The Genotoxic Effects of Titanium Dioxide Nanoparticles (TiO_2 NPs) on *Dugesia dorotocephala* Genomic DNA

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Abstract

The nanotechnology industry is rapidly growing and hence as are its uses in commercial and consumer goods. The Project on Emerging Nanotechnologies cited a 521% increase between 2006 and 2011 in the number of consumer goods containing nanoparticles (www.nanotechproject.org). Nanotechnology can be found in a wide variety of products such as health and fitness, home and garden, automotive, food and beverage, cross cutting, electronics, appliances, and goods for children (www.nanotechproject.org). As the utilization of nanotechnology increases, so does the concern regarding potential toxic effects of nanomaterials in biological systems.

The exponential growth of the nanotechnology industry gave rise to a new area of research- nanotoxicology. The aim of nanotoxicology is to examine the potential toxic effect of nanoparticles within biological systems. Much of the focus of nanotoxicological research has been mammalian centered, creating a deficiency in our knowledge and understanding of genotoxic effects in aquatic ecosystems. Genotoxicity is arguably the most important aspect of the toxic effects induced by nanoparticles.

Planaria are commonly used as bio-indicators to evaluate the toxicity of potentially harmful chemicals due to their sensitivity to environmental changes (Knakievicz et al., 2008). This sensitivity coupled with the ubiquitous nature of planarians throughout various bodies of water makes them a highly valuable model organism on which to conduct genotoxicty studies and to date the use of planarians as bio-indicators of multiple freshwater systems has yet to be utilized. Nanoparticles exhibit increased reactivity due to their increased surface area (Elsaesser and Howard 2012). The increased surface to mass ratio of nanoparticles causes them to be more reactive than normal sized particles of the same compound (Reeves et al. 2008). Moreover due to the decreased radius and increased surface area of nanoparticles, they are able to pass through cell membranes and easily inflict damage within biological systems (Howard 2010).

This study demonstrates that titanium dioxide nanoparticles (TiO_2 NPs) induced apoptotic fragmentation of *Dugesia dorotocephala* genomic DNA. In addation the increased reactivity allows the TiO_2 NPs to rapidly cause damage over a 24-hour period regardless of concentration/ level of exposure and rapidly induce genotoxic damage over a 24-hour period in two planarian colonies with different physical characteristics.

Introduction

The nanotechnology industry is rapidly growing and hence as are its uses in commercial and consumer goods. The Project on Emerging Nanotechnologies cited a 521% increase between 2006 and 2011 in the number of consumer goods containing nanoparticles (www.nanotechproject.org). Nanotechnology can be found in a wide variety of products such as health and fitness, home and garden, automotive, food and beverage, cross cutting, electronics, appliances, and goods for children (www.nanotechproject.org). As the utilization of nanotechnology increases, so does the concern regarding potential toxic effects of nanomaterials in biological systems.

Nanoparticles are engineered microscopic particles that are less than 100 nm when measured in at least one dimension (Petersen and Nelson 2010). Nanoparticles can be classified as carbon based and inorganic nanoparticles (Ju-Nam and Lead 2008). Of particular interest are inorganic metal oxide nanoparticles as they are one of the most widely used (Griffith et al., 2008). Titanium dioxide (TiO_2) nanoparticles fall into the category mentioned above and have already been incorporated into a large number of consumer products and are intentionally used in water treatment to degrade organic matter (Howard 2010).

Nanoparticles exhibit increased reactivity due to their increased surface area (Elsaesser and Howard 2012). The increased surface to mass ratio of nanoparticles causes them to be more reactive than normal sized particles of the same compound (Reeves et al. 2008). Due to the decreased radius and increased surface of nanoparticles, they are able to pass through cell membranes and easily inflict damage within biological systems (Howard 2010). The extent of damage is a result of chemical composition, surface reactivity, nanoparticle shape, radius of curvature, and hydrophobicity and hydrophilicity (Nel et al., 2009).

Introduction of nanoparticles into an aquatic environment can occur in one of four ways: point sources, non-point sources, accidental release, and intentional release. Within aquatic environments nanoparticles can exist as free particles, aggregates on surface water, and in sediments (Baun et al., 2008). Abiotic factors, such as pH and nanoparticle size determine in which form a particle exists within the aquatic environment (Keller et al., 2010). The presence of nanoparticles in aquatic environments has been shown to be detrimental.

Consumption of titanium dioxide nanoparticles by rainbow trout for 8 weeks resulted in an accumulation of TiO_2 nanoparticles in the following vital organs: gut, liver, spleen and brain and were still present in these organs 2 weeks after exposure time (Ramsden et al., 2009). Furthermore following a seven day exposure to a 10 mg/L nanoparticles solution, nanoparticles accumulated in the gills, brain, testis, liver, and blood of adult medaka (Kahru and Dubouruier 2010). Toxicity is attributed to high surface area, leading to greater reactivity and an increased ability to penetrate and accumulate in cells (Xiong et al., 2011).

Bioaccumulation of nanoparticles not only presents a toxicity problem for the biota but, it also exposes humans to the bioaccumulation to nanoparticles through consumption of fish and crustaceans (Baun et al., 2008). Zhang et al. (2008) found that the presence of TiO_2 nanoparticles can facilitate the increased cadmium uptake in carp by 146% . Likewise in separate study a 132% increase in arsenic uptake was observed in the presence of titanium dioxide nanoparticles (Sun et al., 2007). Furthermore metals bind and displace essential metal ions from regulatory enzymes, resulting in the inactivation of the enzyme (Guecheva et al., 2003). Metals can also alter cysteine groups of enzymes or alter the conformation of the enzyme (Guecheva et al., 2003).

Chronic exposure to titanium dioxide nanoparticles results in reproductive impairment in fish. In a 2011 study conducted by Wang and collegues found that there was a 30% decrease in the number of zebra fish eggs laid following a 13 week aquatic exposure to 0.1mg L^{-1} of TiO_2 nanoparticles. Furthermore hatching rates of zebrafish embryos had also decreased when exposed to nanoparticles (Yeo and Kang 2008). Multiple studies have demonstrated that nanoparticles can adversely affect the development of fish embryos. Developmental anomalies such as cardiac malformation, spinal abnormalities, and oedema in yolk were found as a result of fish embryo exposure to silver nanoparticles (Lee et al., 2007; Asharani et al., 2008).

TiO_2 nanoparticles exhibit different physiochemical characteristics in aquatic systems and this can affect the way in which the nanoparticles are able to cause damage as well as impact the extent of damage. Nanoparticles can have properties of dissolved molecules, such as the ability to cross the membranes, and particles, such as active surface area (Velzeboer et al., 2008). For example aggregation of nanoparticles may result

in greater genotoxic damage because of the increased concentration of nanoparticles in the aggregate entering the cell simultaneously. Aggregation may also reduce damage as aggregates of nanoparticles may have a have a difficult time crossing cell membranes due to an increased functional diameter compared to nanoparticles that have not formed aggregates. Conflicting studies have cited results supporting both positions. Engineered nanoparticles have also been found to cause genotoxic responses, such as chromosomal fragmentation, DNA strand breakages, point mutations, oxidative DNA adducts and alterations in the expression of genes (Singh et al., 2009). Exogenous particles, like titanium dioxide nanoparticles, can give rise to reactive oxygen species (ROS)- molecules with unpaired electrons- that cause DNA damage through the formation of carbon sugars radicals as well as radical adducts of heterocyclic bases (Dizdaroglu et al., 2002). Double- and single- stranded chromosomal fragmentation of the genome is also a result of ROS, which is linked to apoptosis and necrosis (Higuchi 2003). Through the use of microarray analysis, it was observed that oxidative stress related genes were expressed after expose to TiO_2 nanoparticles (Horie et al., 2012). It was found that smaller nanoparticles are able to induce oxidative stress and apoptosis in cells through entry into the mitochondria and larger nanoparticles have a larger surface are that can result in the formation of reactive oxygen species (ROS) (Hsiao and Huang 2011). Damage to DNA has the ability to partially or completely inhibit the expression of genes, which has the potential to give rise to a substantial number of genetic defects, diseases, and cell death. Arguably the most detrimental effect of nanoparticles is the damage they cause to DNA resulting in mutations and genomic instability, which can ultimately lead to cancer (Jackson and Loeb 2001).

Planarians are bilaterally symmetrical Platyhelminthes that are distributed through freshwater ecosystems (Reddien and Alavarado 2004). Planarians are valuable bio-indicator species as they are sensitive to toxins, which can be observed as acute toxicity, lethality, teratogenesis, carcinogenesis, and neurotoxicity (Best and Morita 1991). The effect of heavy metals on fresh water organism has been apparent for a while (Knakievicz et al., 2007). In a study conducted by Knakievicz et al., (2007) found that regenerating organisms show greater sensitivity to copper (Knakievicz et al., 2007). In a similar study

exposure of planarians to the heavy metals aluminum and chromium interfered with the regeneration of decapitated planarians (Calervo et al., 1998).

Planarians are able to regenerate in response to an injury. Neoblasts are a totipotent stem cells that have the capacity to regenerate all cell types found in planarians (Reddien and Alavarado 2004). Neoblasts have large nuclei with very little cytoplasm, comprise 30% of the total cell population and are distributed throughout mesenchymal space of body except for pharyngeal region and in front of the photoreceptors (Aboobaker 2011; Reddien and Alavarado 2004). Damaged tissue in planarians can be repaired through cell proliferation to form a blastema, followed by morphallaxis, the remodeling of cells to restore symmetry and proportion (Reddien and Alavarado 2004). Cells in the blastema differentiate over several days to replace missing tissues (Pellettieri et al., 2010). The molecular mechanisms controlling planarian regeneration is not well understood (Aboobaker 2011).

To date a substantial amount of research has been conducted on the genotoxicity of engineered nanoparticles using mammalian models, while there is insufficient research into nanotoxic effects on planarians. Planarians are commonly used as bioindicators to evaluate the toxicity of potentially harmful chemicals as they are sensitive to environmental change (Knakievicz et al., 2008). This sensitivity coupled with the ubiquitous nature of planarians throughout various bodies of water makes them a highly valuable model organism on which to conduct genotoxicity studies.

Materials and Methods

Culturing of Planaria-*Dugesia tigrina* and *Dugesia dorotocephala*

Asexual planaria (2n/3n) purchased from Ward's Natural Science were cultivated in the dark in spring water at 22°C. The planaria were allowed to acclimate to laboratory conditions for 14 days. Planaria were cared for as recommended by Ward's Natural Science (Available at : http://resources.wardsci.com/livecare/planaria/). One week prior to exposure to titanium dioxide exposure, the planaria were not fed.

LC_{50}-24 hour acute toxicity testing

A. Preparation of TiO_2 nanoparticle suspensions

In order to prepare a stable titanium dioxide nanoparticle (TiO_2 NP) suspension, 0.500 g of TiO_2 NPs (Sigma-Aldrich CAS-No. 13463-67-7) were added to 100 mL of spring water. A suspension was then created using a magnetic stirrer and stir bar to distribute TiO_2 NPs throughout the spring water. 0.1 g of bovine serum was added to suspension to stabilize particles in suspension and stirred using a magnetic stirrer and stir bar for 40 minutes.

B.Determination of LC_{50}-24 hour acute toxicity test

Aliquots of the prepared TiO_2 NP suspension was then diluted using spring water as indicated as follows:

Treatment Group	Volume of TiO_2 NP suspension	Volume of spring water	Concentration (M) of TiO_2 NPs
A	24 mL	0 mL	
B	20 mL	4 mL	
C	14 mL	10 mL	
D	10 mL	14 mL	
E	4 mL	20 mL	

TiO_2 NP treatment groups were prepared using petri dishes. To create treatment group A, 24 mL of TiO_2 NP suspension was added to a petri dish. To create treatment group B, 20 mL of TiO_2 NP suspension was added to a petri dish and diluted with 4 mL of spring water. To create treatment group C, 14 mL of TiO_2 NP suspension was added to a petri dish and diluted with 10 mL of spring water. To create treatment

group D, 10 mL of TiO_2 NP suspension was added to a petri dish and diluted with 14 mL of spring water. To create treatment group E, 20 mL of TiO_2 NP suspension was added to a petri dish and diluted with 20 mL of spring water. A control group was created by adding 24 mL of spring water to a petri dish.
Six planarians were added to each treatment group and incubated at 22°C for 24 hours. Following the incubation period the mortality rates were determined by visual inspection using a dissection microscope. The treatment group where half of the planaria were deceased following 24-hour incubation period was taken as the LC_{50}.This procedure was used for both *Dugesia tigrina* and *Dugesia dorotocephala.*

Genotoxicity testing

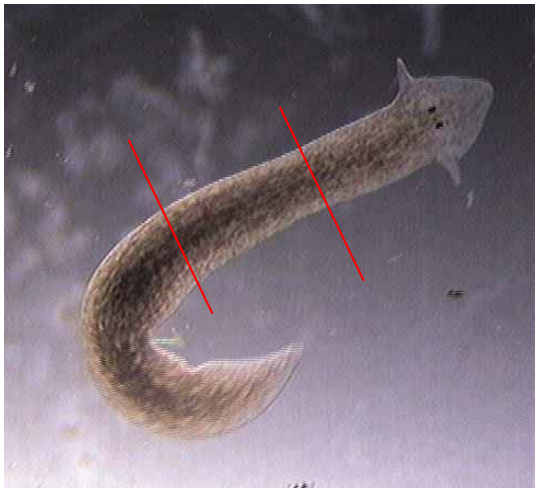
A.Creation of the TiO_2 NP exposure groups

To prepare TiO_2 NP exposure groups which to expose the planaria to, such that genotoxic damage could be qualified, three treatment groups were created using a volume of TiO_2 NP suspension below that of the LC_{50}. Treatment groups were prepared as follows:

Treatment Group	Concentration (M) of TiO_2 NPs	Volume of TiO_2 NP suspension	Volume of spring water
1	6.095×10^{-2}	20 mL	4 mL
2	5.370×10^{-2}	18 mL	6 mL
3	5.460×10^{-2}	16 mL	8 mL
Control	0	0 mL	24 mL

To create treatment group 1, 20 mL of TiO_2 NP suspension was added to a petri dish and diluted with 4 mL of spring water. To create treatment group 2, 18 mL of TiO_2 NP suspension was added to a petri dish and diluted with 6 mL of spring water. To create treatment group 3, 16 mL of TiO_2 NP suspension was added to a petri dish and diluted with 8 mL of spring water. A control group was created by adding 24 mL of spring water to a perti dish. 3 replicates of each treatment group were created.
Following the first trial, a fourth treatment group was created, in which 6 regenerating planaria were incubated in 24 mL of TiO_2 NP suspension due to apparent resilience to NPs.

To initiate planarian regeneration, planaria were placed on ice and two horizontal cuts were made behind the head (as indicated below). 6 planaria were placed in each treatment group.



Each treatment group and incubated at 22°C for 24 hours. Following the incubation period, planaria were removed from TiO_2 NP treatment groups, and transferred to fresh spring water to allow for the removal of TiO_2 NPs. Planaria from each treatment group were placed into separate 1.5 mL microcentrifuge tubes containing 100% ethanol to preserve planaria tissues, and stored at -4°C.

B.Extraction of planaria genomic DNA

To extract DNA from planaria, preserved planaria tissues from each treatment group were placed into separate 1.5 mL microcentrifuge tubes, to create composite samples. Tissue in each 1.5 mL microcentrifuge tube was pulverized using disposable pestle. 180 µl of Buffer ATL was added to each microcentrifuge tube. To digest proteins in tissues, 20 µl of proteinase K was added to each microcentrifuge tube. Each microcentrifuge tube was vortexed for 20 seconds and incubated in a hot water bath at 56°C for 2 hours. Following 2 hour incubation, microcentrifuge containing samples were vortexed for 20 seconds. 200 µl of Buffer AL was added to each sample and thoroughly mixed via vortexing. 200 µl of 100% ethanol was then added to each sample and thoroughly mixed via vortexing.

Each sample was pipetting into a DNeasy Mini column in a 2 mL collection tube. 500 µl of Buffer AW1 was added to each sample and centrifuged for 1 minute at 10,000 rpm. Flow-through was discarded and DNeasy Mini column was placed in a new 2 mL collection tube. 500 µl of Buffer AW2 was applied to each column, and then centrifuged for 3 minutes at 14,000 rpm. Flow-through was discarded and column was transferred to a 1.5 mL microcentrifuge tube. To elute DNA from column, 200 µl of Buffer AE was applied to column and incubated at room temperature for 1 minute. Following incubation, sample was centrifuged for 1 minute at 10,000 rpm. Elution step was repeated an additional time. Column was discarded and harvested DNA was stored at -20°C.

C.Concentrating harvested genomic DNA

To create a concentrated solution of harvested planaria genomic DNA, each sample was centrifuged for 2 minutes at 5,000 rpm, 1 minute at 12,000 rpm, and 2 minutes at 13,000 rpm to pellet DNA. Excess Buffer AE was removed, such that only 50 µl of buffer remained in each sample. Pelleted DNA was re-suspended by vortexing until a homogenous mixture was observed.

Agarose Gel Electrophoresis to Detect Apoptotic Induced DNA Fragmentation

A.Preparation of 1X TAE Solution

To prepare 1000 mL of 1X TAE solution from 50X TAE stock solution:

$$C_1V_1=C_2V_2$$
$$(50X\ TAE)(V_1)=(1X\ TAE)(1000\ mL)$$
$$V_1=20\ mL$$

Diluted 20 mL of 50X TAE stock solution in 980 mL of ddH_20 to prepare 1000 mL of 1X TAE solution

B. Preparation of 1% Agarose Gel

To prepare 1% agarose gel 1.04 g of agarose was weighed into a 250 mL conical flask and then 100 mL OF 1X TAE solution was added. The flask and contents were weighed so that evaporated solvent could be replaced following microwaving. The flask and contents then were microwaved for 1 minute and 30 seconds such that agarose was dissolved completely to yield molten agarose. The flask was re-weighed and ddH_20 was added to replace any solvent lost to evaporation. The molten agarose was allowed to cool on bench top for 5 minutes prior to the

addition of 2 µl of ethidium bromide in order to prevent aerosolization of the ethidium bromide. Molten agarose was then poured into a gel casting tray containing comb and allowed to solidify at room temperature.

C. Loading Agarose Gel and Electrophoresis

The comb was removed from solidified gel and agarose gel was then placed into electrophoresis tank such that the wells of the gel were near the cathode. 700 mL of 1X TAE solution was then added to the electrophoresis tank. To increase weight of digests such that the DNA in the digests sunk into wells of the gel, 3.5 µl of 6X loading dye added to each 1.5 mL eppendorf tube containing the planaria genomic DNA. 5 µl of DNA ladder was loaded into the first well of the gel. 15 µl of 1-C (see below for abbreviation key) was loaded into the second well of the gel. 15 µl of 1-TG1 was loaded into the third well of the gel. 15 µl of 1-TG2 was loaded into the fourth well of the gel. 15 µl of 1-TG3 was loaded into the fifth well of the gel. 15 µl of 2-C was loaded into the sixth well of the gel. 15 µl of 2-TG1 was loaded into the seventh well of the gel. 15 µl of 2-TG2 was loaded into the eighth well of the gel. 15 µl of 2-TG3 was loaded into the ninth well of the gel. 15 µl of 2-TG4 was loaded into the tenth well of the gel. The gel was then electrophoresed at 70V for 30 minutes. Following electrophoresis, the gel was removed from electrophoresis tank and placed on a UV transilluminator to visualize DNA fragmentation.

The procedure was repeated using 30 µl of planaria DNA and an increased electrophoresis voltage of 80V and an increased time of 60 minutes. Following electrophoresis, the gel was removed from electrophoresis tank and placed on a UV transilluminator to visualize DNA fragmentation.

Abbreviation Key

DNA Source	Abbreviation
Trial 1, Control Group	1-C
Trial 1, Treatment Group 1	1-TG1
Trial 1, Treatment Group 2	1-TG2
Trial 1, Treatment Group 3	1-TG3
Trial 2, Control Group	2-C
Trial 2, Treatment Group 1	2-TG1
Trial 2, Treatment Group 2	2-TG2
Trial 2, Treatment Group 3	2-TG3
Trial 2, Treatment Group 4	2-TG4

Results

In order to determine if titanium dioxide nanoparticles (TiO_2 NPs) were able to induce genomic damage in planaria, LC_{50} was determined so that treatment groups could be designed, such that damage to genomic DNA could be assessed without causing mortality. Three treatment groups were created, and one control group, each of which had three replicates. Regeneration in *Dugesia dorotocephala* was initiated, and 6 regenerating planarians were placed in each treatment group, and exposed to TiO_2 NPs for 24 hours. Following exposure the TiO_2 NPs, genomic DNA was harvested from all planarians each treatment group. Harvested genomic DNA was run on agarose gel assess damage. Upon UV transillumination of agarose gel, genomic DNA is visible in Lane 2, and DNA of lower molecular weights are visible in Lanes 3, 4,5,7,8,9, and 10 (Figure 1).

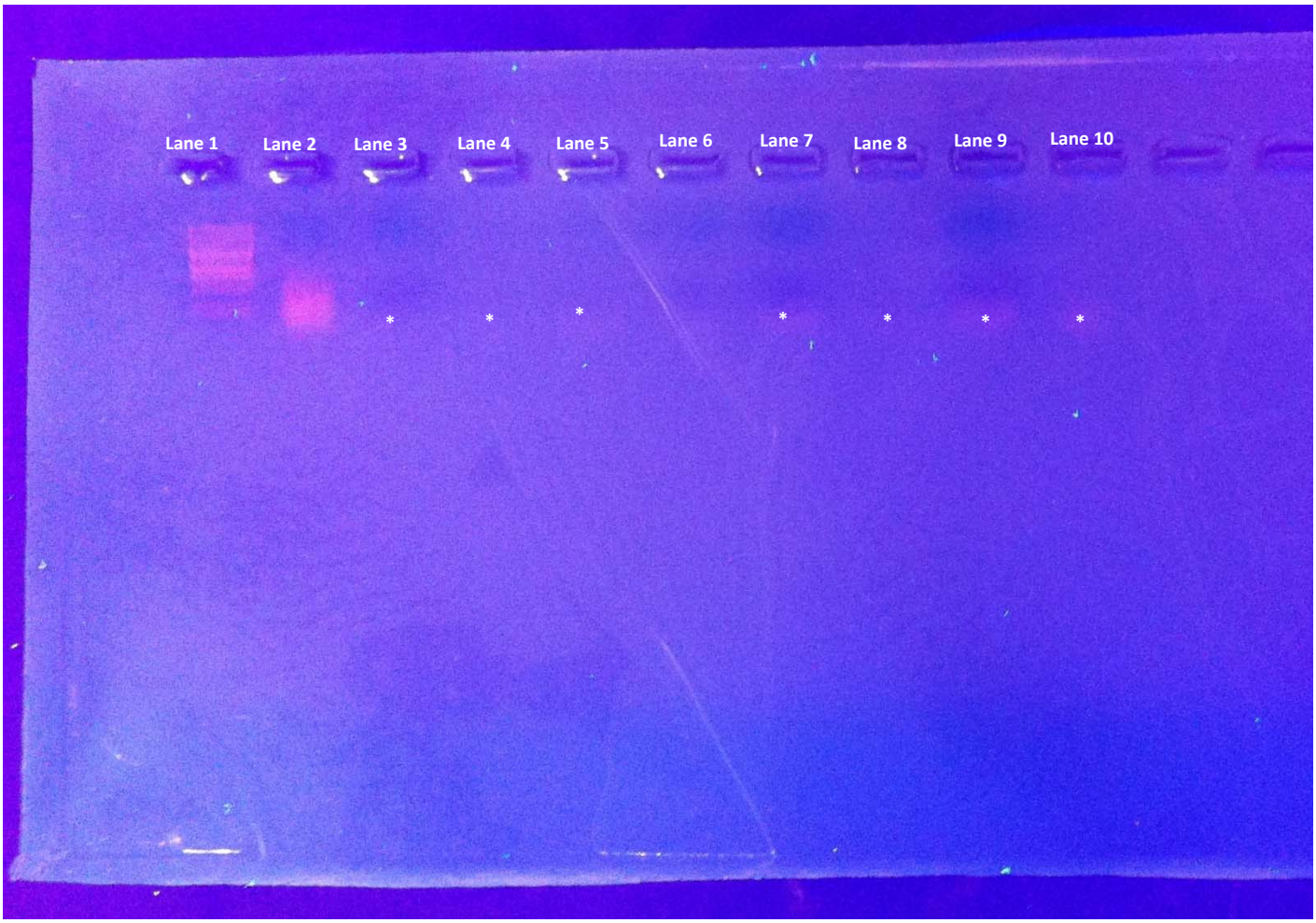


Figure 1. UV transillumination of 1% agarose gel containing composite samples of DNA harvested from regenerating planaria (*Dugesia dorotocephala*) following 24 hour exposure to titanium dioxide nanoparticles (TiO_2 NP). Gel was electrophoresed at 70 V for 30 minutes. Lane 1 contains 1kb DNA ladder. Lane 2 contains composite DNA sample harvested from planaria in the control group of trial 1 (1-C). Lane 3 contains composite DNA sample harvested from planarian in treatment group 1 (20 mL of TiO_2 NP suspension diluted with 4 mL of spring water) of trial 1 (1-TG1). Lane 4 contains composite DNA sample harvested from planaria in treatment group 2 (18 mL of TiO_2 NP suspension diluted with 6 mL of spring water) of trial 1. Lane 5 contains composite DNA harvested from planaria in 1-TG3 (16 mL of TiO_2 NP suspension diluted with 8mL of spring water). Lane 6 contains composite DNA sample harvested from planaria in the control group of trial 2 (2-C). Lane 7 contains composite DNA sample harvested from planaria in treatment group 1 (20 mL of TiO_2 NP suspension diluted with 4 mL of spring water) of trial 2 (2-TG1). Lane 8 contains composite DNA sample harvested from planaria in 2-TG2 (18 mL of TiO_2 NP suspension diluted with 6mL of spring water). Lane 9 contains composite DNA sample harvested from 2-TG3 (16 mL of TiO_2 NP suspension diluted with 8 mL of spring water). Lane 10 contains composite DNA sample harvested from planaria in 2-TG4 (24 mL of TiO_2 NP suspension). Genomic DNA is visible in Lane 2. Genomic DNA is not visible in Lane 6. DNA in Lanes 3,4,5,7,8,9, and 10 (indicated by white asterisk) is smaller in size than the genomic DNA indicating fragmentation.

To increase separation of genomic DNA, and increase visibility of small DNA fragments observed above, a 1% agarose gel was prepared. Genomic DNA is visible in Lane 2 (Figure 2). No DNA was visible in Lanes 2-10 upon UV transillumination (Figure 2).

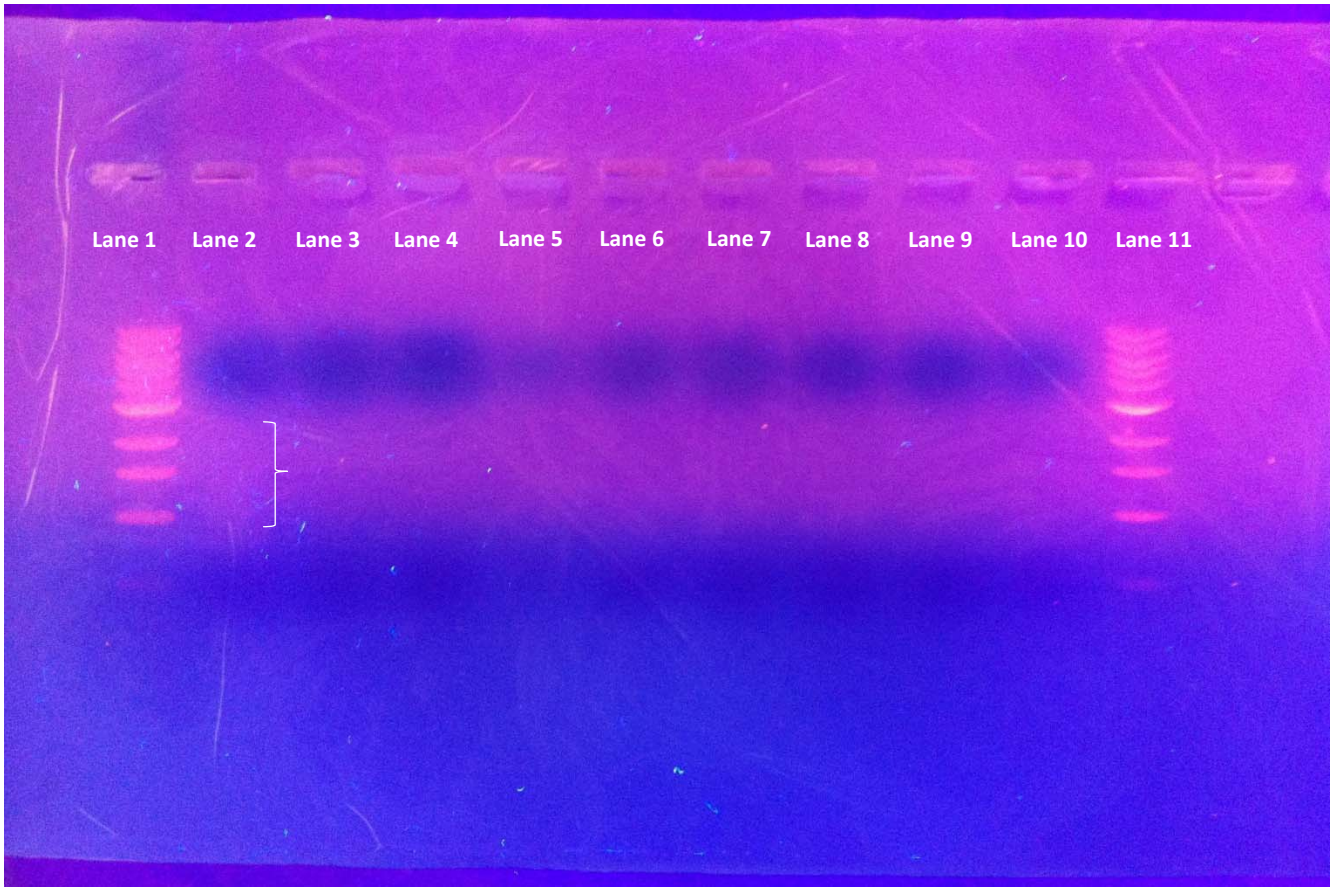


Figure 2. UV transillumination of 1% agarose gel containing composite samples of DNA harvested from regenerating planaria (*Dugesia dorotocephala)* following 24 hour exposure to titanium dioxide nanoparticles (TiO_2 NP). Gel was electrophoresed at 80 V for 60 minutes. Lane 1 contains 1kb DNA ladder (far left). Lane 2 contains composite DNA sample harvested from planaria in the control group of trial 1 (1-C). Lane 3 contains composite DNA sample harvested from planarian in treatment group 1 (20 mL of TiO_2 NP suspension diluted with 4 mL of spring water) of trial 1 (1-TG1). Lane 4 contains composite DNA sample harvested from planaria in treatment group 2 (18 mL of TiO_2 NP suspension diluted with 6 mL of spring water) of trial 1. Lane 5 contains composite DNA harvested from planaria in 1-TG3 (16 mL of TiO_2 NP suspension diluted with 8mL of spring water). Lane 6 contains composite DNA sample harvested from planaria in the control group of trial 2 (2-C). Lane 7 contains composite DNA sample harvested from planaria in treatment group 1 (20 mL of TiO_2 NP suspension diluted with 4 mL of spring water) of trial 2 (2-TG1). Lane 8 contains composite DNA sample harvested from planaria in 2-TG2 (18 mL of TiO_2 NP suspension diluted with 6mL of spring water). Lane 9 contains composite DNA sample harvested from 2-TG3 (16 mL of TiO_2 NP suspension diluted with 8 mL of spring water). Lane 10 contains composite DNA sample harvested from planaria in 2-TG4 (24 mL of TiO_2 NP suspension). Lane 11 contains 1 kb DNA ladder. Genomic DNA is visible in Lane 1 (indicated by white right brace).

All six planarians (*Dugesia tigrina*) in treatment group A did not survive. Treatment group B had a percent mortality of 33%.

Table 1. LC_{50} *Dugesia tigrina*

Treatment Group	Concentration (M) of TiO_2 NPs	No. of live planarians	No. of deceased planarians	Percent Mortality
A	6.339×10^{-2}	0	6	100%
B	6.095×10^{-2}	4	2	33%
C	5.760×10^{-2}	6	0	0%
D	5.560×10^{-2}	6	0	0%
E	5.282×10^{-2}	6	0	0%

All *Dugesia dorotocephala* in treatment groups A,B,C,D and E were alive following 24-hour acute lethality test.

Table 2. LC_{50} *Dugesia dorotocephala*

Treatment Group	Concentration (M) of TiO_2 NPs	No. of live planarians	No. of deceased planarians	Percent Mortality
A	6.339×10^{-2}	6	0	0%
B	6.095×10^{-2}	6	0	0%
C	5.760×10^{-2}	6	0	0%
D	5.560×10^{-2}	6	0	0%
E	5.282×10^{-2}	6	0	0%

Discussion

Dugesia tigrina displayed greater sensitivity to titanium dioxide nanoparticles than *Dugesia dorotocephala.* *Dugesia dorotocephala* appear to be more robust and able to withstand exposure to TiO_2 NPs for a longer period of time than *Dugesia tigrina*.

Examination of regenerating planaria via microscope following 24-hour exposure to TiO_2 NPs revealed that TiO_2 NPs had aggregated within and along the edges of tissues of both *Dugesia tigrina* and *Dugesia dorotocephala* (data not shown). Notably the accumulation of TiO_2 NPs in tissues of *Dugesia tigrina* was greater. Extreme tissue degradation, and a higher incidence of mortality was observed in trial 1. Extreme tissue degradation was not observed in trial 2, nor was a high incidence of mortality. This difference can be attributed to the size difference between the *Dugesia dorotocephala* colonies. The colony of *Dugesia dorotocephala* used in trial 1 were significantly smaller.

DNA of a lower molecular weight is observed is Figure 1 for those lanes containing DNA harvested from planarians exposed to TiO_2 NPs (Lanes 3,4,5,7,8,9, and 10). Genomic DNA harvested from planarians exposed to TiO_2 NPs has a lower molecular weight and hence is smaller than genomic DNA in Lane 1, the control group. This indicates that exposure of planarian genomic DNA to TiO_2 NPs resulted in the apoptotic induced fragmentation of genomic DNA as apoptosis is characterized by the degradation of genomic DNA to oligonucleosomal fragments (Jarvis et al., 1994). NPs induce the activity of caspase 3, DNA fragmentation, reactive oxygen species generation, and oxidative stress (Akhtar et al., 2012).

Contrary to initial expectations, fragmentation of genomic DNA was not visible in the gel that underwent modified electrophoresis (gel run at 80 V for 60 minutes). Increasing separation would, led to DNA fragments becoming undetectable upon UV transillumination as seen in Figure 2. The presence of fragmented DNA is confirmed in Figure 1, thus having utilized the same DNA samples for both agarose electrophoresis gels, this indicates that the DNA fragments have a very low molecular weight, because increasing separation results in the inability to visualize the DNA fragments. This could be confirmed through the use of acrylamide gel electrophoresis to resolve the smaller DNA fragments, as DNA fragmentation yields DNA lengths of less than 180 bp (Huang et al., 1997). Decreased separation would result in the aggregation of small DNA fragments at the same point, allowing ease of visualization (as seen in Figure 1).

DNA fragments of similar molecular weights are observed in Figure 1. Concentration of TiO_2 NPs does not appear to be a factor in the amount of DNA damage induced. Across all treatment groups, in both trials, DNA fragments of similar molecular weights were resolved. This is significant as nanoparticles exhibit increased reactivity due to their increased surface area (Elsaesser and Howard 2012). The increased surface to mass ratio of nanoparticles causes them to be more reactive than normal sized particles of the same compound (Reeves et al. 2008). Moreover due to the decreased radius and increased surface area of nanoparticles, they are able to pass through cell membranes and easily inflict damage within biological systems (Howard 2010). The increased reactivity allows the TiO_2 NPs to rapidly cause damage over a 24-hour period regardless of concentration/ level of exposure.

Trial 1 and 2 utilized separate colonies, indicating that induction of DNA damage is consistent.

The planarians used for trial 2 were larger, seemingly more robust, and exhibited a 0% mortality rate during preliminary acute toxicity testing, whereas the planarians used in trial 1 exhibited 66% mortality rate (data not shown). Upon examination of genomic DNA following exposure to TiO_2 NPs, DNA fragments of similar sizes were observed in DNA harvested from planaria in both trials, reiterating that increased reactivity of TiO_2 NPs were able to rapidly induce genotoxic damage over a 24-hour period in two colonies with different physical characteristics.

Conclusion

TiO_2 NPs induce apoptotic fragmentation of *Dugesia dorotocephala* genomic DNA. The increased reactivity allows the TiO_2 NPs to rapidly cause damage over a 24-hour period regardless of concentration/ level of exposure. TiO_2 NPs were able to rapidly induce genotoxic damage over a 24-hour period in two colonies with different physical characteristics.

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