (B, D and F).

X–ray microanalysis

Back – scattered electron microscopy was used to detect the presence of heavy elements with a high atomic number in the sample and further SEM/EDS analysis was carried out to produce maps and spectrums of elements present. Samples were carbon coated for spectrum analysis causing the carbon peaks seen in figures 11B and 12B. Titanium (11E and 12E, represented in yellow) was found in aggregates attached to the surface of the cells at both concentrations of 300µM and 1000µM, but not in control samples (data not shown) following 24 and 48 hours of exposure. Titanium was also found in the media at both concentrations of 300µM and 1000µM, but was more commonly observed attached to the surface of the neuroblastoma cells. X-ray microanalysis could not be carried out on cells exposed to copper due to excessive cell death and detachment from the coverslip.

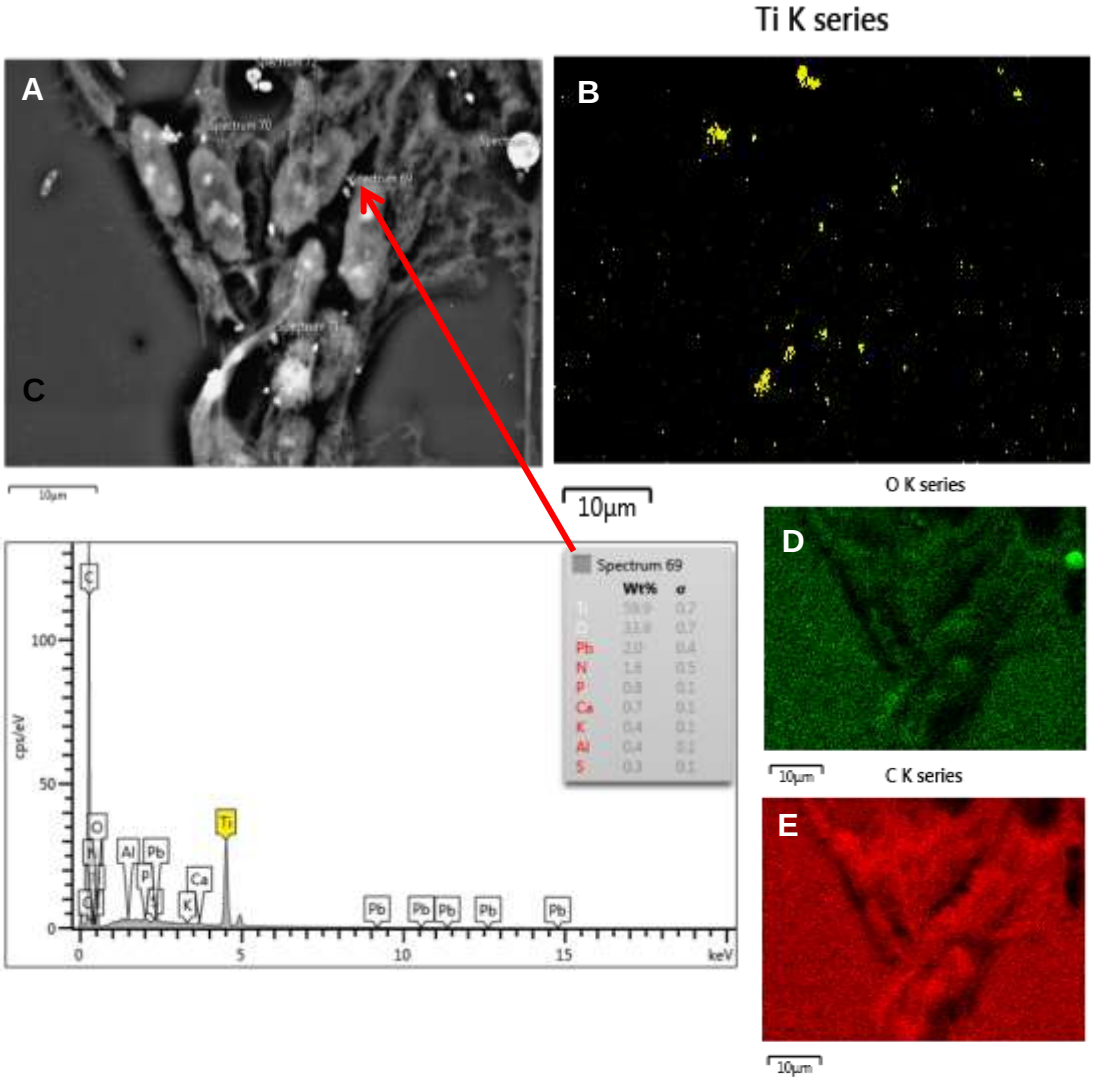


Figure 10. (A) SH-SY5Y cell following 48 hour exposure to 300µM (23.9598 µg / ml) TiO_2 nanoparticles. (C) EDS spectrum showing peaks of C (E), O (D), Ti (B), Pb, Ca, P, Na, As, S, Cl, Al, K and Si at location 69 as shown by red arrow.

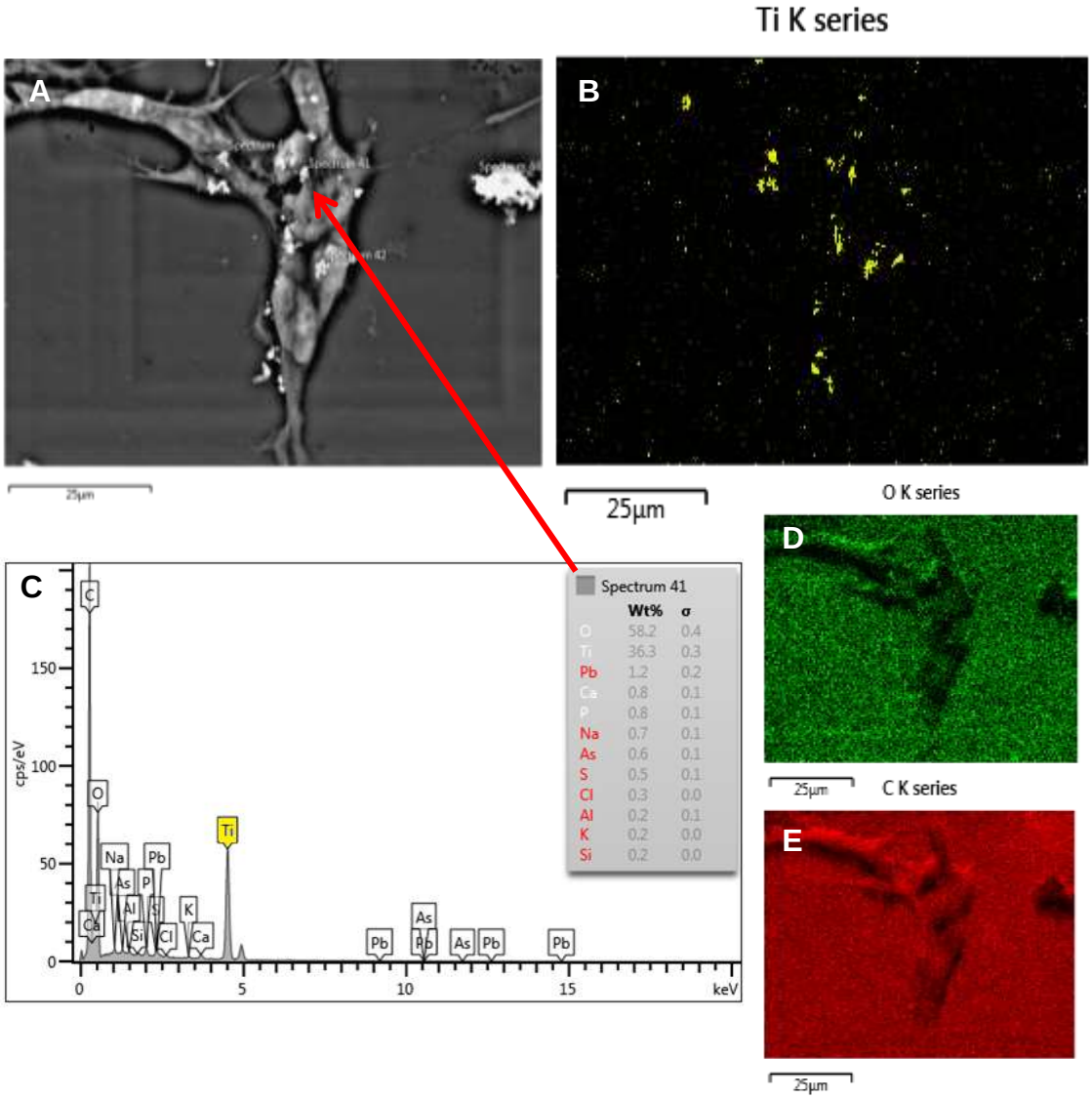


Figure 11. (A) SH-SY5Y cell following 48 hour exposure to 1000µM (79.866 µg / ml) TiO_2 nanoparticles. (C) EDS spectrum showing peaks of C (E), O (D), Ti (B), Pb, Ca, P, Na, As, S, Cl, Al, K and Si at location 41 as shown by red arrow.

Discussion

Despite extensive *in vitro* and *in vivo* research into the safety of Titanium dioxide nanoparticles, their mechanisms and levels of toxicity are still poorly understood, particularly in the nervous system, with conflicting and variable results throughout the literature. This study assessed the cytotoxic and genotoxic potential of TiO_2 nanoparticles on the developing nervous system using undifferentiated SH-SY5Y cells. The cells are derived from human brain tumours, however are often used in neurotoxicity assessments due to their dopaminergic properties and biochemical characteristics similar to neurons (Filograna *et al.,* 2015).

This study found that TiO_2 nanoparticles, even at concentrations much higher than environmentally relevant (3000µM and 10,000µM), had no impact on mitochondrial function, as determined by MTT assay, with no significant difference of cell viability

between treated cells and the control (P>0.05). Despite no significant difference in cell viability it can be seen in figures 6 and 7 that 300µM and 1000µM respectively, have an absorbance higher than that of the control. This indicates a potential interference of TiO_2 with the assay. Nanomaterials are known to interact with colorimetric and fluorometric dyes used in assays such as the MTT, often giving false results for cytotoxicity, (Doak *et al.,* 2009; Wörle-Knirsch *et al.,* 2006). Blanks containing TiO_2 were used in this assay to account for changes in absorbance caused by TiO_2 nanoparticles, however it is unclear how much TiO_2 was present in the wells containing the SH-SY5Y cells. As seen in figures 10 and 11, the nanoparticles can be seen adhered to the cells rather than dispersed in the media. If the TiO_2 has a greater tendency to attach to the cells than remain dispersed in the media, then TiO_2 will remain in wells even after media is removed causing an increased absorbance respective to the blanks. It is understood that different cytotoxicity assays can give different results depending on cell line, assay and agent used. In order to avoid false indications of toxicity and obtain reliable results, multiple assays should ideally be used (Fotakis and Timbrell, 2006). If not for the time constraints on the study, a neutral red retention assay measuring lysosomal function would have been carried out alongside the MTT assay.

Conflicting results have been reported throughout the literature concerning the affect TiO_2 nanoparticles can have on cell viability, with some studies obtaining positive results (Acar *et al.,* 2015; Coccini *et al.,* 2015; Gosch *et al.,* 2010; Wang *et al.,* 2007) and some reporting negative results (Petkovic *et al.,* 2011; Valdiglesias *et al.,* 2013; Wagner *et al.,* 2009; Zhang and Sun, 2004). The variability in results between studies may be due to experimental differences. Differences in experimental conditions such as temperature of laboratory, spectrophotometer sensitivity and batch of culture medium have been shown to influence the results of nanoparticles with intermediate levels of toxicity, such as TiO_2, causing varied results between laboratories where the same methods and procedures were used (Lanone *et al.,* 2009). The cell line used may also have an effect on levels of toxicity reported. Some cells are much more sensitive than others (Filograna *et al.,* 2015). The crystalline structure, particle size, coating and interaction with the media can influence the surface charge, sedimentation, aggregation and hence toxicity of TiO_2 nanoparticles (Holmberg *et al.,* 2013; Jugan *et al.,* 2012; Li *et al.,* 2004; Ma *et al.,* 2010; Liu *et al.,* 2011; Marquez-Ramirez *et al.,* 2012; Oberdörster *et al.,* 2007; Petkovic *et al.,* 2011).

The presence or absence of fetal bovine serum (FBS) in media can also alter cell viability. A portion of SH-SY5Y cells grown in medium without serum underwent apoptosis and had altered cell morphology, indicating the need for consistency between experimental procedures. FBS also changes the mean size of nanoparticle aggregates, reducing levels of aggregation (Macleod *et al.,* 2001). The TiO_2 nanoparticles used in this study were suspended in water. It has been shown that nanoparticles suspended in water are prone to aggregation due to the hydration and reduction of electrostatic repulsion (Ates *et al.,* 2013; Marquez-Ramirez *et al.,* 2012). Aggregate size increases with concentration causing the degree of dispersion caused by media to become less pronounced (Doak *et al.,* 2009). This may explain with no cytotoxicity was observed at concentrations as high as 3000µM and 10,000µM; despite the use of FBS; aggregates may have been too large to enter the cells readily. Hackenberg *et al., (*2010) prepared anatase TiO_2 nanoparticle dilutions in the same way as this study, but found that sonication did not prevent aggregation.

Despite the negative results obtained from the MTT assay, positive results were obtained from the alkaline comet assay. As seen in figure 7, cells treated with 3000µM TiO_2 nanoparticles had a % tail DNA significantly higher than that of the control ($P<0.05$). This increase in % tail DNA is an indicator of DNA damage and can be seen in figure 8. The percentage of DNA in the tail is the amount of DNA that has migrated out of the nucleus and is directly proportional to the amount of DNA damage the agent has caused (Kumaravel and Jha, 2006). Since the alkaline comet assay is not specific for double strand breaks, this DNA damage could be excision repair sites or single strand breaks. To determine the type and extent of genotoxic damage caused by the TiO_2 nanoparticles other assays such as the micronucleus assay and ɣ-H2AX foci detection assay can be used (Jugan *et al.,* 2012; Trouiller *et al.,* 2009). Other studies both *in vivo* and *in vitro* have also reported TiO_2 nanoparticle induced genotoxic damage (Botelho *et al.,* 2014; Ghosh *et al.,* 2010; Jugan *et al.,* 2012; Petkovic *et al.,* 2011; Shukla *et al.,* 2011; Trouiller *et al.,* 2009; Valdiglesias *et al.,* 2013; Vevers and Jha, 2008) although mostly at concentrations lower than those used in this study. Trouiller *et al.,* (2009) found genotoxic effects using a similar concentration to that used in this study. They found that orally administered TiO_2 nanoparticles at 600µg/ml induced micronuclei, DNA deletions and ɣ-H2AX foci formation, an indicator of double strand breaks in mice *in vivo*. Similar to his study, Valdiglesias *et al.,* (2013) and Ursini *et al., (*2014) found that TiO_2 nanoparticles were able to induce DNA damage yet had little effect on cell viability. They found that anatase TiO_2 nanoparticles were able to induce genotoxic damage assessed by the comet assay. These results were however obtained at much lower, environmentally relevant concentrations (80-150µg/ml) indicating the variability of *in vitro* nanoparticle toxicity results. Studies have also reported negative results for TiO_2 induced genotoxicity. Naya *et al.,* (2012) found no increase in % tail DNA after intratracheal instillation in rats at concentrations as high as 5.0mg/kg body weight. Similarly Hackenberg *et al.,* (2011) also found that TiO_2 nanoparticles did not induce genotoxicity assessed by the comet assay, despite reaching the nucleus of lymphocytes and Linnainmaa *et al.,* (1997) found TiO_2 nanoparticles did not increase micronuclei number.

The production of reactive oxygen species (ROS) is often held accountable for the genotoxic damage caused by the TiO_2 nanoparticles due to the high correlation between ROS levels and genotoxicity, and the negative results often obtained by mutation assays, ruling mutation out as a mechanism for genotoxicity (Chen *et al.,* 2014; Jugan *et al.,* 2012; Shukla *et al.,* 2011). TiO_2 has been shown to significantly reduce levels of glutathione, reduce catalase activity, induce mitochondrial common deletion and increase levels of reactive oxygen species, and oxidise pyrimidine and purine bases, indicators of oxidative stress (Jaeger *et al.,* 2012; Jugan *et al.,* 2012; Reeves *et al.,* 2008; Saquib *et al.,* 2012; Sekar *et al.,* 2011; Trouiller *et al.,* 2009).

One proposed mechanism for ROS mediated genotoxicity is the inactivation of NER and BER DNA repair pathways by reactive oxygen species (Chen *et al.,* 2014). Valdiglesias *et al.,* (2013) however found no evidence for ROS formation or oxidative damage despite finding positive results for dose-dependent apoptosis, altered cell cycle and DNA strand breaks suggesting TiO_2 nanoparticles may induce genotoxic damage by means other than ROS. Similarly Demir *et al.,* (2015) found significant TiO_2 nanoparticle induced DNA damage assessed by the comet assay, but negative results from the modified comet assay using FPG enzyme suggesting the DNA damage was not induced by oxidative stress. A modified comet assay or electron

spin resonance could be carried out to determine if the genotoxic damage seen in this study was due to oxidative damage (Reeves *et al.,* 2008). Mitcohondrial DNA damage, measured by mitochondrial common deletion could have also been employed in this study as a biomarker of oxidative stress induced genotoxicity, (Jaeger *et al.,* 2012).

Many factors may influence the variability in genotoxic results obtained such as cell lines and media used. Electrostatic interactions are important for DNA adsorption. DNA is a negatively charged polymer and is more likely to adhere to TiO_2 nanoparticles if they are positively charged. The pH of the surrounding media has been shown to influence the charge of TiO_2, with it becoming more negatively charged at pH >7 and positively charged at pH<6. The concentration of NaCl in media can influence TiO_2 DNA adsorption, and cause variable results (Zhang *et al.,* 2014). Another reason for the varied results obtained for TiO_2 genotoxicity across the literature is particle size and crystalline structure. Smaller nanoparticles have been found to have a higher genotoxic potential than larger nanoparticles and anatase nanoparticles more so than rutile due to their photocatalytic properties (Chen *et al.,* 2014).

Field emission scanning electron microscopy was used to assess the morphology of the cells following 24 and 48 hour exposure to 300µM and 1000µM TiO_2 nanoparticles. No clear morphological differences were observed in any of the cells. This experiment had a low cell count preventing and quantitative analysis. If this experiment were to be repeated, cell counts could be carried out in each treatment condition. These results are similar to Valdiglesias *et al.,* (2013) who also reported no cytotoxic effect or morphological alterations in SH-SY5Y cells exposed to TiO_2. X-ray microanalysis was used to determine the presence and location of the TiO_2 nanoparticles. As seen in figures 8 and 9, TiO_2 was present in the treated cell samples. The TiO_2 was more commonly found attached to the cells rather than in the surrounding media (data not shown). This is due to weak attractive forces such as Van der Waals forces and capillarity interactions taking place between single and aggregates of TiO_2 and the SH-SY5Y cell membranes. Treated cells show a tendency to increase adhesive properties during exposure, with Van der Waals and electrostatic forces increasing with incubation time (da Rosa, 2013).

Previous studies have shown that neuronal cells are capable of internalising both single and aggregates of TiO_2 nanoparticles in a time dependant manner (Kenzaoui *et al.,* 2012; Huerta-Garcia *et al.,* 2014; Marquez-Ramirez *et al.,* 2012; Valdiglesias *et al.,* 2013). The mechanism of TiO_2 uptake is still unclear although previous studies have suggested mechanisms may include endocytosis, cytoskeleton protein interaction (Marquez-Ramirez *et al.,* 2012), destabilisation of the membrane (Valant *et al.,* 2012) and the binding of biomolecules from culture medium to nanoparticles to form nanomaterial-protein coronas, masking the nanoparticles identity (Bramini *et al.,* 2014; Cedervall *et al.,* 2007; Doak *et al.,* 2009; Kim *et al.,* 2007; Lynch and Dawson, 2008; Michaelis *et al.,* 2006).

It is unclear in this study whether cells were internalised in the images obtained from x-ray microanalysis as they do not reveal whether the TiO_2 nanoparticles are attached to the surface of the cells or inside them. SH-SY5Y cells used in this study grow as a mixture of both floating and adherent cells (ATCC, 2014). Cells may adhere to the surface of the cover slips after exposure to the TiO_2, trapping TiO_2

nanoparticles underneath the cells. This can cause problems using simple imaging techniques as it is unclear whether the nanoparticles are inside the cells, attached to the cell membrane or underneath them. More advanced methods such as flow cytometry and transmission electron microscopy could be used to determine internalisation. Such methods would have been used if not for the time and financial restraints on this study.

Floating cells pose a problem for all assays and experiments in this study. Dead cells have been seen to detach from the well surface, particularly cells treated with copper (positive control). This suggests that as a cell becomes more damaged, it is more likely to detach. All media was removed and replaced for all experiments. This may have resulted in the removal of healthy or damaged cells at all concentrations.

In vitro cytotoxicity assays can be used to predict human toxicity, providing a cheaper and quicker alternative to *in vivo* tests and reducing the number of animals used in experiments (Fotakis and Timbrell, 2006). The results obtained from this study and other studies must be obtained from environmentally relevant concentrations to evaluate potential risk to human health. It is unclear exactly what concentrations of TiO_2 nanoparticles humans are exposed to on a daily basis, and of this how much accumulates in the brain. *In vivo* mammalian studies in mice have found TiO_2 in the hippocampus in concentrations ranging from 0.13 to 0.3 µg/ml following 500µg exposure (Wang *et al.,* 2008a and Wang *et al.,* 2008b). It has also been estimated that the average 3-4 year old male in the western world may consume 2mg TiO_2/kg BW per day (Weir *et al.,* 2012). Since little data is available to predict the amount of TiO_2 human brains may be exposed to care has to be taken when determining safety levels. In order to utilise the data from *in vitro* toxicity assays which reflects intrinsic toxic potential, more *in vivo* studies are necessary to determine expressed toxicity which takes into account appropriate route of exposure, metabolism, excretion and DNA repair capabilities of the tissue.

Conclusion

The vast use of TiO_2 nanoparticles in medical, food and industrial processes causes humans to be exposed to the nanoparticles on a daily basis. Despite *in vivo* and *in vitro* toxicity tests on multiple target organs, systems and cells, there is still a clear lack of understanding regarding the mechanisms and extent of TiO_2 nanoparticle toxicity. In conclusion, the results from this study show that although TiO_2 nanoparticles did not reduce cell viability, they can, at high concentrations, induce significant DNA damage. These results contribute to the knowledge of titanium dioxide nanoparticle impacts on human health. Further research is needed to determine mechanisms of toxicity.

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References

Acar, M.S., Bulut, Z.B., Ates, A., Nami, B., Koçak, N and Yildiz, B. (2015). Titanium dioxide nanoparticles induce cytotoxicity and reduce mitotic index in human amniotic fluid-derived cells. *Human and Experimental Toxicology.* 34, 174-182. doi: 10.1177/0960327114530742.

ATCC. (2014). *SH-SY5Y (ATCC® CRL-2266™).* Available: http://www.atcc.org/products/all/CRL-2266.aspx#culturemethod. Last accessed 3rd March 2016.

Ates, M., Daniels, J., Arslan, Z. and Farah, I.O. (2013). Effects of aqueous suspensions of titanium dioxide nanoparticles on *Artemia salina*: assessment of nanoparticle aggregation, accumulation, and toxicity. *Environmental Monitoring Assessment*. 185, 3339-3348. doi: 10.1007/s10661-012-2794-7.

Borm, P.J.A. and Berube, D. (2008). A tale of opportunities, uncertainties and risks. *Nanotoday*. 3, (1-2) 56-59. doi: 10.1016/S1748-0132(08)70016-1.

Botelho, M.C., Costa, C., Silva, S., Costa, S., Dhawan, A., Oliveira, P.A. and Teixeira, J.P. (2014). Effects of titanium dioxide nanoparticles in human gastric epithelial cells in vitro. *Biomedicine & Pharmacotherapy.* 68, (1) 59-64. doi: 10.1016/j.biopha.2013.08.006.

Bourikas, K., Kordulis, C. And Lycourghiotis, A. (2014). Titanium Dioxide (Anatase and Rutile): Surface Chemistry, Liquid-Solid Interface Chemistry, and Scientific Synthesis of Supported Catalysts. *Chemical Reviews.*114, (19) 9754-9823. doi: 10.1021/cr300230q.

Boyle, D., Al-Bairuty, G.A., Ramsden, C.S., Sloman, K.A., Henry, T.B. and Handy, R.D. (2013). Subtle alterations in swimming speed distributions of rainbow trout exposed to titanium dioxide nanoparticles are associated with gill rather than brain injury. *Aquatic Toxicology.* 126, 116-127. doi: 10.1016/j.aquatox.2012.10.006.

Bramini, M., Ye, D., Hallerbach, A., Raghnaill, M.N., Salvati, A., Aberg, C. and Dawson, K.A. (2014). Imaging Approach to Mechanistic Study of Nanoparticle Interactions with the Blood-Brain Barrier. *ACS Nano.* 8 (5) 4304-4312. doi: 10.1021/nn5018523.

Brun, E., Carrière, M. and Mabondzo, A. (2012). *In vitro* evidence of dysregulation of blood-brain barrier function after acute and repeated/long-term exposure of TiO_2 nanoparticles. *Biomaterials*. 33, (3) 886-896. doi: 10.1016/j.biomaterials.2011.10.025.

Cedervall, T., Lynch, I., Foy, M., Berggård, T., Donnelly, S.C., Cagney, G., Linse, S. and Dawson, K.A. (2007). Detailed Identification of Plasma Proteins Adsorbed on Copolymer Nanoparticles. *Angewandte Chemie International Edition*. 46, 5754-5756. doi: 10.1002/anie.200700465.

Chen, T., Yan, J. and Li, Y. (2014) 'Genotoxicity of titanium dioxide nanoparticles', *Journal of Food and Drug Analysis*, 22**,** (1), pp. 95–104. doi: 10.1016/j.jfda.2014.01.008.

Coccini, T., Grandi, S., Lonati, D., Locatelli, C. and De Simone, U. (2015). Comparative cellular toxicity og titanium dioxide nanoparticles on human astrocyte and neuronal cells after acute and prolonged exposure. *NeuroToxicology.* 48, 77-89. doi: 10.1016/j.neuro.2015.03.006.

Czajka, M., Sawicki, K., Sikorska, K., Popek, S., Kruszewski, M and Kapka-Skrzypczak, L. (2015). Toxicity of titanium dioxide nanoparticles in central nervous system. *Toxicology in Vitro*. 29, (5) 1042-1052. doi: 10.1016/j.tiv.2015.04.004.

D'Agata, A., Fasulo, S., Dallas, L.J., Fisher, A.S., Maisano, M., Readman, J.W. and Jha, A.N. (2013). Enhanced toxicity of 'bulk' titanium dioxide compared to 'fresh' and 'aged' nano-TiO_2 in marine mussels (*Mytilus galloprovincialis). Nanotoxicology*. 8, (5) 549-558. doi: 10.3109/17435390.2013.807446.

da Rosa, E.L. S. (2013). Kinetic effects of TiO_2 fine particles and nanoparticles aggregates on the nanomechanical properties of human neutrophils assessed by force spectroscopy. *BioMed Central Biophysics.* 6, (11) 1-10. doi: 10.1186/2046-1682-6-11.

Demir, E., Akça, H., Turna, F., Aksakal, S., Burgucu, D., Kaya, B., Tokgün, O., Vales, G., Creus, A. and Marcos, R. (2015). Genotoxic and cell-transforming effects of titanium dioxide nanoparticles. *Environmental Research.* 136, 300-308. doi: 10.1016/j.envres.2014.10.032.

Doak, S.H., Griffiths, S.M., Manshian, B., Singh, N., Williams, P.M., Brown, A.P. and Jenkins, G.J.S. (2009). Confounding experimental considerations in nanogenotoxicology. *Mutagenesis.* 1-9. doi: 10.1093/mutage/gep010.

Elder, A., Gelein, R., Silva, V., Feikert, T., Opanashuk, L., Carter, J., Potter, R., Maynard, A., Ito, Y., Finkelstein, J. And Oberdörster, G. (2006). Translocation of Inhaled Ultrafine Manganese Oxide Particles to the Central Nervous System. *Environmental Health Perspectives.* 114, (8) 1172-1178. doi: 10.1289/ehp.9030.

Federici, G., Shaw, B.J. and Handy, R.D. (2007). Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. *Aquatic Toxicology*. 84, (4) 415-430. doi: 10.1016/j.aquatox.2007.07.009.

Filograna, R., Civiero, L., Ferrari, V., Codolo, G., Greggio, E., Bubacco, L., Beltramini, M. and Bisaglia, M. (2015). Analysis of the Catecholaminergic Phenotype in Human SH-SY5Y and BE(2)-M17 Neuroblastoma Cell Lines upon Differentiation. *PLOS ONE*. 10, (8). doi: 10.1371/journal.pone.0136769.

Fotakis, G. and Timbrell, J.A. (2006). In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters.* 160, (2) 171-177. doi: 10.1016/j.toxlet.2005.07.001.

Ghosh, M., Bandyopadhyay, M. and Mukherjee, A. (2010). Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: Plant and human lymphocytes. *Chemosphere.* 81, (10) 1253-1262. doi: 10.1016/j.chemosphere.2010.09.022.

Gottschalk, F., Sonderer, T., Scholz, R.W. and Nowack, B. (2009). Modelled Environmental Concentrations of Engineered Nanomaterials (TiO_2, ZnO, Ag, CNT, Fullerenes) for Different Regions. *Environmental Science and Technology.* 43, (24) 9216-9222. doi: 10.1021/es9015553.

Hackenberg, S., Friehs, G., Froelich, K., Ginzkey, C., Koehler, C., Scherzed, A., Burghartz, M., Hagen, R. and Kleinsasser, N. (2010). Intracellular distribution, geno- and cytotoxic effects on nanosized titanium dioxide particles in the anatase crystal phase on human nasal mucose cells. *Toxicology Letters.* 195, 9-14. doi: 10.1016/j.toxlet.2010.02.022.

Hackenberg, S., Friehs, G., Kessler, M., Froelich, K., Ginzkey, C., Koehler, C., Scherzed, A., Burghartz, M. and Kleinsasser, N. (2011). Nanosized Titanium Dioxide Particles do not Induce DNA Damage in Human Peripheral Blood Lymphocytes. *Environmental and Molecular Mutagenesis.* 52, (4) 264-268. doi: 10.1002/em.20615.

Handy, D.R. and Shaw, B.J. (2007). Toxic effects of nanoparticles and nanomaterials: Implications for public health, risk assessment and the public perception of nanotechnology. *Health, Risk & Society.* 9, (2) 125-144. doi: 10.1080/13698570701306807.

Holmberg, J.P., Ahlberg, E., Bergenholtz, J., Hassellöv, M. and Abbas, Z. (2013) 'Surface charge and interfacial potential of titanium dioxide nanoparticles: Experimental and theoretical investigations', *Journal of Colloid and Interface Science*, 407, 168–176. doi: 10.1016/j.jcis.2013.06.015.

Hu, R., Zheng, L., Zhang, T., Gao, G., Cui, Y., Cheng, Z., Cheng, J., Hong, M., Tang, M and Hong, F. (2011). Molecular mechanism of hippocampal apoptosis of mice following exposure to titanium dioxide nanoparticles. *Journal of Hazardous Materials.* 191, (1-3) 32-40. doi: 10.1016/j.jhazmat.2011.04.027.

Huerta-García, E., Pérez-Arizti, J.A., Márquez-Ramírez, S.G., Delgado-Buenrostro, L., Chirino, Y.I., Iglesias, G.G. and López-Marure, R. (2014). Titanium dioxide nanoparticles induce strong oxidative stress and mitochondrial damage in glial cells. *Free Radical Biology and Medicine.* 73, 84-94. doi: 10.1016/j.freeradbiomed.2014.04.026.

Jacobasch, C., Völker, C., Giebner, S., Völker, J., Alsenz, H., Potouridis, T., Heidenreich, H., Kayser, G., Oehlmann, J. and Oetken, M. (2014). Long-term effects of nanoscaled titanium dioxide on the cladoceran *Daphnia magna* over six generations. *Environmental Pollution.* 186, 180-186. doi: 10.1016/j.envpol.2013.12.008.

Jaeger, A., Weiss, D.G., Jonas, L. and Kriehuber, R.(2012). Oxidative stress-induced cytotoxic and genotoxic effects of nano-sized titanium dioxide particles in human HaCaT keratinocytes. *Toxicology.* 296, (1-3) 27-36. doi: 10.1016/j.tox.2012.02.016.

Jugan, M-L., Barillet, S., Simon-Deckers, A., Herlin-Boime, N., Sauvaigo, S., Douki, T. and Carriere, M. (2012). Titanium dioxide nanoparticles exhibit genotoxicity and impair DNA repair activity in A549 cells. *Nanotoxicology.* 6, (5) 501-513. doi: 10.3109/17435390.2011.587903.

Kenzaoui, B.H., Bernasconi, C.C., Guney-Ayra, S. and Juillerat-Jeannerat, L. (2012). Induction of oxidative stress, lysosomal activation and autophagy by nanoparticles in human brain-derived endothelial cells. *Biochemical Journal*. 441, (3) 813-821. doi: 10.1042/BJ20111252.

Kim, H.R., Andrieux, K., Gil, S., Taverna, M., Chacun, H., Desmaële, D., Taran, F., Georgin, D. and Couvreur, P. (2007). Translocation of Poly(ethylene glycol-*co*-hexadecyl)cyanoacrylate Nanoparticles into Rat Brain Endothelial Cells: Role of Apolipoproteins in Receptor-Mediated Endocytosis. *Biomacromolecules.* 8, (3) 793-799. doi: 10.1021/bm060711a.

Kumaravel, T.S. and Jha, A.N. (2006). Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutation Research*. 605, 7-16. doi:10.1016/j.mrgentox.2006.03.002

Langer, R. and Weissleder, R., (2015). Scientific discovery and the future of medicine. *Nanotechnology. JAMA*, 313, 135-136. doi: 10.1001/jama.2014.16315.

Lanone, S., Rogerieux, F., Geys, J., Dupont, A., Maillot-Marechal, E., Boczkowski, J., Lacroix, G. and Hoet, P. (2009). Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. *Particle and Fibre Toxicology.* 6, (14). doi: 10.1186/1743-8977-6-14.

Lee, K.P., Trochimowicz, H.J. Reinhardt, C.F. (1985). Pulmonary response of rats exposed to titanium dioxide (TiO_2) by inhalation for two years. *Toxicology and Applied Pharmacology.* 79, (2) 179-192.

Li, W., Ni, C., Lin, H., Huang, C.P. and Shah, S.I. (2004). Size dependence of thermal stability of TiO_2 nanoparticles. *Journal of Applied Physics.* 96, (11) 6663-6668. doi: 10.1063/1.1807520.

Liga, M.V., Bryant, E.L., Colvin, V.L. and Li, Q. (2011) 'Virus inactivation by silver doped titanium dioxide nanoparticles for drinking water treatment', *Water Research*, 45, (2) 535–544. doi:10.1016/jwatres.2010.09.012.

Linnainmaa, K., Kivipensas, P. and Vainio, H. (1997). Toxicity and cytogenetic studies of ultrafine titanium dioxide in cultured rat liver epithelial cells. *Toxicology in Vitro.* 11, (4) 329-331, 333-335. doi: 10.1016/S0887-2333(97)00000-3.

Liu, X., Chen, G. and Su, C. (2011). Effects of material properties on sedimentation and aggregation of titanium dioxide nanoparticles of anatase and rutile in the aqueous phase. *Journal of Colloid and Interface Science.* 363, (1) 84-91. doi: 10.1016/jcis.2011.06.085.

Lynch, I. and Dawson, K.A. (2008). Protein-nanoparticle interactions. *Nanotoday.* 3, (1-2). 40-47. doi: 10.1016/S1748-0132(08)70014-8.

Ma, L., Liu, J., Wang, J., Duan, Y., Yan, J., Liu, H., Wang, H., Hong, F. (2010). Oxidative stress in the brain of mice caused by translocated nanoparticulate TiO_2 delivered to the abdominal cavity. *Biomaterials.* 31, (1) 99-105. doi: 10.1016/j.biomaterials.2009.09.028.

Macleod, M.R., Allsopp, T.E., McLuckie, J. and Kelly, J.S. (2001). Serum withdrawal causes apoptosis in SHSY 5Y cells. *Brain Research.* 889, (1-2) 308-315. doi: 10.1016/S0006-8993(00)03173-5.

Magdolenova, Z., Bilaničová, D., Pojana, G., Fjellsbø, L.M., Hudecova, A., Hasplova, K., Marcomini, A. and Dusinska, M. (2012). Impact of agglomeration and different dispersions of titanium dioxide nanoparticles on the human related *in vitro* cytotoxicity and genotoxicity. *Journal of Environmental Monitoring*. 14, (2) 455-464. doi: 10.1039/C2EM10746E.

Márquez-Ramírez, S.G., Delgado-Buenrostro, N.L., Chirino, Y.I., Iglesias, G.G. and López-Marure, R. (2012). Titanium dioxide nanoparticles inhibit proliferation and induce morphological changes and apoptosis in glial cells. *Toxicology.* 302, (2-3). 146-156. doi: 10.1016/j.tox.2012.09.005.

Michaelis, K., Hoffmann, M.M., Dreis, S., Herbert, E., Alyautdin, R.N., Michaelis, M., Kreuter, J. and Langer, K. (2006). Covalent Linkage of Apolipoprotein E to Albumin Nanoparticles Strongly Enhances Drug Transport into the Brain. *The Journal of Pharmacology and Experimental Therapeutics.* 317, (3) 1246-1253. doi: 10.1124/jpet.105.097139.

Naya, M., Kobayashi, N., Ema, M., Kasamoto, S., Fukumuro, M., Takami, S., Nakajima, M., Hayashu, M. and Nakanishi, J. (2012). *In vivo* genotoxicity study of titanium dioxide nanoparticles using comet assay following intratracheal instillation in rats. *Regulatory Toxicology and Pharmacology.* 62, (1) 1-6. doi: 10.1016/j.yrtph.2011.12.002.

Oberdörster, G., Stone, V. and Donaldson, K. (2007). Toxicology of nanoparticles: A historical perspective. *Nanotoxicology.* 1, (1) 2-25. doi: 10.1080/17435390701314761.

Petković, J., Žegura, B. and Stevanović, M. (2011). DNA damage and alteration in expression of DNA damage responsive genes induced by TiO_2 nanoparticles in human hepatoma HepG2 cells. *Nanotoxicology*. 5, (3) 341-353. doi: 10.3109/17435390.2010.507316.

Ramsden, C.S., Smith, T.J., Shaw, B.J. and Handy, R.D. (2009). Dietary exposure to titanium dioxide nanoparticles in rainbow trout, (*Oncorhynchus mykiss*): no effect on growth, but subtle biochemical disturbances in the brain. *Ecotoxicology*. 18, (7) 939-951. doi: 10.1007/s10646-009-0357-7.

Reeves, J.F., Davies, S.J., Dodd, N.J.F. and Jha, A.N. (2008). Hydroxyl radicals ($^{\bullet}OH$) are associated with titanium dioxide (TiO_2) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. *Mutation Research.* 640, 113-122. doi:10.1016/j.mrfmmm.2007.12.010.

Roco, M.C. (2003). Nanotechnology: convergence with modern biology and medicine. *Current Opinion in Biotechnology.* 14, (3) 337-346. doi: 10.1016/S0958-1669(03)00068-5.

Rothen-Rutishauser, B.M., Schűrch, S., Haenni, B., Kapp, N. and Gehr, P. (2006). Interaction of Fine Particles and Nanoparticles with Red Blood Cells Visualised with Advanced Microscopic Techniques. *Environmental Sciences and Technology.* 40, (14) 4353-4359. doi: 10.1021/es0522635.

Saquib, Q., Al-Khedhairy, A.A., Siddiqui, M.A., Abou-Tarboush, F.M., Azam, A. and Masarrat, J. (2012). Titanium dioxide nanoparticles induced cytotoxicity, oxidative stress and DNA damage in human amnion epithelial (WISH) cells. *Toxicology in Vitro.* 26, (2) 351-361. doi: 10.1016/j.tiv.2011.12.011.

Seigrist, M., Stampfli, N., Kastenholz, H. and Keller, C. (2008). Perceived risks and perceived benefits of different nanotechnology foods and nanotechnology food packaging. *Appetite.* 51, (2) 283-290. doi: 10.1016J.appet.2008.02.020.

Sekar, D., Falcioni, M.L., Barucca, G. And Falcioni, G. (2011). DNA Damage and Repair Following *In Vitro* Exposure to Two Different Forms of Titanium Dioxide Nanoparticles on Trout Erythrocyte. *Environmental Toxicology.* 29, (1) 117-127. doi: 10.1002/tox.20778.

Shukla, R.K., Sharma, V., Pandey, A.K., Singh, S., Sultana, A. and Dhawan, A. (2011). ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. *Toxicology in vitro.* 25, (1) 231-241. doi: 10.1016/j.tiv.2010.11.008.

Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P. and Schiestl. R.H. (2009). Titanium Dioxide Nanoparticles Induce DNA Damage and Genetic Instability *In vivo* in Mice. *Cancer Research.* 69, (22) 8784-8789. doi: 10.1158/0008-5472.CAN-09-2496.

Ursini, C.L., Cavallo, D., Fresegna, A.M., Ciervo, A., Maiello, R., Tassone, P., Buresti, G., Casciardi, S. and Lavicoli, S. (2014). Evaluation of cytotoxic, genotoxic and inflammatory response in human alveolar and bronchial

epithelial cells exposed to titanium dioxide nanoparticles. *Journal of Applied Toxicology.* 34, (11) 1209-1219. doi: 10.1002/jat.3038.

Valant, J., Brobne, D. and Novak, S. (2012). Effect of ingested titanium dioxide nanoparticle on the digestive gland membrane of terrestrial isopods. *Chemosphere*. 87 (1) 19-25. doi: 10.1016/j.chemosphere.2011.11.047.

Valdiglesias, V., Costa, C., Sharma, V., Kiliç, G., Pásarao, E., Teixeira, J.P., Dhawan, A. and Laffon, B. (2013). Comparative study on effects of two different types of titanium dioxide nanoparticles on human neuronal cells. *Food and Chemical Toxicology.* 57, 352-361. doi: 10.1016/j.fct.2013.04.010.

Vevers, W.F. and Jha, A.N. (2008). Genotoxic and cytotoxic potential of titanium dioxide (TiO_2) nanoparticles on fish cells in vitro. *Ecotoxicology.* 17, (5) 410-420. doi: 10.1007/s10646-008-0226-9.

Vignardi, C.P., Hasue, F.M., Sartório, P.V., Cardoso, C.M., Machado, A.S.D., Passos, M.J.A.C.R., Santos, T.C.A., Nucci, J.M., Hewer, T.L.R., Watanabe, L-S., Gomes, V. and Phan, N.V. (2015). Genotoxicity, potential cytotoxicity and cell uptake of titanium dioxide nanoparticles in the marine fish *Trachinotus carolinus* (Linnaeus, 1766). *Aquatic Toxicology.* 158, 218-229. doi: 10.1016/j.aquatox.2014.11.008.

Wagner, S., Műnzer, S., Behrens, P., Scheper, T., Bahnemann, D. And Kasper, C. (2009). Cytotoxicity of titanium and silicon dioxide nanoparticles. *Journal of Physics: Conference Series*. 170, (1) 12-22. doi: 10.1088/1742-6596/170/1/012022.

Wang, J.J., Sanderson, B.J.S. and Wang, H. (2007). Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis.* 628, (2) 99-106. doi: 10.1016/j.mrgentox.2006.12.003.

Wang, J., Liu, Y., Jiao, F., Lao, F., Li, W., Gu, Y., Li, Y., Ge, C., Zhou, G., Li, B., Zhao, Y., Chai, Z. and Chen, C. (2008a). Time-dependant translocation and potential impairment on central nervous system by intranasally instilled TiO_2 nanoparticles. *Toxicology.* 254, (1-2) 82-90. doi: 10.1016/j.tox.2008.09.014.

Wang, J., Chen, C., Liu, Y., Jiao, F., Li, W., Lao, F., Li, Y., Li, B., Ge, C., Zhou, G., Gao, Y., Zhao, Y. And Chai, Z. (2008b). Potential neurological lesion after nasal inhalation of TiO_2 nanoparticles in the anatase and rutile crystal phases. *Toxicology Letters.* 183, (1-3) 72-80. doi: 10.1016/j.toxlet.2008.10.001.

Weir, A., Westerhoff, P., Fabricius, L., Hristovski, K. and Goetz, N. von (2012) 'Titanium dioxide Nanoparticles in food and personal care products', *Environmental Science & Technology*, 46, (4) 2242–2250. doi: 10.1021/es204168d.

Wohlfart, S., Gelperina, S. and Kreuter, J. (2012). Transport of drugs across the blood-brain barrier by nanoparticles. *Journal of Controlled Release.* 161, (2) 264-273. doi: 10.1016/j.jconrel.2011.08.017.

Wörle-Knirsch, J.M., Pulskamp, K. and Krug, H.F. (2006). Oops They Did It Again! Carbon Nanotubes Hoax Scientists in Viability Assays. *Nano Letters*. 6, (6) 1261-1268. doi: 10.1021/n1060177c.

Ze, Y., Zheng, L., Zhao, X., Gui, S., Sang, X., Su, J., Guan, N., Zhu, L., Sheng, L., Hu, R., Cheng, J., Cheng, Z., Sun, Q., Wang, L. and Hong, F. (2013) 'Molecular mechanism of titanium dioxide nanoparticles-induced oxidative injury in the brain of mice', *Chemosphere*, 92, (9) 1183–1189. doi: 10.1016/j.chemosphere.2013.01.094.

Zhang, A-P. and Sun, Y-P. (2004). Photocatalytic killing effect of TiO_2 nanoparticles on Ls-174-t human colon carcinoma cells. *World Journal of Gastroenterology.* 10, (21) 3191-3193. doi: 10.3748/wjg.v10.i21.3191.

Zhang, X., Wang, F., Liu, B., Kelly, E.Y., Servos, M.R. and Liu, J. (2014) 'Adsorption of DNA Oligonucleotides by titanium dioxide Nanoparticles', *Langmuir*, 30**,** (3) 839–845. doi: 10.1021/la404633p.