5-2015

Mitochondrial Dysfunction and Loss of Glutamate Uptake in Primary Astrocytes Exposed to Titanium Dioxide Nanoparticles

Christina L. Wilson
University of Nebraska-Lincoln, cldavis0313@huskers.unl.edu

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By

Christina L. Wilson

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Chemical Engineering

Under the Supervision of Professor Srivatsan Kidambi

Lincoln, Nebraska

May, 2015

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University of Nebraska, 2015

Adviser: Srivatsan Kidambi

The proper function of astrocytes is critical for brain health as they are the most abundant cells in the brain which monitor ion homeostasis, recycle neurotransmitters and respond to tissue damage therefore disruption in astrocyte function can result in overall detrimental effects and has been linked with neurodegenerative diseases. Titanium Dioxide (TiO_2) nanoparticles are currently the second most produced engineered nanomaterial in the world with vast usage in consumer products leading to recurrent human exposure. Animal studies indicate significant nanoparticle accumulation in the brain while cellular toxicity studies demonstrate negative effects on neuronal cell viability and function. However, the toxicological effects of nanoparticles on astrocytes have not been extensively investigated. Therefore, we determined the toxic effect of three different TiO_2 nanoparticles (rutile, anatase and commercially available P25 TiO_2 nanoparticles) on primary rat cortical astrocytes by evaluating events related to astrocytes functions and mitochondrial dysregulation: (1) glutamate uptake and glutamate transporter gene expression; (2) redox signaling mechanisms by measuring ROS production; and (3) the expression patterns of dynamin-related proteins (DRPs) and mitofusins 1 and 2, whose

expression is central to mitochondrial dynamics. Anatase, Rutile and P25 were found to have LC_{50} values of 88.22±10.56ppm, 136.0±31.73ppm and 62.37±9.06ppm respectively indicating nanoparticle specific toxicity. All three TiO_2 nanoparticles induced a significant loss in glutamate uptake and down-regulation of glutamate transporter expression, which is indicative of loss in vital astrocytes function. TiO_2 nanoparticles also induced an increase in reactive oxygen species generation, decrease mitochondrial membrane potential and decrease mitochondrial dehydrogenase activity, suggesting mitochondria damage. TiO_2 nanoparticle exposure altered expression patterns of DRPs at low concentrations (25ppm, 50ppm) and apoptotic fission at high concentrations (100ppm). Collectively, our data provide compelling evidence that TiO_2 nanoparticles exposure has potential implications in astrocytes-mediated neurological dysfunction.

Acknowledgments

None of this would be possible without the kind support of all my friends, family and coworkers and there is not enough paper in the world to thank everyone who has played a part in the completion of this project but I will thank a few people that had very specific roles and beg all others to understand that everything they did means so much to me. I would first thank my husband, Peter, for his love and caring as he shouldered the extra work while I spent many hours and late nights researching and writing. I thank my parents, Chuck and Pam, for their encouragement and prayers over many years and imparting to me a love of learning and good work ethics. I also thank my siblings for their encouraging words and always being available to encourage me over the span of this project. Also a big thanks to Holly Campbell, Katie Jones, Allison Crow and Amanda Godsey for all your encouragement, chocolate and cups of tea You girls always knew just what I needed when I needed it.

I also express my gratitude to the professionals that have helped me along the way. Most of all a big thanks to Dr. Srivatan Kidambi, my mentor, for imparting to me the skills necessary for this project and providing funds, lab space and supplies needed to practice these skills. Thank you for always encouraging me to push beyond perceived limits. I would also thank Dr. William Velander and Dr. Ravi Saraf for being on my committee and providing many discussions full of wisdom and insight. Also, I wish to give thanks to Dr. Oleh Khalimonchuk of the UNL Biochemistry Department for mentoring me on this project and for guidance in exploring cellular mitochondria. I can’t express enough the gratitude I have for my colleagues Dr. Amita Davery, Vaishaali Natarajan and Stephen

Hayward for their many discussions, training sessions and help in order to bring about this project.

Finally I thank all the individuals that allowed me to use their equipment and provided funding for this project. Han Chen and the UNL Center for Biotechnology Microscopy Core Facilities, thank you for assistance in obtaining the TEM images. I also thank Dr. Concetta Dirusso for the use of her fluorescent plate reader. I wish to give thanks to Dr. Yusong Li for the use of the DLS machine. Thank you to Dr. Velander for the use of his colorimetric plate reader. Thank you to the Molecular Mechanism of Disease for honoring me as a fellow and providing me with opportunities to present my work and receive valuable feedback at a national conference for development of this and other projects. Finally a huge thanks to the University of Nebraska-Lincoln for awarding me the Othmer Fellowship and the Milton Mohr Fellowship and supporting my work with opportunities to present and receive feedback from researchers across the university.

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List of Abbreviations

Adenosine Triphosphate (ATP)

Blood Brain Barrier (BBB)

Dulbecco's Modified Eagle Medium (DMEM)

Dynamic Light Scattering (DLS)

Dynamin Related Proteins (DRPs)

Enhanced Green Fluorescent Protein (eGFP)

Glial Fibrillary Acidic Protein (GFAP)

Glutamate Aspartate Transporter (GLAST)

Institutional Animal Care and Use Committee (IACUC)

Microtubule-associated protein 2 (MAP-2)

Mitochondrial Membrane Potential (MMP)

Mitofusin (Mfn)

Oxygen (O)

Reactive Oxygen Species (ROS)

Titanium (Ti)

Titanium Dioxide (TiO_2)

Transmission Electron Spectroscopy (TEM)

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1 Chapter 1 Introduction

1.1 Overview of Astrocytes in the Central Nervous system

The brain is composed of two main cell types; neuron and glial cells. The most studied of the brain cells are neurons because of their known importance to organism function through signal transduction in the central and peripheral nervous systems. However, most of the tissue is made up of a variety of glial cells (microglia, oligodendrocytes, and astrocyte). Astrocytes, also known as astroglia, are the most abundant glial cell named so due to their distinct stellate-shape. They are found throughout the central nervous system stretching into every region to contact all brain and blood vessel cells. Originally named "brain glue" and assumed to only provide for tissue structural integrity, much research over the past few decades has revealed a number of vital roles in brain heath and dysfunction. Proper intercellular communications between astrocytes and neurons is vital to prevent brain dysfunction and imminent neurodegeneration.[1]

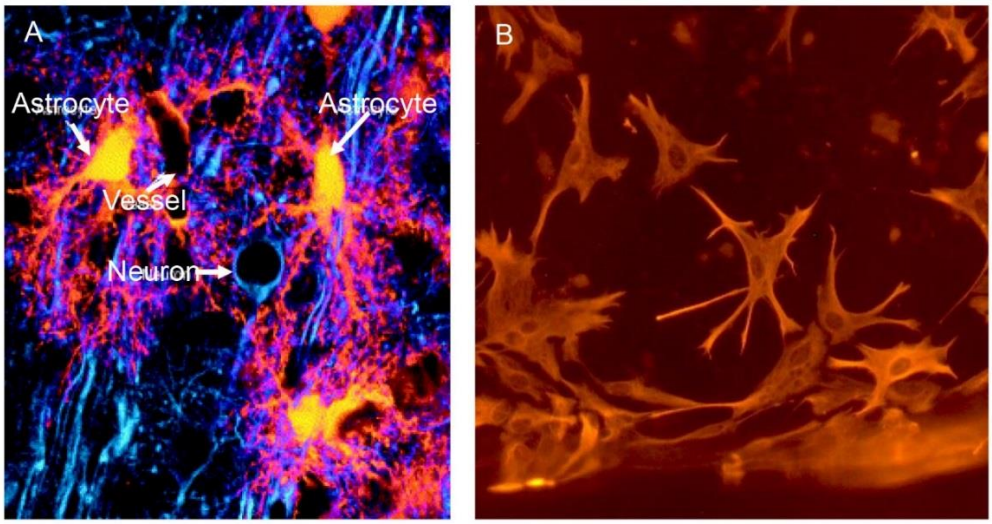


Figure 1: Astrocytes in vivo and in vitro. *(A) Image by Wang and Nedergaard showing the complex spatial organization of brain tissue with neurons (MAP-2; blue) and a blood vessels contacted by astrocytes (eGFP; yellow) stretching out into all parts of the tissue to collect signals and correct deviation from tissue homeostasis.[2] Hippocampal astrocytes in monoculture (GFAP; red) with their distinct star-like shape.*

Recent studies have observed that astrocytes perform many vital roles in synaptic formation/maturation, ion homeostasis and energy production. Studies in animal and *in vitro* models strongly suggest that astroglia provide important chemical compounds useful in the formation, maturation and turnover of neuronal synapses through a number of mechanisms including their superior efficiency of cholesterol metabolism, provision of growth factors and axon pruning.[3] Astrocytes regulate extracellular ions through a series of ion channels, hemichannels and receptors which trigger mechanisms of uptake and/or blood brain barrier (BBB) manipulation. This assists in the passage of ions out or osmosis of water into the brain through the BBB for the purpose of restoring ideal ionic conditions for neuronal function.[4] Manipulation of the BBB also allows astrocytes to encourage transport of nutrients, especially oxygen and glucose, to provide for brain energy production and consumption. This is a significant task as the brain consumes 20% of the

total blood glucose and oxygen in support of cerebral activity. Astrocytes also metabolize key substrates recognized as alternative sources of energy, such as glycogen and lactate, with greater efficiency than neurons thereby providing for their high energy demands.[5] These roles are highly regulated by the active signaling of neurons and neuronal activity, which makes the understanding of astrocyte function and dysfunction an exciting avenue of study in regard to neurological disease and prospective therapies.

1.2 Overview of Titanium Dioxide Use and Toxicity Studies

Titanium dioxide is a naturally occurring complex of titanium which for decades has been collected, synthesized and utilized in a variety of industrial and commercial applications. The most common forms of TiO_2 utilized in industry are anatase and rutile, each having unique atomic and crystalline form (**Fig. 2**) providing them with different physicochemical properties. Therefore, each has strengths for a variety of applications. Overall, both forms are well known for desirable properties such as good fatigue strength, thermal stability, resistance to corrosion, as well as excellent optical, catalytic and semiconductor properties.[6] Due to its high refractive index, TiO_2 has been used to provide white color in a variety of products such as paint, sunscreen, toothpaste, pharmaceuticals and a number of food items.[7] Other industries have recognized excellent catalytic properties of TiO_2 which has been useful for self-cleaning thin films, water remediation and energy production.[6]

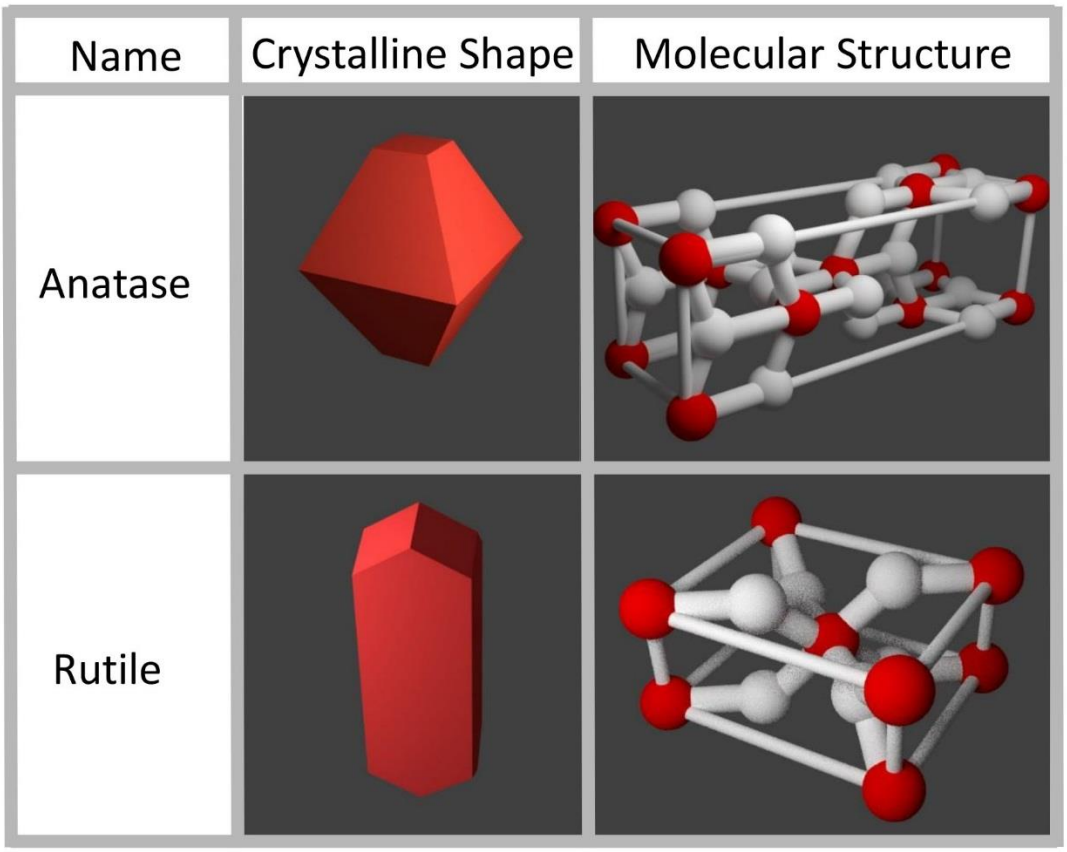


Figure 2: Nanoparticle crystal structure and atomic structure models. *This schematic shows the crystalline shape and atomic structure of the two most common forms of TiO_2 (structures assembled in Blender, adapted from Barnard et al[8] and Bourikas et al[9], red and white balls represent titanium and oxygen atoms, respectively.) The desirable physicochemical properties and toxicological profiles are strongly dependent on nanoparticle crystalline form.*

Early application of TiO_2 utilized "fine" particles, a particle with diameter of 0.1-3um in size, which maintained all the desirable properties of the bulk material and several studies showed these particles to be biocompatible with human and animal systems, even to the point of utilizing fine TiO_2 particles as a negative control in toxicity studies.[10] As technological advances allowed for easier and less expensive manufacture of nanomaterials, materials with at least one dimension <100nm, manufacturers began to substitute "fine" particles for "ultrafine" or "nanoparticles" due to enhancement of desirable properties resulting from the increased surface area of the smaller particles. As

of 2012, a study showed that TiO_2 nanoparticles were the second most produced engineered nanomaterial in the world.[11] In 2013 the project on emerging nanotechnologies consumer products inventory listed 1628 products or product lines, 10% of these products utilized titanium nanotechnology.[12] The increased utilization, thereby increased environmental exposure and combined with the switch to the use of smaller particle sizes, the question has been raised concerning whether particle size affects toxicity of nanoparticles. This study explores the potential toxicity in organisms receiving TiO_2 nanoparticle exposure from their environment. Therefore a thorough understanding of nanoparticle exposure and current toxicity knowledge was necessary.

Several reviews have been written in recent years highlighting important studies regarding TiO_2 nanoparticle toxicity in animal models through the common routes of exposure: Ingestion, absorption, inhalation, and injection. [13, 14] In rainbow trout exposed to TiO_2 in their diet (0-100mg/kg) for 8 weeks, Ramden and coworkers observed Ti accumulation in the gill, gut, liver, brain and spleen causing some biochemical disturbances, such as changes in Cu and Zn and Na+ levels, and showing low clearance from tissues such as the brain, liver, intestines and gills.[15] Similarly, Wang and co-workers demonstrated that a single oral administration of TiO_2 nanoparticles in rats (5g/kg) resulted in nanoparticle distribution and accumulation in various tissues including the stomach, liver, kidneys, and brain.[16] In hairless mice receiving topical treatment TiO_2 nanoparticle treatment Ti was found to accumulate mostly in the muscle, liver, lung, spleen and heart with a small but significant amount of Degussa P25 crossing the blood brain barrier (BBB) to accumulate in the brain, resulting in tissue damage.[17] A multispecies study showed that mice, rats and hamsters showed pulmonary inflammation and tissue lesions after chronic

inhalation of high concentrations of TiO_2 with the most drastic results shown in mice, then rats, and then hamsters.[18] Interestingly, damage and accumulation were noted in mice brains after nasal inhalation of TiO_2 nanoparticles suggesting particles may not require access through the BBB but may be transported through nasal passages into each of the major brain regions.[19] Finally, 28 days after intravenous exposure to a single acutely non-toxic dose of (5mg/kg) a significant accumulation of nanoparticles was observed in the liver and spleen. The low dose did not result in the increase of blood cytokines or tissue damage.[20] Overall, animal models show that regardless of entry route TiO_2 can accumulate in many major organs, including the brain, in most cases resulting in tissue damage and biochemical disruption. However the complexity of the *in vivo* model limits the biochemical knowledge regarding tissue specific disturbances. Therefore, we turn to *in vitro* models for more specific focus on the reaction of individual cell types in contact with TiO_2 nanoparticles.

Several reviews have also been written regarding toxicity results in cell culture models with exposure to TiO_2 nanoparticles. Here I will summarize some of the major results and common themes noted among different cell types associated with tissues previously noted to be affected by nanoparticle accumulation.[21] [22] A majority of *in vitro* studies have focused on epithelial cells especially associated with skin, lung and intestine since those incorporate the common routes of entry for TiO_2 nanoparticles. In human lung cancer cells derived from the endothelium (A549 cells) anatase TiO_2 nanoparticle exposure was observed to result in concentration dependent decreased proliferation, DNA damage, decreased mitochondrial membrane potential (MMP) and increased expression of apoptosis markers Caspase 3 and 9.[23] Human pulmonary epithelial cell line NCI-H292

were utilized to show that Toll like receptor 4, an innate immune response to foreign material, plays a key role in the uptake of P25 TiO_2 nanoparticles and subsequent inflammatory response.[24] This observation reveals that innate receptors can increase sensitivity to TiO_2 nanoparticles contributing to the cell type specific variability in response to nanoparticle presence. Study in keratinocytes, HaCaT cells, show uptake of anatase and rutile particles into the cells cytoplasm with anatase nanoparticles observed in the nucleus and extensive mitochondrial damage accompanied with increased reactive oxygen species (ROS) production.[25] A few studies have observed the cytotoxicity of TiO_2 nanoparticles to liver cells, the liver being a vital organ for detoxification of the blood and a major site of nanoparticle accumulation, and observed that in 24 hours of exposure TiO_2 nanoparticles are not cytotoxic even at high concentrations of 250 μg/ml in BRL3A rat liver cells.[26] However a study in L-02 human hepatocytes exposed to low levels of TiO_2 nanoparticles (1μg/ml) after 24 hours resulted in increased oxidative stress but not chromosome damage or apoptosis[27] suggesting that a stress response is triggered in the cells at concentrations below the cytotoxic or genotoxic level. In mouse fibroblasts, a common cell type which synthesizes extracellular matrix, nanoparticle internalization and generation of ROS was observed in low concentrations (0-30μg/ml) while significant cell death was observed only in response to high concentration (300 and 600μg/ml).[28] The various responses and sensitivities to TiO_2 is to be expected since cells perform different roles in tissues, making some cell types more sensitive to alterations in the cellular environment than others. It is therefore important to study the reaction of as many cell types as possible especially in tissues which are highly sensitive to injury such as the brain.

2 Chapter 2 Nanoparticle Characterization in Culture Media

2.1 Introduction

Nanotoxicologists agree that nanomaterial toxicity is strongly dependent on the intrinsic and aggregating properties of nanomaterials therefore, for the sake of consistency and reproducibility of results, a basic nanoparticle characterization is an important part of any nanotoxicity study.[29] In the beginning of this emerging field, lack of uniformity in term definitions, nanoparticle characterization, and treatment methods made it difficult to compare results, leading to disagreements regarding the safety of nanomaterial usage. However, in the early 2000's several leaders in the field established reviews to define terms and establish methods bringing standardization to the field.[29, 30] Along with the benchmarks they established, these researchers emphasized the importance of physicochemical properties, especially size, shape and structure, on nanoparticle toxicity and therefore strongly advised that researchers provide physicochemical properties of nanomaterials. Some of these properties may be conveyed by the manufacturer, contingent on whether the material was purchased or made in house. However characterization of nanoparticles under testing conditions, such as aggregation in administration solution, should be performed by the research and can be helpful for retrospective interpretation of data.

In this study we characterized three commercially available TiO_2 nanoparticles (Anatase, Rutile and P25) by transmission electron microscopy (TEM) and dynamic light scattering (DLS) in order to provide physicochemical characterization and to compare our nanoparticles to previously published work for the nanoparticles we would utilize in future toxicity studies. TEM was performed to observe the particles for nanoparticle shape on a

per particle basis. DLS was utilized to report the aggregate size and zeta potential (aggregate state) in physiologically relevant serum-free (neuron media) and serum containing (astrocyte media) cell culture medium.

2.2 Materials and Methods

2.2.1 Preparation of Nanoparticle Suspensions

The nanoparticles utilized for this study were Degussa P25 [Sigma Aldrich, St. Louis, MO], Anatase, or Rutile [Mk Nano, Mississauga, Ontario, Canada] TiO_2 nanoparticles. Prior to suspension all nanoparticles were sterilized by UV treatment overnight. Stock suspensions of 1000ppm were made in autoclaved 1X PBS by sonication [FS30D Fisher Scientific] for 30minutes and stored at 4°C. For experiments, the stock suspensions were diluted in culture media and sonicated for 30minutes.

2.2.2 Transmission Electron Microscopy

Nanoparticles were suspended in deionized water, deposited on carbon coated copper grids [Ted Pella, Redding, CA] and visualized with a Hitachi H7500 Transmission Electron Microscope.

2.2.3 Culture Media Preparation

Culture media was prepared as for cell culture studies. In short, for astrocyte culture media, DMEM [MP Biomedicals], 10% Heat Inactivated Horse Serum [PAA Lab], 1% HEPES [Media Tech], 1% penicillin-streptomycin [Life Technologies], and 1% L-Glutamine [Life Technologies] were combined, pH adjusted to 7.4 with 1M HCl or NaOH, and filter sterilized with a 0.22μm filter [Thermo Scientific]. For neuron culture media, Neurobasal media [Life Tech] with 2% B27-Supplement [Invitrogen], 1% 200mM L-Glutamine, 0.0055mg/ml glucose [Sigma-Aldrich] and 1 % penicillin-streptomycin was

combined, pH adjusted to 7.4 with 1M HCl or NaOH, and filter sterilize with a 0.22μm filter. These were stored at 4°C until needed for measurements.

2.2.3 Mean Effective Diameter and Zeta Potential

ZetaPALS [Brookhaven instrument, Holtsville, NY] was utilized to determine nanoparticle effective diameter and Zeta potential [PALS Zeta Potential Analyzer, Smoluchowski model] at the treatment concentrations of 25ppm, 50ppm and 100ppm. Nanoparticle solutions were made with culture media at pH 7.4 as outlined for cell culture experiments. All size measurements were performed at 25°C, at a scattering angle of 90° and analyzed via intensity and volumetric distribution.

2.3 Results and Discussion

2.3.1 Nanoparticle Crystalline Structure

TEM was employed to characterize the individual crystal shapes and sizes of the TiO_2 nanoparticles (**Fig 3**) in the absence of solution based interactions for a per particle analysis. Anatase TiO_2 nanoparticles were observed to be the characteristic spherical crystal structure while rutile nanoparticles had typical rod-like crystal structure as has been observed in previous studies.[31, 32] The Anatase and Rutile TiO_2 nanoparticles utilized for this study were both reported as 50nm in diameter by the manufacturer, thus allowing for observations of crystal structure influence on nanoparticle toxicity independent of particle size. P25 nanoparticles are the third variation of TiO_2 nanoparticles chosen for this study due to their popular use commercially, industrially and in toxicity studies. P25, a 3:1 mixture of anatase and rutile, has structural characteristics of both anatase and rutile and was reported to be 21nm in diameter. Our images had similar shape as previously reported P25 nanoparticles.[33]

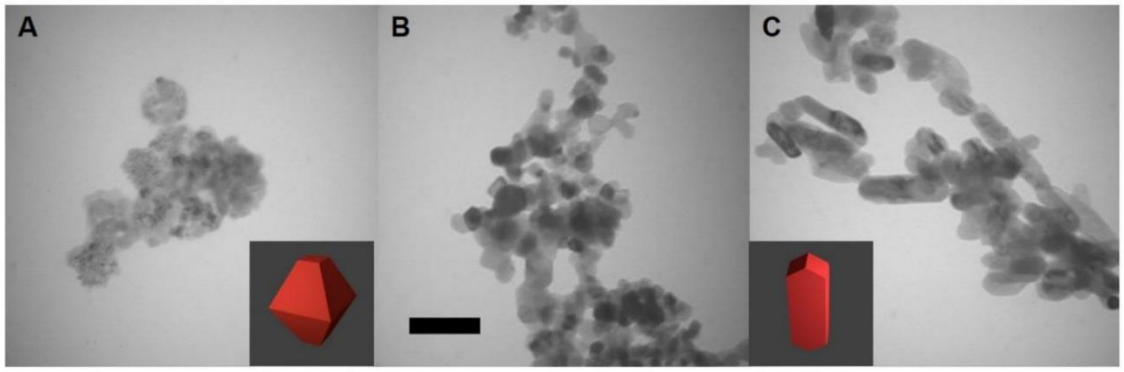


Figure 3: Nanoparticle characterization images of per particle form. *TEM images of Anatase (A), P25 (B), and Rutile (C) TiO_2 nanoparticles. Scale bar = 100 nm*

2.3.2 Nanoparticle Dependent Aggregation Profile in Physiologically Relevant Media

In addition to characterizing the TiO_2 particles in a per particle basis, in the absence of solution interactions, we then performed DLS and Zeta potential analysis to uncover the solution dependent behavior of the particles. Previous reports have shown that TiO_2 nanoparticles agglomerate in cell culture media.[34] To compare the level of agglomeration of the TiO_2 nanoparticles in physiologically relevant conditions, in the presence of proteins and divalent ions, we studied the agglomeration of the TiO_2 nanoparticles in astrocyte and neuron culture medium. The nanoparticle suspensions were characterized using DLS for hydrodynamic diameter of the aggregates and zeta potential to discern nanoparticle charge and colloidal stability. As shown in **Table. 1**, at a concentration of 50ppm in astrocyte culture media P25, anatase and rutile nanoparticles aggregated to average diameter of approximately 370nm, 550nm, and 360nm, respectively and at a concentration of 50ppm in neuron culture media the average diameter was approximately 380nm, 530nm, and 340nm respectively. We observed that the average hydrodynamic diameter depended on crystalline form, but not on concentration, indicating that the relative concentrations would

be reliable for our study and that the cells treated with serum vs serum free media would be interacting with similar nanoparticle aggregates. It was observed that the type of crystal structure of the particles (anatase vs rutile) and the relative composition of the three forms of nanoparticles have a greater influence over aggregation size than the media composition. This is similar to other studies where it was observed that nanoparticles aggregate to diameters in the range of 300-600nm in media containing serum and it has also been previously reported to have a nanoparticle form dependence.[31, 32, 35] However, our results show that the serum-free media, caused a similar aggregation to that of the serum media, while literature varies greatly on the aggregate diameter in serum free conditions.[31, 36] According to the manufacturer the specifics of the B-27 supplement added to the neuron media is confidential but they disclose that it contains some proteins, including BSA, which has been speculated to play a role in particle aggregation in previous studies[37], therefore our "serum free" media is different than the previously referenced "serum free" solutions studied which were protein free.

Zeta potentials were also measured for the three TiO_2 nanoparticles (**Table. 1).** The zeta potential values did not significantly change depending on forms of the nanoparticles, concentration, or medium. In all cases the weakly negative net charge of the nanoparticles highlighted their inherent colloidal instability driven by favorable aggregation forces. Our observed zeta potential results, in both mediums, were similar to previous studies in serum containing media[32, 35] and very different from the serum free study[36] which observed a strongly positive zeta potential. Our characterization provides us with valuable information about the intricate characteristics of the nanoparticles, including size, charge and driving

forces for aggregation, that provide valuable insight for data analysis and comparison to previous studies.

2.4 Conclusion

We conclude from our observations that the nanoparticles we purchased are similar to TiO_2 (P25, anatase and rutile) nanoparticles utilized in previous toxicity studies. These nanoparticles have similar structure as visualized with TEM and similar aggregation states in protein containing, physiologically relevant cell culture media as observed with DLS as has been observed in previously published toxicity studies.

Table 1: Nanoparticle aggregate characterization in physiologically relevant cell culture media. *Particle size provided by manufacturer indicated in methods. Hydrodynamic diameter, zeta potential, and conductance at three concentrations (25ppm, 50ppm and 100ppm) measured in serum containing (Astrocyte) and serum free (Neuron) culture media. Data are expressed as the mean ± standard deviation of two independent samples.*

Nanoparticle	Size		Astrocyte Culture Media			Neuron Culture Media		
	(nm)	ppm	Hydrodynamic Diameter (nm)	Zeta potential (mV)	Conductance (µS)	Hydrodynamic Diameter (nm)	Zeta potential (mV)	Conductance (µS)
P25 (70% Anatase & 30% rutile)	~ 21	25	374.3 ± 26.4	-10.7 ± 4.6	22247	375.5 ± 19.5	-8.6 ± 4.0	13058
		50	367.6 ± 15.3	-8.5 ± 4.9	21753	380.1 ± 18.5	-10.2 ± 3.9	15128
		100	337.9 ± 14.7	-9.2 ± 4.3	21479	404.5 ± 27.9	-10.6 ± 3.9	15034
Rutile	~ 50	25	366.9 ± 15.0	-11.4 ± 6.2	19607	326.0 ± 16.1	-9.1 ± 4.0	14682
		50	363.5 ± 19.2	-12.1 ± 3.6	23040	340.8 ± 22.3	-10.5 ± 1.4	14680
		100	362.4 ± 18.4	-8.1 ± 3.4	19834	448.7 ± 27.6	-9.7 ± 2.6	14175
Anatase	~ 50	25	541.2 ± 51.0	-10.4 ± 5.0	20032	492.1 ± 22.8	-6.4 ± 1.1	12218
		50	555.9 ± 41.3	-9.4 ± 4.1	22269	532.8 ± 37.3	-7.2 ± 1.7	11301
		100	545.3 ± 47.8	-10.8 ± 3.2	19831	556.4 ± 34.7	-9.7 ± 2.8	14488

*Composition includes 10% Heat inactivated horse serum, 1 % 1 M hepes buffer, 1 % L-glutamine, and 1% penicillin-streptomycin in DMEM.
** Composition includes 2% B27-Supplement, 1% 200mM L-Glutamine, 0.0055 mg/ml glucose and 1 % penicillin-streptomycin in Neurobasal Media

3 Chapter 3 Toxicity and Functional Impairment in Primary Astrocytes Cultured With Titanium Dioxide Nanoparticles

3.1 Introduction

Titanium Dioxide (TiO_2) nanoparticles are the second most produced engineered nanomaterial in the world with a vast majority utilized for cosmetics, including sunscreen, and consumer products.[11, 38] The unique properties of TiO_2 nanoparticles have also proven useful in applications of air and water purification and energy storage[7], providing increased opportunities for commercial and industrial exposure to these nanoparticles. This increased human and environmental contact with TiO_2 nanoparticles has led to an intense scrutiny of its biocompatibility resulting in many studies, suggesting a need for concern.[13, 39]

Animal and cell studies indicate the possibility of harmful effects resulting from human exposure to TiO_2 nanoparticles. Wang and co-workers demonstrated that oral administration of TiO_2 nanoparticles in rats resulted in nanoparticle distribution and accumulation in various tissues including the brain.[16] Intranasal exposure of TiO_2 nanoparticles also demonstrated high accumulation of the nanoparticles in different regions of the brain resulting in the increase of Glial Fibrillary Acidic Protein (GFAP) positive cells, oxidative stress, and brain tissue damage.[19]Wu and co-workers exposed TiO_2 nanoparticles to PC12 cell lines and observed decreased cell viability, generation of reactive oxygen species (ROS), loss of mitochondrial membrane potential (MMP) and increased expression of biomarkers associated with apoptosis/cell death.[32] Long *et al.* demonstrated a cell-dependent effect due to exposure to TiO_2 nanoparticles wherein BV2

microglia cell lines had a faster decrease in viability compared to N27 neuronal cell lines suggesting that microglia are more susceptible to TiO_2 nanoparticles.[40] Together, these *in vitro* and *in vivo* studies indicate that exposure to TiO_2 nanoparticles results in a certain degree of toxicity. However, the majority of previous studies have focused on neurons and neuronal cell lines leaving a significant gap of knowledge regarding toxicity mechanisms in other brain cells including astrocytes.

Astrocytes are distinct stellate-shaped brain cells found throughout the central nervous system that perform many vital roles for proper brain function. Recent studies demonstrated that astrocytes receive signals from neurons, actively respond to neuronal activity with cytosolic calcium elevations evoked by neurotransmitters, such as glutamate, gamma-aminobutyric acid (GABA), norepinephrine, dopamine, acetylcholine, serotonin, adenosine triphosphate (ATP), and nitric oxide[41] and are the primary cell type for some neurotransmitter recycling. For instance, glutamate, an essential excitatory neurotransmitter utilized for neuronal signal transduction, is an especially important task of astrocytes as neurons lack an essential enzyme for net conversion of glutamate from glucose and therefore rely on astrocyte metabolism to provide sufficient quantities for function. Furthermore, neurons cannot efficiently clear glutamate from the synaptic cleft after a signal transduction event and therefore rely on astrocyte clearance for the prevention of excitotoxicity. Through glutamate recycling astrocytes also play into the overall brain energy production as glutamate enters and exits the citric acid cycle by conversion to α-ketoglutarate thereby producing adenosine triphosphate (ATP), the major biomolecule which supplies energy for all cellular processes.[42] Another interesting role is their response to and eventual restoration of homeostasis after tissue injury.[43]Hence, astrocytes might

possess the key to understanding the underlying mechanisms that mediate brain injury due to exposure to TiO_2 nanoparticles. Therefore, it is important to understand the mechanisms of toxicity in astrocytes as it affects their ability to function.

One mechanism that has been observed in numerous previous *in vivo* and *in vitro* studies is the increase in intercellular ROS and oxidative stress after treatment with TiO_2. Oxidative stress occurs when intracellular levels of ROS exceed the innate antioxidant actions of the cell. This can result in oxidative damage to proteins, lipids and DNA and has been implicated in a number of neurodegenerative diseases.[44] In this study we sought to assess the impact of low, physiologically relevant concentrations of TiO_2 nanoparticles on cellular activity of primary cortical astrocytes. We utilized three commercially employed TiO_2 nanoparticles (P25, Anatase, and Rutile), to investigate nanoparticle specific perturbation in an explicit range of concentrations mimicking TiO_2 nanoparticle accumulation. Additionally, we evaluated the effect of TiO_2 nanoparticles exposure on ROS production, a strong indicator of perturbations in normal function. Our findings demonstrate toxic effects of TiO_2 nanoparticles on cellular function and health in primary astrocytes.

3.2 Materials and Methods

3.2.1 Isolation, Seeding and Treatment of Primary Neurons and Astrocytes

Primary Cortical Neurons and Astrocytes were prepared from 1-3day-old Sprague-Dawley rat pups [Charles River, Sulzfeld, Germany] in compliance with UNL's IACUC protocol 1046 and according to protocol[45, 46] with slight modifications. In short, the tissue was digested with 0.025% Trypsin [Life Technologies] and 0.0016% DNase [Roche] which was quenched by Astrocyte culture media (DMEM [MP Biomedicals], 10% Heat

Inactivated Horse Serum [PAA Lab], 1% HEPES [Media Tech], 1% penicillin-streptomycin [Life Technologies], 1% L-Glutamine [Life Technologies]). The inactive trypsin was removed by centrifugation at 1700rpm for 5minutes, then cells were suspended in plating media and gently triturated with glass pipette. The cells were then passed through a 70μm cell filter, centrifuged, suspended in neuron culture media (Neurobasal media [Life Tech] with 2% B27-Supplement [Invitrogen], 1% 200mM L-Glutamine, 0.0055 mg/ml glucose [Sigma-Aldrich] and 1 % penicillin-streptomycin) or Astrocyte culture media and counted by trypan blue stain with a hemocytometer. Neurons were seeded on poly-L-Lysine coated tissue culture plates at a cell density of 2,000 cells per cm^2 in Neuron culture media and Astrocytes were seeded on poly-L-lysine coated tissue culture plates at a cell density of 500 cells per cm^2. The first media change was performed on day in vitro (DIV) two in both cell types and every four days following until experiments were performed. Neurons received half media changes per well in order to preserve the sensitive neurite network and astrocytes received full media changes with vigorous shaking to remove other glial cell types. Neurons were utilized on DIV 10-12 after extensive neurite networks had been established. Astrocytes were utilized on DIV 3-5 after obtaining 70% confluence.

3.2.2 Lethal Concentration Assay

The concentration lethal to 50% of astrocytes (LC_{50}) was determined utilizing the MTT [3-(4,5-dimethyldiazol-2-yl)2,5 diphenyl Tetrazolium Bromide] [Life Technologies] Assay. It is a colorimetric assay that evaluates the mitochondrial conversion of the MTT salt. In short, after 24hours of nanoparticle exposure the culture media was aspirated and 5mg/ml MTT working solution in DMEM incubated on live cells at 37°C for 3hours. Lysis buffer (0.1N HCl in Isopropanol) was added in a 5:1, lysis buffer to MTT solution, ratio.

The absorbance values were read at 570/630nm in an AD340 plate reader [Beckman Coulter, Indianapolis, IN].

3.2.3 Cellular Morphology with Phase Contrast Imaging

Morphology of live cells were assessed using an Axiovert 40 CFL [Zeiss, Germany] and images taken with a Progres C3 [Jenoptick] camera.

3.2.4 Gene Expression

Total RNA was isolated using Trizol [Life Technologies] according to the manufacturer's instructions. The quality and quantity were determined by ND-1000 spectrophotometer [NanoDrop Technologies Wilmington, DE] and reverse transcribed using iSciptTM cDNA synthesis kit [Bio-Rad Laboratories] by manufacturer's instructions.

Quantitative Real Time PCR was performed using SYBR Green Master Mix [Applied Biosystems, Foster City, CA] in an epgradient S Mastercycler [Eppendorf]. The PCR program was set to the following: 10minutes at 95°C, 40-60 cycles of 95°C for 15seconds followed by annealing at 60°C for 15seconds and elongation at 72°C for 60seconds, and finally 95°C for 15seconds to end. The primers of interest obtained from Integrated NDA Technologies [Coralville, IA] with the following sequences: GLAST (Forward 5'-CTACTCACCGTCAGCGCTGT-3' and Reverse 5'-AGCACAAATCTGGTGATGCG-3'). GAPDH (Forward 5' ATG ATT CTA CCC ACG GCA AG 3' and Reverse 5' CTG GAA GAT GGT GAT GGG TT 3') was measured as reference. The ΔΔCT method was utilized for analysis of each sample. Results reported as normalized to the average expression of untreated cells.

3.2.5 Glutamate Uptake Assay

The uptake of [3H] glutamic acid was used to determine change in glutamate uptake experienced by TiO_2 nanoparticle treated astrocytes. The treatment media was removed and replaced by serum free high glucose DMEM containing 50μM glutamate and 18.5kBq of [3H] glutamic acid [Perkin Elmer]. Uptake was terminated after 15minutes by removal of working solution. Cells were washed twice with ice-cold PBS. Cells were then lysed in 10mM NaOH containing 0.1% Triton X-100 and 300μl of lysate was assayed for 3H by liquid scintillation counting. The protein content was assayed using Bradford assay. Results were reported as CPM/ μg protein.

3.2.6 Reactive Oxygen Species Generation

CM-H2DCFDA is a fluorescent indicator activated by the presence of ROS. The culture media was aspirated and the cells washed with warm 1X PBS. 10μM CM-H2DCFDA [Life Technologies] in DMEM was added to each well and incubated at 37°C for 30minutes. Cells were washed 3 times with PBS followed by fluorobrite media [Life Technologies] added to each well. The bottom of the well was read at excitation 495nm and emission 529nm with a SLFA plate reader [Biotek, Winooski, VT]. The results were reported as a fraction of the average untreated intensity.

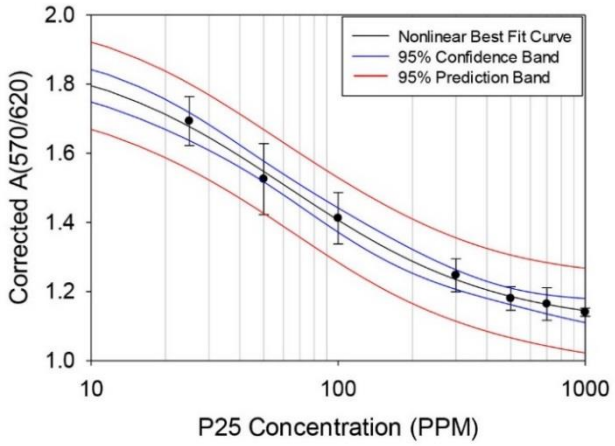
3.2.7 Statistical Analysis

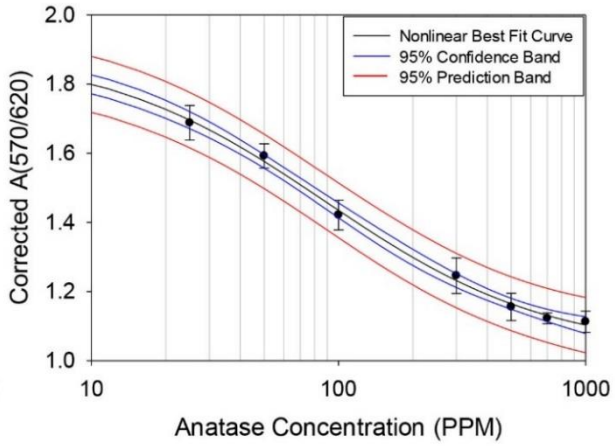
All data is presented as the mean ± the standard deviation. Statistical comparisons between treatments utilized Sigma Plot ANOVA (Dunnett's Method) for analysis. Pool size was as indicated in figure description.

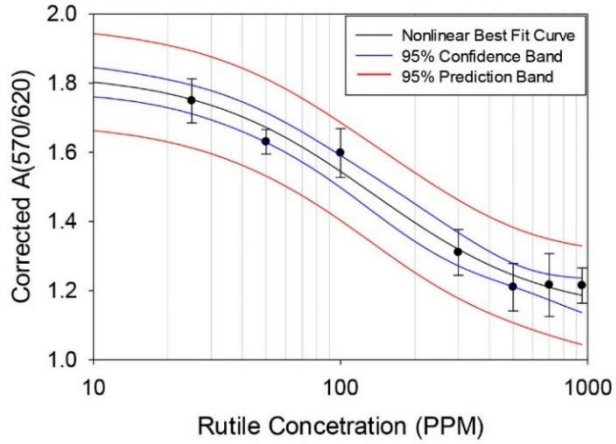
3.3 Results and Discussion

3.3.1 Determining Lethal Concentration in Primary Astrocytes

We utilized the lethal concentration assay at 24hours (hr) TiO_2 nanoparticle exposure, to determine concentrations of acute toxicity to primary rat cortical astrocytes. As seen in **Figure 4**, the LC_{50} values of P25, anatase and rutile TiO_2 nanoparticles were 62.37±9.06ppm, 88.22±10.56ppm, and 136.0±31.73ppm, respectively. Our results are in agreement with other studies indicating that anatase crystalline phase is more toxic than rutile.[32] This result provided us with values of acute toxicity that were useful in determining concentrations for further studying mechanisms of toxicity. Therefore, all subsequent studies were carried out using three different concentrations of TiO_2 nanoparticles (25ppm, 50ppm, and 100ppm) with 24hr exposure that is reflective of the LC_{50} data. These concentrations are in a relevant sub-acute toxic range compared to previous studies that have been carried out using higher concentrations to study the effect of TiO_2 nanoparticle exposure on neuronal cells.[21] Previous reports have used high concentrations of the nanoparticles and were thus unable to discern mechanistic response beyond loss of viability.[21]







Nanoparticle	LC_{50} value (PPM)
P25	62.37 ± 9.06
Anatase	88.22 ± 10.56
Rutile	136.0 ± 31.73

Figure 4: Lethal concentration. *Lethal concentration was quantified at 0ppm, 25ppm, 50ppm, 100ppm, 300ppm, 500ppm, 700ppm, and 1000ppm nanoparticle. P25 was observed to be the most lethal nanoparticle after 24hr treatment followed by Anatase and Rutile as determined by calculating the LC_{50} values utilizing sigma plot analysis. (N = 6).*

3.3.2 Nanoparticles Induce Loss of Viability and Morphology in Astrocytes

To compare the effect of TiO_2 nanoparticles to primary neuron and astrocyte viability and morphology phase contrast imaging **(Fig. 5 and 6).** Changes in cellular morphology represent a strong qualitative indicator of cellular injury and viability loss.[47] Neurons appeared to be insensitive to nanoparticle presence as a change in morphology and viability was not observed in 24hr (Data not shown). Even after 5 days in culture an insignificant loss in morphology and viability was observed for all nanoparticle forms and all concentrations except slight loss of neurite outreach in neurons treated with 100ppm P25 and anatase nanoparticles, indicating that the neurons were mostly indifferent to the presence of nanoparticles (**Fig 5**). However, we observed concentration- and phase-dependent changes in morphology and viability of astrocytes in the presence of TiO_2 nanoparticles after 24hr (**Fig 6**). Exposure to 100ppm of P25 and anatase demonstrated the most profound changes in cell morphology and viability, as compared to 100ppm rutile. In addition, the change in morphology, and subsequently cell viability, was concentration dependent and correlated well with the LC_{50} values. Similar results have been observed in other cell lines and mixed cultures that observed reduced cell size and rounded shape prior to cell detachment.[36, 47] Overall, these experiments validated that we were working in the sub-acute realm and that astrocytes experienced more sensitivity to TiO_2 nanoparticle presence than neurons.

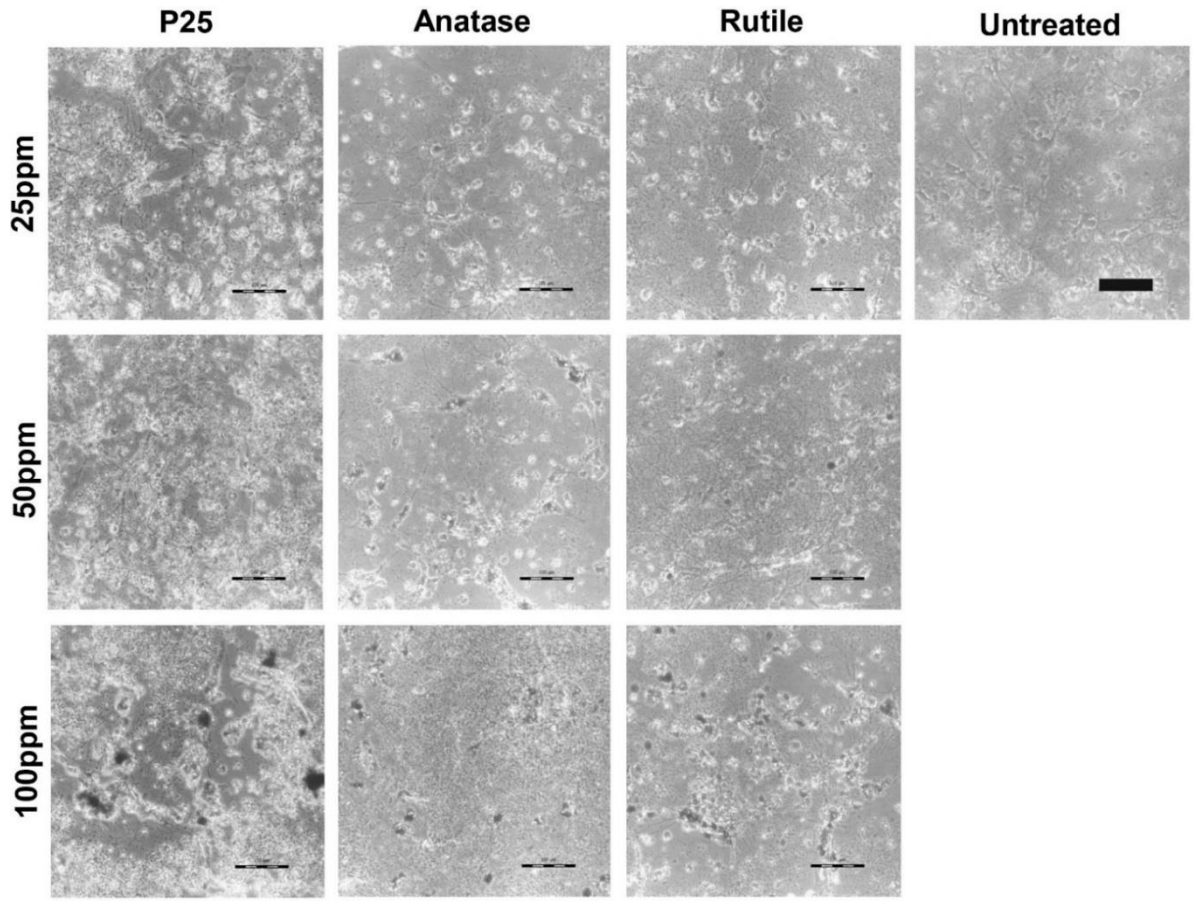


Figure 5: Effect of TiO_2 nanoparticles on the morphology and viability of Neurons 5days after TiO_2 treatment. *Day 1-4 did not exhibit drastic change in morphology and viability (as indicated by the survival of delicate neurite networks). By day 5 with nanoparticles the neurite network of neurons treated with P25 and Anatase 100ppm appeared to break down while cells treated with Rutile 100ppm nanoparticles still have many neurites intact. Cells treated with 25ppm and 50ppm of all three nanoparticles types show much less change in morphology after 5days. Scale bar = 200μm.*

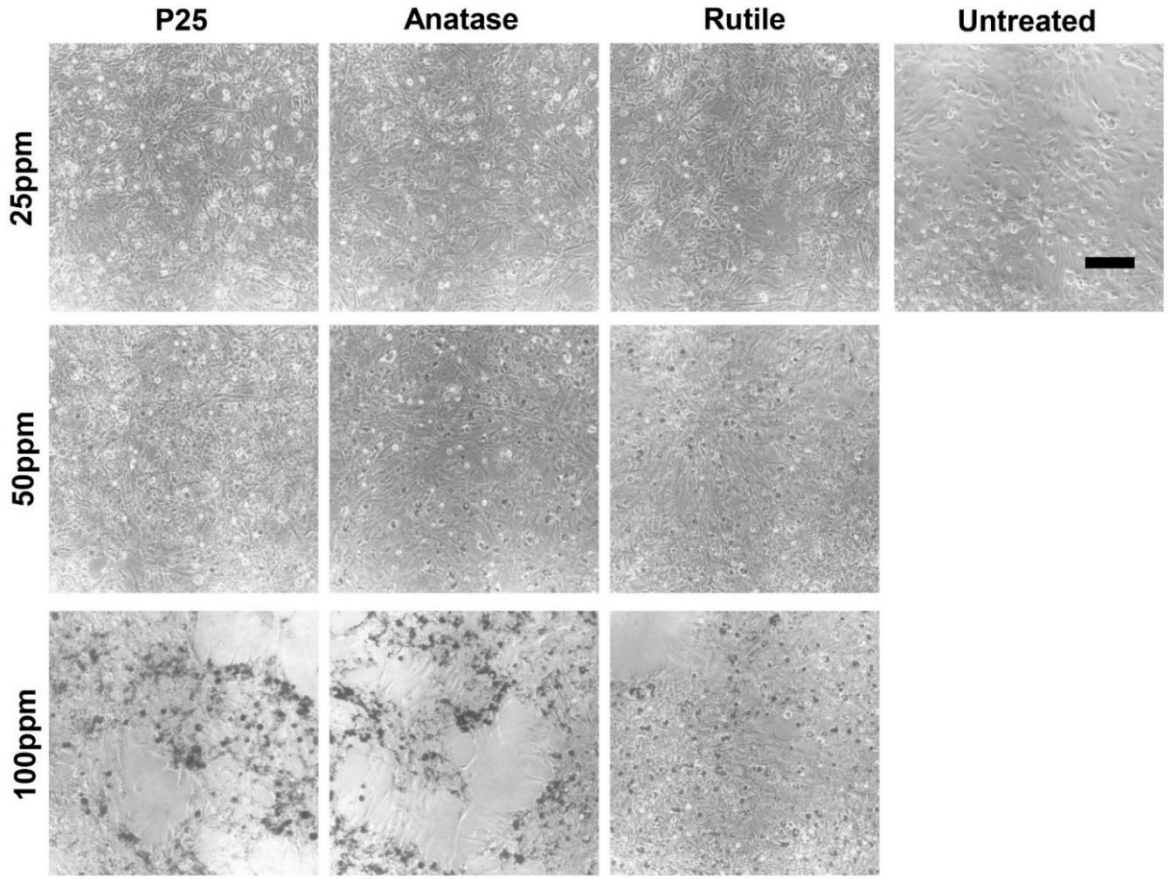


Figure 6: Effect of TiO_2 nanoparticles on the morphology and viability of astrocytes. *Change in astrocyte viability and morphology was assessed with phase contrast images 24hr after TiO_2 treatment. A concentration- and form-dependent trend was observed with exposure to P25 and anatase 100ppm showing the greatest change in morphology and exposure to rutile 100ppm TiO_2 nanoparticles less change. Astrocytes treated with all three nanoparticles at concentrations 25ppm and 50ppm show much less change in morphology than 100ppm. Scale bar = 200μm.*

3.3.3 Nanoparticles cause Loss of Glutamate Uptake and Glutamate Transporter Expression

To study the effect of TiO_2 nanoparticle exposure on primary astrocyte function, we measured glutamate uptake and GLAST gene expression (**Fig 7**). Astrocytes are the major cell of the brain to clear and process glutamate for future neuron function and prevention of neural excitotoxicity therefore loss of this function could have negative effect on overall brain health.[1, 42] We quantified the glutamate uptake by astrocytes using radiolabelled glutamate upon 24hr exposure to three concentrations (25ppm, 50ppm, and 100ppm) and types (P25, anatase, and rutile) of TiO_2 nanoparticles (**Fig 7A**) and observed significant concentration and type dependent loss. The exposure of astrocytes to 25ppm, 50ppm, and 100ppm of P25 resulted in approximately 30% reduction in glutamate uptake. Astrocytes treated with 50ppm and 100ppm of anatase resulted in 45% reduction in glutamate uptake while 25ppm did not affect glutamate uptake in astrocytes compared to untreated cells. The exposure of astrocytes to rutile resulted in negligible reduction in glutamate uptake in 25ppm and 50ppm while 100ppm resulted in 40% reduction in glutamate uptake. These data indicate that TiO_2 nanoparticle exposure exerts both concentration- and type-dependent effect on glutamate uptake of astrocytes, with P25 causing the highest damage to astrocytes biology even at low concentration of 25ppm.

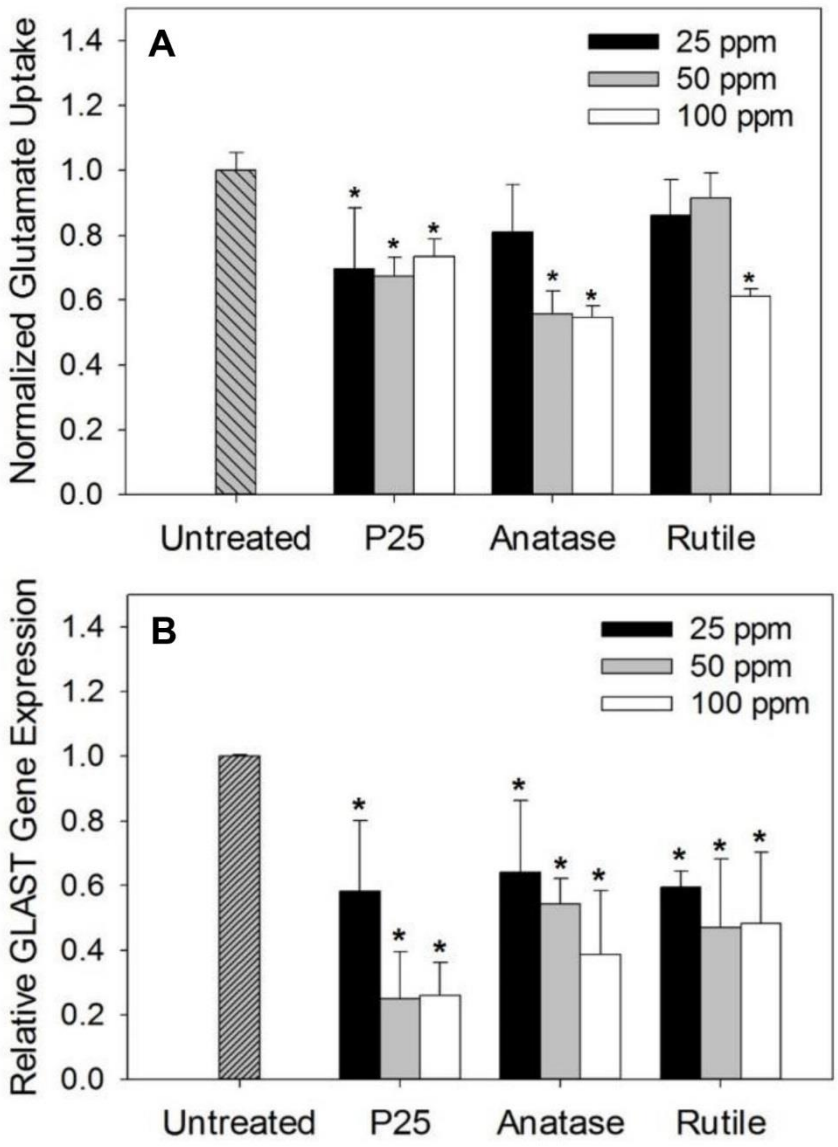


Figure 7: Effect of TiO_2 nanoparticles on glutamate uptake. *Decrease in glutamate uptake and mRNA expression of glutamate transporter GLAST was observed indicating loss of important cellular functions in astrocytes by 24hr TiO_2 nanoparticles exposure. N = 3, "*" indicates $P<0.05$.*

To further investigate the mechanisms leading to reduction in glutamate uptake, we measured the gene expression levels of glutamate/aspartate transporter (GLAST). Glutamate homeostasis is maintained by neuron-astrocyte interaction via several glutamate transporters and is a key metabolic function of astrocytes and neurons at the mitochondrial

level.[48] Glutamate transporters are classified under two categories, namely the Excitatory Amino Acid Transporters (EAATs) and the Vesicular Glutamate Transporters (vGLUTs) which are responsible for maintaining low physiological extracellular glutamate levels.[49] Five subtypes have been identified in rodents and humans including GLAST that is predominately expressed in astrocytes and is required for regulating the glutamate uptake behavior of these cells.[50]

To understand the effect of TiO_2 nanoparticle exposure on glutamate transport, we investigated the relative gene expression of GLAST (**Fig 7B**) after 24hr of exposure to the different TiO_2 nanoparticles. We observed significant concentration- and type-dependent down-regulation of GLAST gene expression. The exposure of astrocytes to 25ppm, 50ppm, and 100ppm of P25 resulted in 40%, 75%, and 75% fold down-regulation of GLAST expression, respectively. The same concentrations of anatase TiO_2 nanoparticles resulted in 35%, 45%, and 60% fold down-regulation of GLAST expression, respectively. Lastly, exposure of astrocytes to 25ppm, 50ppm, and 100ppm of rutile resulted in 40%, 50%, and 50% fold down-regulation of GLAST expression, respectively. From these data, we hypothesize that exposure to TiO_2 nanoparticles results in significant damage to the glutamate transporter expression resulting in the reduction in glutamate uptake in astrocytes. This is suggestive of the damage that TiO_2 nanoparticles elicit on astrocytes function. The ability to produce, uptake and recycle glutamate is a vital role of astrocytes in the brain as they interact with neurons for healthy brain functions.[1, 42] Hence, the compromise of this function through TiO_2 nanoparticle exposure is indicative of potentially detrimental effect to brain function and increased risk toward neurodegenerative process.

3.3.4 Nanoparticles Induce Oxidative Stress in Primary Astrocyte

To further investigate the damage caused by TiO_2 nanoparticle exposure in primary astrocytes, we determined intracellular levels of ROS when exposed to TiO_2 nanoparticles for 24hr. ROS generation has previously been observed to accompany loss of glutamate and glutamate transporter expression linking induced mitochondrial stress to loss of astrocyte function and could therefore be an attribute of TiO_2 nanoparticle toxicity.[51] Concentration- and type-dependent increase in ROS production (as indicated by enhanced CM-H2DCFDA fluorescence) was observed following the 24hr exposure of primary astrocytes to TiO_2 nanoparticles (**Fig 8**). The exposure of astrocytes to 100ppm of P25 and anatase resulted in the highest CM-H2DCFDA fluorescence while exposure of 100ppm of rutile did not lead to a significant increase in ROS, as compared to untreated cells. The higher production of ROS in P25 and anatase indicate that primary astrocytes are under high stress.[52] This observation is comparable to other studies that have demonstrated a similar effect of TiO_2 nanoparticle exposure on oxidative stress in both animal and cell models.[53, 54]

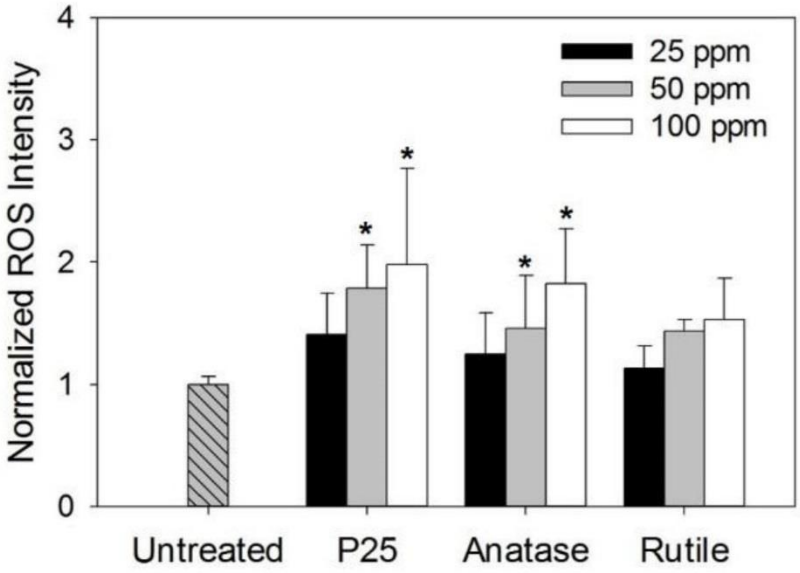


Figure 8: Oxidative stress induced by TiO_2 nanoparticles after 24hr. *ROS generation was observed to have a concentration and form dependence. Significant ROS generation was observed in the cells treated with 100ppm and 50ppm P25 and Anatase nanoparticles after 24hr indicative of oxidative stress in primary astrocytes. N = 5, "*" indicates P<0.05.*

3.4 Conclusion

Overall, we observed that culture of primary astrocytes with commercially and industrially relevant TiO_2 nanoparticles at low concentrations resulted in concentration- and type-dependent cytotoxic effects in viability, morphology and cell function. The glutamate uptake and GLAST gene expression were significantly compromised due to TiO_2 nanoparticles exposure even at concentrations as low as 25ppm. These alterations correlate with an increase in the amount of intracellular ROS production. Therefore, we conclude that TiO_2 nanoparticles in sub-acute toxic concentrations could contribute to loss of astrocyte function and, potentially, neurodegeneration.

4 Chapter 4 Mechanisms of Toxicity of Titanium Dioxide Nanoparticles in Culture with Primary Astrocytes

4.1 Introduction

Mitochondria are essential organelles in the cell primarily known for their involvement in cellular bioenergetics and metabolism. More recently, they have been recognized for organelle communication and initiation of programmed cell death. The signaling mechanisms from mitochondria are especially fascinating since they prevent the cell from committing to biological processes that cannot be supported by the metabolic actions of the mitochondria. ROS is a byproduct of ATP production and also used as signals to regulate a wide variety of cellular processes such as oxygen sensing, autophagy, and immune response.[55] Although ROS is a common component of cellular processes it is typically matched by antioxidant molecules to maintain a tolerable level for prevention of oxidative damage. However, ROS imbalance can lead to elevated intracellular ROS which is an accepted early sign of altered mitochondrial function. Chronic elevated levels of intracellular ROS can lead to a state of oxidative stress which has been implicated in cell death and a variety of neurodegenerative diseases.[56, 57] Therefore, disturbances in mitochondrial function is insightful to understanding TiO_2 nanoparticles toxicity and their potential role in neurodegeneration.

A unique and vital feature of mitochondria is the constant dynamic nature utilized to rejuvenate and meet the energy requirements of the cell. The balance of merging (fusion) and dividing (fission) maintains the mitochondrial dynamics facilitating cell bioenergetics demand and eliminating injured mitochondria thereby assuring sufficient energy supply for proper cell function. Fusion, mediated by membrane-anchored dynamin family members

mitofusins (Mfn 1 and 2), is required for mitochondrial (mt) DNA maintenance because it allows mtDNA exchange and protects the mtDNA from damage during stress.[58, 59] Fission, mediated by cytosolic dynamin family member Dynamin-related protein 1 (Drp1), is essential for mitochondrial distribution and selective elimination of damaged mtDNA. Mitochondria utilize fission in response to extensive and persistent mitochondrial damage, as abundant fission can result in the release of factors to initiate the cascade for cellular apoptosis.[60]

The disruption of this delicate balance has been observed to have positive and negative effects in response to cellular stressors. Excessive fission, characterized by overexpression of fission marker Drp1, is commonly a sign of irreparable damage as the cell is set in motion toward apoptotic programmed cell death.[61] The production and release of pro-apoptotic factors such as Bcl-2 and cytochrome C is strongly dependent on the production of Drp1 as shown by delayed release when the Drp1 gene expression is eliminated.[62] Excessive fusion, characterized by an increase in fusion markers, has been observed to promote mitochondrial function and prolong cellular survival in response to specific low levels of stress including stressors with potential to induce apoptosis.[59] Furthermore, Wang *et al* observed overexpression of Mfn2, resulting in increased mitochondrial fusion, to prevent glutamate excitotoxicity in primary rat motor neurons, showing the mitochondrial fusion as pro-survival in the presence of stress. However, Uo *et al* found that eliminating the expression of Drp1 in primary cortical neurons induces hyperfused mitochondrial morphology and a significant decrease in cell viability, solidifying the importance of the careful balance maintained by the mitochondria.[63] Thus, studies of mitochondrial dynamics

in brain cells emphasizes that the careful balance must be maintained for cellular function and survival which is strongly stressor dependent.

In the presence of stress induced by TiO_2 nanoparticles, two previous studies have observed mitochondrial injury and altered bioenergetics function in astrocyte cell lines. These studies were performed with 96% anatase nanoparticles at moderately high concentrations (20μg/ml) in C6 and U373 cell lines, a mouse and human glioma cell line, observing internalization of nanoparticles, increased intracellular ROS, decreased mitochondrial membrane potential, and apoptosis.[47, 54] However, no studies have observed the effect of TiO_2 nanoparticles on mitochondrial dynamics in primary astrocytes. Therefore, the aim of this study was to understand the effect of three commercially available nanoparticles (P25, Anatase and Rutile) at sub-acute toxic concentrations (25ppm, 50ppm, and 100ppm) on the mitochondrial health and dynamics of primary rat cortical astrocytes. We monitored mitochondrial health by observing the change in mitochondrial membrane potential and mitochondrial dehydrogenase activity and observed change in mitochondrial dynamics by monitoring change in Drp1, Mfn1 and Mfn2 gene expression after 24 hour incubation with low levels of TiO_2 nanoparticles.

4.2 Materials and Methods

4.2.1 Mitochondrial Membrane Potential (MMP)

MMP was determined using tetramethylrhodamine (TMRM) [Life Technologies], a cationic red dye which accumulates in healthy, active mitochondria marking depolarization. The stock solution was diluted in fluorobrite DMEM to a final concentration of 20nM, added to cells, and incubated in the dark at room temperature for 45minutes. Afterward, the dye was removed and cells were washed 3 times with warm

1XPBS. The fluorescence was read at emission 590nm and excitation 573nm. Results are reported as the normalized intensity to the untreated cells.

4.2.2 Mitochondrial Dehydrogenase Activity

Mitochondrial Dehydrogenase Activity was determined utilizing the MTT [3-(4,5-dimethyldiazol-2-yl)2,5 diphenyl Tetrazolium Bromide] [Life Technologies] Assay. It is a colorimetric assay that evaluates the mitochondrial conversion of the MTT salt. In short, after 24hr nanoparticle exposure the culture media was aspirated and 5mg/ml MTT working solution in DMEM incubated on live cells at 37°C for 3hr. Lysis buffer (0.1N HCl in isopropanol) was added in a 5:1, lysis buffer to MTT solution, ratio. The absorbance values were read at 570/630nm in an AD340 plate reader [Beckman Coulter, Indianapolis, IN].

4.2.3 Gene Expression

Total RNA was isolated using Trizol [Life Technologies] according to the manufacturer's instructions. The quality and quantity was determined by ND-1000 spectrophotometer [NanoDrop Technologies Wilmington, DE] and reverse transcribed using iSciptTM cDNA synthesis kit [Bio-Rad Laboratories] by manufacturer's instructions.

Quantitative Real Time PCR (RT-PCR) was performed using SYBR Green Master Mix [Applied Biosystems, Foster City, CA] in an epgradient S Mastercycler [Eppendorf]. The PCR program was set to the following: 10minutes at 95°C, 40-60 cycles of 95°C for 15seconds followed by annealing at 60°C for 15seconds and elongation at 72°C for 60seconds, and finally 95°C for 15seconds to end. The primers of interest obtained from Integrated NDA Technologies [Coralville, IA] with the following sequences: Mfn1

(Forward 5'-TCGTGCTGGCAAAGAAGG-3' and Reverse 5'-CGATCAAGTTCCGGGTTCC-3'), Mfn2 (Forward 5'-CGATGTGGTAGTGAGGTTGG-3' and Reverse 5'-CTCCCATCTTCCACCATTCC-3'), Drp1 (Forward 5'-GAACTACCTTCCGCTGTATCG-3' and Reverse 5'-CGACCACCATCTCCAATTCC-3'). GAPDH (Forward 5' ATG ATT CTA CCC ACG GCA AG 3' and Reverse 5' CTG GAA GAT GGT GAT GGG TT 3') was measured as reference. The ΔΔCT method was utilized for analysis of each sample. Results reported as normalized to the average expression of untreated cells.

4.2.4 Statistical Analysis

All data is presented as the mean ± the standard deviation. Statistical comparisons between treatments utilized Sigma Plot ANOVA (Dunnett's Method) for analysis. Pool size was as indicated in the figure description.

4.3 Results and Discussion

4.3.1 Mitochondrial Function Disruption

We investigated the effect of TiO_2 nanoparticle exposure on MMP (**Fig 9A**) and mitochondrial dehydrogenase activity (**Fig 9B**), assessing the depolarization of mitochondrial membrane and the activity of succinate dehydrogenase, important markers of mitochondrial health.[26, 47] We observed a 25% decrease in MMP in astrocytes exposed to P25 in all three concentrations while only 100ppm of anatase demonstrated 25% loss in MMP. Interestingly, rutile did not have any effect on the MMP even when exposed to 100ppm concentration. Mitochondrial dehydrogenase activity was measured to determine the damaging effects induced by TiO_2 nanoparticle exposure to primary astrocytes. The exposure of astrocytes to 25ppm, 50ppm, and 100ppm of P25 resulted in 10%, 20%, and

25% loss in mitochondrial dehydrogenase activity, respectively. Anatase nanoparticles caused a 10%, 15%, and 25% loss in mitochondrial dehydrogenase activity with 25ppm, 50ppm, and 100 ppm, respectively. Finally, exposure to rutile nanoparticles of 25ppm, 50ppm, and 100ppm lead to a loss of 5%, 10%, and 15% of mitochondrial dehydrogenase activity, respectively. From this data, we observed that exposure to TiO_2 nanoparticles results in significant mitochondrial stress leading to loss of mitochondria health and function.

4.3.2 Mitochondrial Dynamics Disruption

Imbalances in mitochondrial dynamics are known to impede cellular bioenergetics and contribute to numerous neurodegenerative diseases.[64] To investigate the effect of nanoparticle treatment on mitochondrial dynamics, we measured the relative gene expressions of Mfn1, Mfn2, and Drp1, markers associated with mitochondrial fusion and fission events (**Fig 10**). Mfn1 and Mfn2 are essential proteins for the fusion process while Drp1 is an essential protein for the fission process. Changes in transcript levels of Mfn1 and Mfn2 have been reported to correlate with changes in mitochondrial dynamics.[65] At low concentrations of P25 (25ppm and 50ppm), we observed a significant up-regulation of Mfn1 and Mfn2 transcripts while no significant change in Drp1 was observed. These findings imply a tendency toward fusion in response to TiO_2 nanoparticle treatment. In astrocytes exposed to 100ppm of P25, anatase, and rutile nanoparticles, Drp1 was up-regulated, which indicates the activation of the fission process leading to mitochondrial fragmentation and cell death. These results indicate that TiO_2 nanoparticles induce stress on the mitochondria which has a concentration dependent response similar to that proposed by Van der Bliek[66]. However, further study will need to be conducted to know if the

prolonged stress from low concentration TiO_2 nanoparticles can eventually lead to apoptosis and result in neurodegeneration.

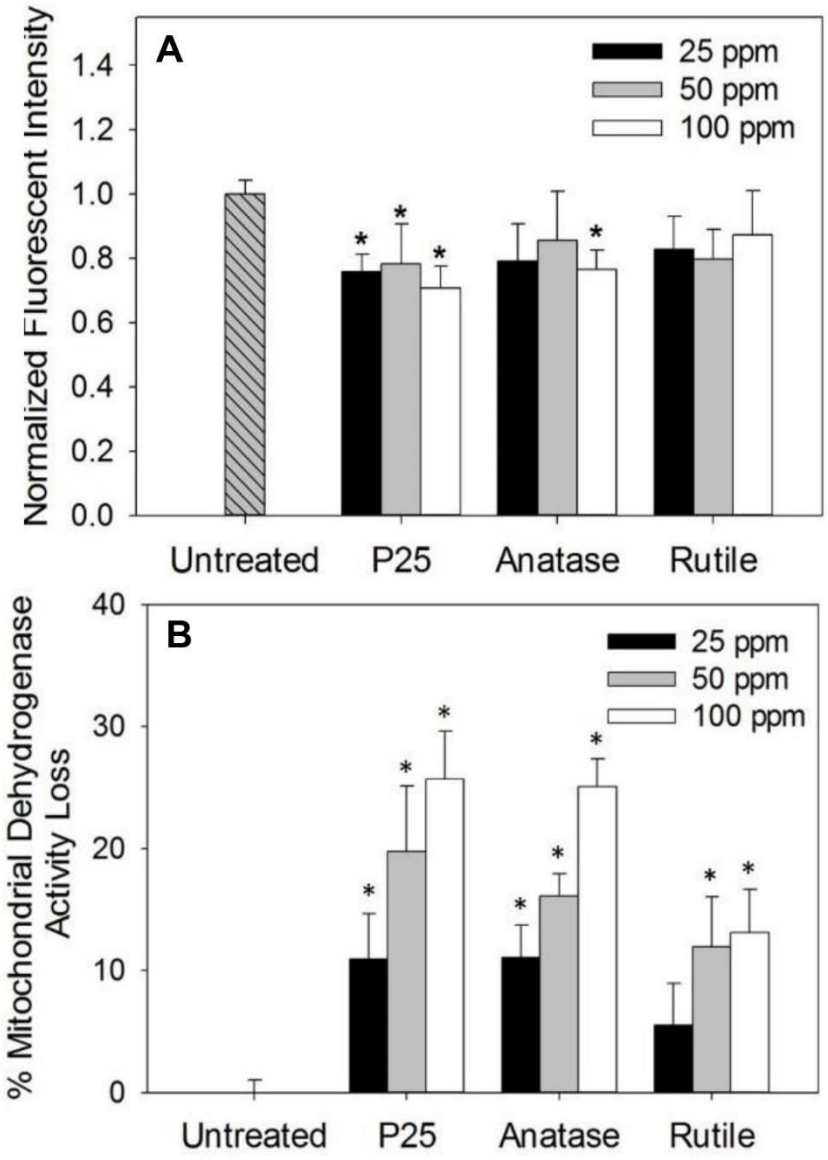


Figure 9: Mitochondrial Stress triggered in Astrocytes after 24hr exposure. *Decrease in MPP (A) and mitochondrial dehydrogenase activity loss (B) indicate loss of mitochondrial health due to nanoparticle stress. N=5, "*" indicates P<0.05 compared to untreated.*

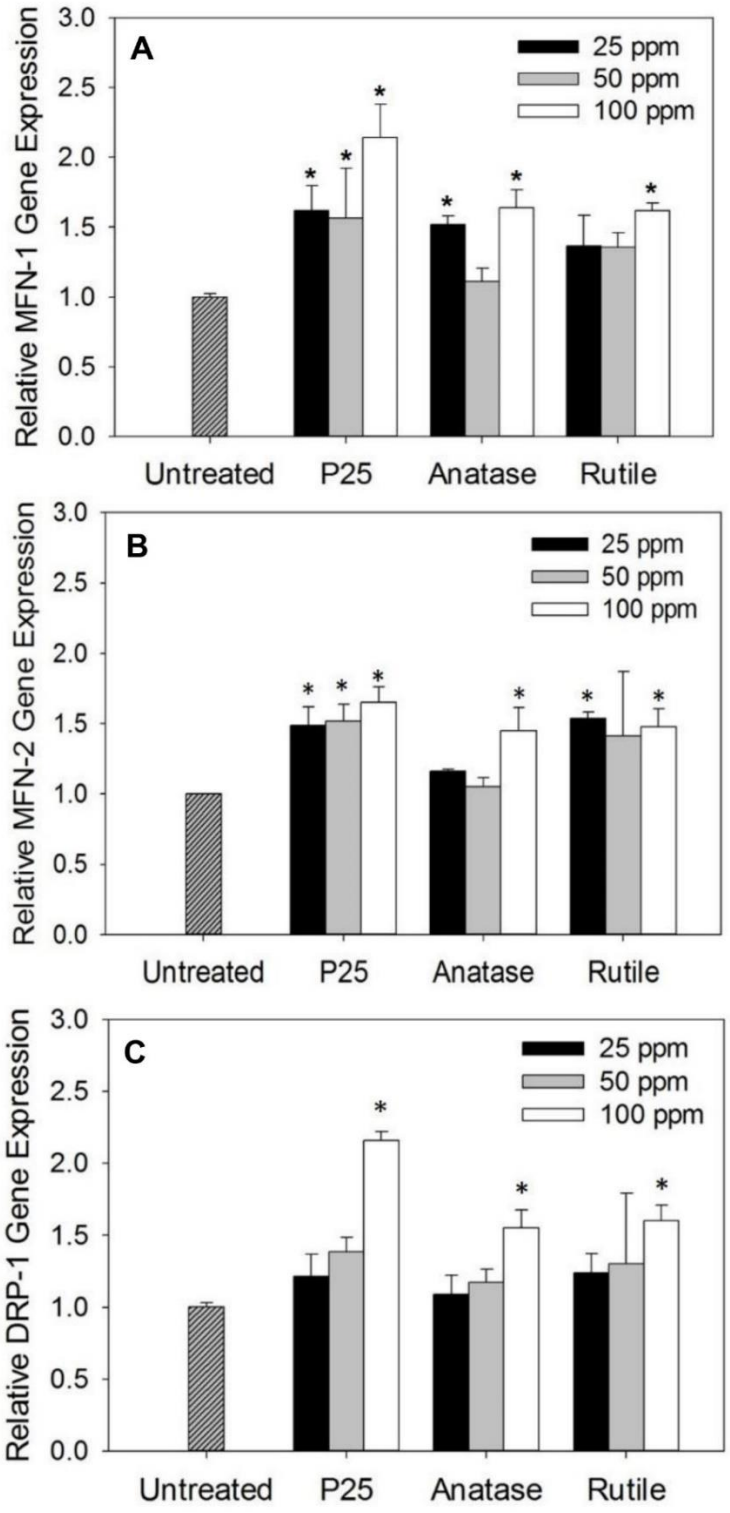


Figure 10: Mitochondrial dynamics interruption in astrocytes after 24hr exposure. *Increase in Mfn1 and 2 and Drp1 indicate a deviation from normal mitochondrial dynamic balance toward hyperfusion in 25ppm and 50ppm treated cells and fission in 100ppm treated cells. N = 3 "*" indicates P<0.05.*

4.4 Conclusion

We observed evidence of concentration dependent decrease in mitochondrial health and response of mitochondrial dynamics after 24hr exposure to TiO_2 nanoparticles. Low concentration (25ppm and 50ppm) nanoparticles, although causing very little change in mitochondrial health, brought about changes in DRPs' expression, indicative of mitochondrial fusion. However, exposure to high concentration (100ppm) TiO_2 nanoparticles caused marked decrease in mitochondrial health and resulted in DRPs' expression patterns reflecting on increased fission. These results confirm that mitochondrial dynamics are disrupted by stress induced by low levels of TiO_2 nanoparticles which may play a role in loss of important astrocyte functions and neurodegeneration.

5 Chapter 5 Recommendation for Future Work

This thesis has assessed and confirmed the negative sub-toxic effect of TiO_2 nanoparticles in primary rat astrocytes *in vitro*. At concentrations below that which would induce significant cell death within 24hr, astrocytes experience disruption of mitochondrial dynamics and function, generation of ROS and loss of glutamate uptake and transporter expression. These observations are important preliminary observations to uncover the role that stress induced by low concentration TiO_2 may play in the onset and progression of neurodegenerative disease however there are many other observation which would further support and provide greater understanding which I will describe here after.

(1) Further understanding of the effect of TiO_2 on bioenergetics impairment of astrocytes. We have observed early indication of mitochondrial function impairment and dynamics disruption in primary astrocytes. However, it would be informative to look more into this subject through other avenues further probing mitochondrial function impairment. The extent of mitochondrial function impairment could further be quantified by loss in ATP production, glucose uptake and lactate production since these are crucial functions of the mitochondria which astrocytes and neurons rely on for energy to support vital cellular processes. Furthermore, we could visualize the extent of mitochondrial dynamics impairment at low concentration in 24 hours by viewing mitochondrial morphology and quantifying protein. This would indicate the extent that the gene expression correlates to the physical changes in the organelle. This study could also be extended beyond 24 hours to find the time requirement for low concentration exposure to result in protein and mitochondrial structure change. As change in gene expression is thought to be an early sign proceeding morphology change this result would validate this

practice. The time dependent exposure study would also indicate whether the hyperfusion experienced by astrocytes will lead to apoptosis in the chronic presence of low concentration TiO_2 nanoparticles.

(2) Further understanding of the effect of TiO_2 on other cell types of the brain. Other cell types of the brain, such as microglia and oligodendrocytes, perform very specific roles in neuron function and development and could also be disrupted by the presence of sub-toxic concentrations of TiO_2. Therefore the level of functional disruption should also be assessed in them. Microglia are the macrophages of the brain and therefore seek out foreign materials and initiate immune response in brain tissue. They are the key defense against infection, inflammation and neurodegeneration and would then play a key role in response to disruption caused by low levels of TiO_2.[67] A few studies have assessed the increase in ROS generation and increased gene expression in pathways associated with inflammation, cell proliferation, and oxidative stress in microglia and mediated toxicity of TiO_2 nanoparticles in neurons.[35, 40, 68] Oligodendrocytes support the survival and growth of neurons by expressing growth factors and providing insulation to the axon of neurons through the myelin sheath.[69] The dysfunction of oligodendrocytes is most commonly known for its presence in multiple sclerosis and leukodystrophies however studies show that it may be implemented in other pathways leading to improper neuronal development and function. One study observed significant uptake of TiO_2 nanoparticles by oligodendrocytes cell line, OLN93, but further knowledge of the impact this has on the cell function could be crucial to understanding if nanoparticles have an impact in brain development and neurodegeneration.[70] As the reaction of cell types to the presence of TiO_2

can be widely varied it is important to understand how each cell type responds in order to understand the true impact of TiO_2 nanoparticles in the brain.

(3) The effect of TiO_2 nanoparticles on neuron-astrocyte communication. Another important future avenue for this study is to understand the ramifications of TiO_2 nanoparticles on cellular communications, especially those between neurons and astrocytes. Since astrocytes receive communication from neurons for restoration of homeostasis and tissue repair, the addition of neuron signals to the culture may alter the astrocyte response and sensitivity to TiO_2 nanoparticles.[71] Current methods available for co-culture of neurons and astrocytes include transwell, conditioned media and random co-culture.[46, 72] Each of these methods have their strengths as far as the extent of cellular communication allowance. Transwell and conditioned media experiments only allow for chemical communication over a distance while random co-culture allows for physical contact and short range communication. There are also strengths and weaknesses in each method for mimicking the cell-cell-nanoparticle interaction. Transwell and conditioned media can allow for only the cellular signal in response to nanoparticles to reach the second cell type while random co-culture would complicate communication by signal and nanoparticle interaction while providing the closest indication of *in vivo* interaction. Therefore, a co-culture study with nanoparticles would most likely benefit from a combination of results utilizing different multiple co-culture methods.

There is much still to be learned about the interaction of nanoparticles in the brain and how this may contribute to a number of developmental and neurodegenerative disorders. These studies are important to the understanding of potential impact and treatment as well as insightful into the basic understanding of brain function.

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